TEMPROAL CHANGE IN SOIL MICROBIOME
UNDER INFLUENCE OF EIGHT YEARS OF MANURE AMENDMENT IN CULTIVATED SOILS

By

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I dedicate this dissertation to our Lord, Jesus Christ. All the achievements in our lives are because of His blessings.

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Four and a half years of studying in OSU seems like yesterday. I will always remember this journey. It will be hard to leave OSU. Rather, I would say “until next time”. I bleed orange, always.
Abstract: Animal manure is one type of organic amendment that is widely used in sustainable agricultural management. It is established that manure affects soil quality, productivity, and biomass, including soil microbiome. Many studies characterize soil microbiome in responsive to soil amendments (e.g. inorganic- and organic-fertilizer); few studies, however, reveal impact of manure with different phase (e.g. solid vs. liquid) and animal origin (e.g. bovine vs. swine) on soil microbiome. We establish change in soil microbiome that was fertilized with none, solid beef manure (BM), and swine effluent (SE) for eight years. Illumina HiSeq platform was used to survey bacterial community by deep sequencing 16S hypervariable region 4 (V4). Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), were measured for soil fertility. Mantel test and Pearson correlation coefficient were analyzed to reveal the overall and specific relationship of soil property with microbiome and phylum, respectively. We find that soil microbiome are different under long-term influence of BM and SE fertilization. Results indicate that TC is the key driving factor shaping BM fertilized soil microbiome; whereas K is the main determinant in SE- and non-fertilized soil microbiome. Core microbiome (Actinobacteria, Proteobacteria, Firmicutes, Acidobacteria, Chloroflexi, Gemmatimonadetes, and Planctomycetes) remained stable in non-fertilized soils over time. Phyla Actinobacteria and Firmicutes were enriched in BM; while Gemmatimonadetes and Nitrospirae were responsive to SE. Many of predominant phyla found in our studies (e.g. Actinobacteria, Proteobacteria, Firmicutes, and Chloroflexi) are copiotrophic bacteria, which potentially play important roles in nutrients cycling in soils. We characterize soil microbiome shaped by eight years of manure amendment, identify core microbiome, and determine soil property as the main driving factor in microbiome. Our studies fill the gap of knowledge on impact of long-term soil management on microbiome.
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CHAPTER I

REVIEW OF LITERATURE

1.1. Brief review of historical bacterial classification

In a world where most of the study objects are difficult to be classified, cultivated or isolated, the frustration is real and the needs for novel methods are rising. The traditional bacterial classification used to be hindered due to lacks of morphological and physiological variations (Sinclair, 1939; Fox et al., 1980; Woese, 1987). The accuracy of early taxonomy heavily relies on exact morphologic and phenotypic matchings to strains, supposing organisms are isolatable, which is another road block itself (Breed et al., 1957; Tortoli et al., 2001). On top of that, various judgments and experiences of researchers also affect phenotypic classification results. Zuckerkandl and Pauling propose to characterize bacteria on the basis of genetic sequences, an approach known as phylogenetics (Zuckerkandl and Pauling, 1965). Phylogenetic analysis ensures bacterial classification results to be unbiased and comparable, and, more importantly, circumvent the uncultivable barrier (Woese et al., 1980; Moyer et al., 1995; Marchais et al., 2009; Slabbinck et al., 2010; Navarro et al., 2016).
1.2. Revolution brought by genetic sequencing

Since the 1980s, a revolution has been undergoing in microbiology because there are more accessible nucleic acids sequencing techniques (Jensen, 1985; Olsen et al., 1986). Genetic sequencing offers perspectives and approaches for one to look into the microbiome world differently. George Fox et al. elevated the importance and feasibility of bacterial evolutional study via ribosomal RNAs (rRNAs) sequencing (Fox et al., 1980). rRNAs are widely recognized and used as molecular chronometers, which are molecules with randomly changed sequences over time (Woese, 1987). Bacteria are placed into their evolutionary positions on the basis of rRNA sequence. Furthermore, Woese and colleagues came up with “The Woesian Revolution”, proposing three domains in microorganism world: Bacteria, Eukarya, and Archaea, established on rRNA sequences (Woese et al., 1990).

1.3. rRNAs sequencing on bacterial taxonomy

The success of employing rRNA sequence in bacterial evolution inspired and boosted bacterial taxonomy studies (Lane et al., 1985; Pace et al., 1986). rRNA is an ideal candidate for bacterial taxonomy because 1) it has highly conserved structure and function (Dubnau et al., 1965; Tran and Rudney, 1996; Morosyuk et al., 1999); 2) it exists ubiquitously in organisms (Woese, 1987; Bottger, 1989); 3) it is sufficiently variable in size and domain (Noller, 1984; Noller et al., 1985); and 4) it is accessible for direct sequencing (Gray et al., 1984; Lane et al., 1985; Noller et al., 1985; Bottger, 1989). Dubnau and colleagues studied genetic relationships among ribosomal and transfer RNAs, and four antibiotic resistant genes, in eight strains belonged to genus Bacillus. They found that rRNAs (e.g., 4S, 16S and 23S) were homogenous and stable during evolutionary change (Dubnau et al., 1965).
1.4. 16S rRNA sequencing on phylogeny

In 1990, Woese and colleagues classified all the organisms on Earth into the Tree of Life by using the 16S rRNA (16S) sequence for Bacteria and Archaea and the 18S for Eucarya (Woese et al., 1990). Small subunit rRNA, 16S in particular, is mostly used for bacterial classification even at genus level (Bottger, 1989; Fox et al., 1992; Kolbert and Persing, 1999; Clarridge, 2004; Harmsen and Karch, 2004). The average length of 16S is approximately 1.5 kb, which is long enough to provide sufficient information for bacterial classification (Clarridge, 2004). Although mostly conserved, bacterial 16S has nine hypervariable regions (V1-V9) that can be used for taxonomy (VandePeer et al., 1996). Several universal primers are available to target and amplify hypervariable regions (Chen et al., 1989; Weisburg et al., 1991; Baker et al., 2003).

1.5. V4 region on 16S

The V4 region (ca. 250 bp) in 16S is widely used for bacterial phylogenetic study. Indeed, studies reported that V4 generated the most accurate bacterial community and species richness estimates as well as the lowest error rate of sequencing reads (Youssef et al., 2009; Kozich et al., 2013). Youssef and colleagues constructed a nearly-full 16S library containing 1,132 clones and performed pyrosequencing. They truncated the library to obtain eight encompassing fragments, namely V1-V2, V3, V4, V5-V6, V6, V6-V7, V7, and V7-V8. They compared species richness estimates on the basis of candidate fragments to the estimate generated by the nearly full-length 16S fragment. They found that estimates generated via V4, V5-V6 and V6-V7 were close to results based on nearly full-length region. Other regions, however, either over- (V1-V2 and V6) or under estimated (V3, V7, and V7-V8) the species
richness. Kozich and colleagues sequenced V3-V4 (ca. 430 bp), V4, and V4-V5 (ca. 375 bp) regions via Illumina MiSeq platform. They found that V4 region had the lowest error rates (0.01%) compared to V3-V4 (0.15%) and V4-V5 (0.50%). Furthermore, Kozich et al. found that paired end reads had the best coverage in V4 region with almost no overlapping, whereas V4-V5 and V3-V4 had 125 and 70 bp overlapping, respectively. Error rate correlates with the length of fragment and overlapping region. Generally, a shorter fragment and less overlapping lead to lower error rate. Furthermore, by comparing to the mock community, they concluded that V4 was the best region to estimate bacterial community.

1.6. Other genetic markers for bacterial phylogeny

In addition to 16S, other genetic markers such as hsp65, groEL, and 16S-23S spacer regions, have been applied for phylogenetic studies. Gene hsp65 (ca. 360 bp) encodes a 65-kDa heat-shock protein (Hsp65). Kapur et al. reported eight allelic variables in hsp65 found in 11 Mycobacterium avium complex (MAC) (Kapur et al., 1995). They suggested to apply the nucleotide polymorphism of hsp65 in rapid identification of MAC. Swanson and colleagues continued with the method of using hsp65 to rapidly assign MAC species (Swanson et al., 1996; Swanson et al., 1997). There are a total of 25 alleles in hsp65 that can be used for MAC classification and over 50 nucleotide polymorphisms for other mycobacterial species delineation (Kolbert and Persing, 1999). Furthermore, hsp65 are more polymorphic than 16S in MAC organisms, making hsp65 more suitable for MAC differentiation (Swanson et al., 1997).

Although 16S is the most widely used for bacterial classification, it fails to classify certain bacteria within species due to the highly conserved sequence. Other gene markers, similar to hsp65 for MAC, are more useful to differentiate particular bacteria. For instance, groEL (ca.
360 bp) that encodes 60kDa heat-shock protein (Hsp60) is used to elucidate relationships among *Bartonella* species (Marston et al., 1999; Zeaiter et al., 2002). Zeaiter and colleagues isolated *groEL* from 10 *Bartonella* strains to study their phylogenetic relationships by using parsimony, neighbor-joining (NJ) and maximum-likelihood (ML) methods, respectively. They reported the first reliable classification of *Bartonella* species and subspecies on the basis of *groEL* sequencing.

The polymorphism of 16S in *Mycobacterium* is too low to distinguish certain species, e.g. *M. kansasii* from *M. gastri* (Roth et al., 1998). The gene that encodes rRNA in bacteria is formed in the order of 16S-23S-5S, with two noncoding spacers in between (Condon et al., 1992). 16S-23S rRNA internal transcribed spacer (ITS) sequence contains more variable sequences than 16S, thus poses as a better candidate for *Mycobacterium* classification (Frothingham and Wilson, 1993; Vandergiessen et al., 1994; Desmet et al., 1995; Roth et al., 1998). Roth and colleagues isolated 16S-23S from 60 strains to represent 13 *Mycobacterium* species. They found allele variations to separate fast and slowly growing mycobacteria. They suggested that 16S-23S sequence was better used to classify *Mycobacterium*, especially for slowly growing ones, which presented more genetic variations in 16S-23S fragment. Similarly, Luz and colleagues isolated 16S-23S from 7 *Salmonella enterica* subspecies. They constructed phylogenetic trees, classified, and studied relationships within *S. enterica* subspecies on the basis of 16S-23S sequencing. They concluded that the variations found in 16S-23S could be used as genetic markers for *S. enterica* subspecies classification (Luz et al., 1998).
1.7. Application and limitations of 16S sequencing

Applying 16S sequencing on phylogenetics has been well appreciated because it provides culture-independent, unbiased, and informative results. Researchers exploit 16S sequencing in many areas including pathogen detection, novel and/or unculturable species classification, environmental ecology, and bacterial community survey (Choi et al., 1996; Fuhrman and Davis, 1997; Clarridge, 2004; Petti et al., 2005; Sogin et al., 2006). In spite of many advantages, limitations of 16S are also worth discussing. To start off, all the nine hypervariable regions present different levels of variation among bacteria; that is, some hypervariable regions are more sensitive and specific on distinguishing certain bacteria, yet some are not. Moreover, there is no single hypervariable region that can adequately classify all the bacteria (Chakravorty et al., 2007). To address the sensitivity and specificity issues especially for clinical practice, Chakravorty et al. suggested to sequence particular hypervariable regions that were highly efficient for specific bacterial identifications. They conducted a study to sequence eight fragments (V1 to V8) from 110 different bacterial species, including common pathogenic blood borne bacteria, Centers for Disease Control and Prevention (CDC) defined select agents, and environmental bacteria. They generated dendrograms on the basis of sequences. They found that V2 and V3 regions were the best at differentiating all the 110 bacteria at genus level with an exception of enterobacteriaceae. V1 was the best at identifying *S. aureus*, and V6 was able to detect all the CDC-defined select agents. Other regions such as V4, V5, V7, and V8, however, were not as informative. One of their conclusions seemed to contradict studies suggesting that V4 region was the best at bacterial taxonomy. The reason could lay in the fact that V4 region was not sensitive to those 110 bacteria in Chakravorty et al.’s study. V4 region, on the other hand, is a better candidate for bacterial community survey. Because V4 fragment has shorter
reads, less, if not no, overlap and can concordantly estimate community structure (Youssef et al., 2009; Kozich et al., 2013). Reciprocally, while V1-V2 region over-estimated richness in bacterial community, such region provided sufficient information to classify clinical related bacteria (e.g. P. aeruginosa, B. cepacia, S. pyogenes, and M. tuberculosis) at species level (Youssef et al., 2009; Salipante et al., 2013).

16S surveys have been widely used to study microbial phylogenetic diversity and community ecology (Giovannoni et al., 1990; Pace, 1997; Brinkmeyer et al., 2003; Knittel et al., 2005). High-throughput sequencing (HTS) techniques generate millions of reads that are assigned to operational taxonomic units (OTUs). Biodiversity within and between groups are compared and measured on the basis of OTUs (Schloss and Westcott, 2011). High-throughput sequencing leads microbial ecological studies into a new era by circumventing the uncultivable barrier, providing massively parallel genetic tags, and ensuring a high OTU coverage (Balint et al., 2016). Microbial ecologists are able to culture-independently study microbial community composition on the basis of hundreds samples. Limitations of applying HTS on microbial community surveys also exist. One of them is that 16S sequencing may distort bacterial community composition due to various sequencing depths (Poretsky et al., 2014). Additionally, primer bias also causes misrepresentation of certain bacteria (Sipos et al., 2007). Poretsky et al. surveyed the planktonic bacterial community in Lake Lanier in Georgia, USA on the basis of V1-V3 amplicons (Poretsky et al., 2014). They found that 16S gene survey presented fairly concordant bacterial community structure compared to whole genome shotgun (WGS) result, but they indicated that 16S had lower resolution in lowly abundant taxa such as Planctomycetes. The relatively low resolution of 16S survey generated different, if not skewed, community composition. More importantly, the alignments and comparisons of 16S among bacteria are
subject to the influences of gene recombination and horizontal gene transfer (HGT), which may also obscure bacterial taxonomy (Staley, 2006).

Other biases caused by polymerase chain reaction (PCR) and sequencing include 1) no knowledge of exact gene copy number in each organism (Eickbush and Eickbush, 2007), 2) primer-template mismatches (Ihrmark et al., 2012), and 3) generation of chimeric molecules (Ashelford et al., 2006). Biases affect studies on different perspectives, for instance, the accuracy of relative abundance is under the influences of hypervariable region selection, chimeric formation and primer bias; whereas richness estimate is affected PCR and sequencing errors (Wang and Wang, 1996; Li et al., 2009; Oliver et al., 2015). Positive control (e.g., mock community) and negative control (e.g., tagging negatives) can be applied to reduce biases.

The variation of 16S copy numbers (1-15) misleads diversity estimates in bacterial community (Lee et al., 2009; Kembel et al., 2012). To address this problem, Kembel and colleagues determined copy numbers by comparing 16S reads to genomes of closely related isolates with known copy numbers (Kembel et al., 2012). While Kembel’s algorism normalizes 16S copy numbers, the method is less useful when genetic diversities occur among microorganisms, that is, when bacteria are less genetically related. Perisin and colleagues developed another method named 16Stimator, an approach to predict 16S copy numbers when repetitive amplicons occurs during sequences assembly (Perisin et al., 2016). They used 16Stimator to estimate comparative copy numbers from 12 endophytic bacterial isolates. Their method not only asserts the usage of genomes on copy number variations between taxa, which gives insights on a clearer picture on absolute abundance of microorganisms in the community; 16Stimator also bypass the limitation of relying only on highly similar genomes.
1.8. Platforms for NGS

Platforms such as Roche 454, Illumina, ABI SOLiD, Ion Torrent, and Pacific Biosciences (PacBio) are major technologies for next generation sequencing (NGS). Roche 454 sequencing technique employs a pyrosequencing method to generate high-throughput sequencing reads (400-600 megabases per 10 hours) (Voelkerding et al., 2009). Pyrosequencing is a DNA sequencing method invented by Ronaghi, Uhlen and Nyren in 1998 (Ronaghi et al., 1998). Pyrosequencing is a real-time DNA sequencing technique, sequencing by synthesis (SBS), that measures the pyrophosphate emission during nucleotide assembly by using bioluminescence (Nyren, 2015). Illumina sequencing is also a SBS approach that detects the fluorescently labeled reversible terminator after the addition of each dNTP (Bentley et al., 2008). Ion Torrent sequencing, on the other hand, detects the release of proton caused by dNTP incorporation (Rothberg et al., 2011). PacBio utilizes the process named single molecule real time (SMRT) sequencing, which attaches DNA fragment and DNA polymerase to the bottom of zero-mode waveguides (ZMWs) (Eid et al., 2009). NGS technologies produce high-throughput parallel synthetic reads by attaching one short DNA fragment and adaptor to one bead, then immobilizing each bead in one well in a slide (Quail et al., 2012). Besides PCR amplicons, wells also have DNA polymerase and buffers, and a slide is filled with NTPs. A light signal is emitted for detection when one nucleotide is added to the DNA strand (Mardis, 2013). Bead location and the type of nucleotide can be determined based on the light signal. After one detection, the NTPs buffer is washed away, ready for next NTP addition and detection. The sequencing results are generated on the basis of signal density per cycle.
Sequencing instruments used to be bulky in size and time, they were costly to run and less accessible to small laboratories. New platforms, on the other hand, are tailored to be benchtop size, reagents and run times efficient, and budget friendly since 2010 (Loman et al., 2012). The widely used benchtop HTS platforms include the Roche 454 GS Junior (smaller version of 454 GS FLX), Illumina MiSeq (counterpart of Illumina HiSeq), and Ion Torrent Personal Genome Machine (PGM). Loman and colleagues evaluated and compared performances of 454 GS Junior, Illumina MiSeq and PGM by sequencing genome from E. coli isolates of O104:H4 (Loman et al., 2012). They reported that 454 GS Junior generated the longest reads (average of 522 bases), yet had the lowest throughput; PGM had the 4 times more throughput but the shortest reads (average of 121 bases). MiSeq on the other hand generated the highest throughput and longest reads, which sequenced 7 strains after one run. Additionally, MiSeq had the lowest sequencing error rate and 454 GS Junior had the best de novo assemblies. The authors stated that admittingly performance variations exist among platforms, almost all the technologies can meet the research expectations. Comparing platforms provides more information for researchers to select the platform that best fits their purposes.

1.9. Other methods for phylogenetic study

16S sequencing is convenient in classifying bacteria that exist virtually anywhere, especially when taxa are genetically diverse to each other. In 1994, Stackebrandt and Goebel proposed a demarcation suggesting that species would be distinguished from known ones without carrying DNA-DNA hybridization (DDH) when the homology of 16S gene was lower than 97% (Stackebrandt and Goebel, 1994). However, speciation on the basis of 16S offers little information at species level when sequence similarity is higher than 97% (Ash et al., 1991;
Amann et al., 1992; Fox et al., 1992; Staley, 2006). Nevertheless, 16S sequencing poses as one of the most valuable approaches to bacterial polyphasic studies. Performing 16S survey as the first step helps to decide the necessity of carrying DDH, because DDH is a time-consuming procedure and few laboratories can perform properly. Techniques, such as DDH, genomic sequencing, and multilocus sequence analysis (MLSA), are needed for further classification at species and subspecies level when the similarity of 16S is higher than 97% (Hayashi, 2004; Gevers et al., 2005; Staley, 2006; Glaeser and Kampfer, 2015).

The DDH used to be, if not still, considered as the gold standard for species classification. This method is superior when species are closely related (Stackebrandt and Goebel, 1994). The newest definition of a prokaryotic species is written as “a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions” (Stackebrandt et al., 2002). Organisms are considered as the same species when they 1) carry genomes that have 70% or higher DNA-DNA reassociation and within 5 °C or less ΔTm and 2) have comparable phenotypic descriptions (Vandamme et al., 1996; Stackebrandt et al., 2002; Gevers et al., 2005). The rationale of the species definition is that DNA similarity couples with thermal stability in DNA duplex; the thermal stability decreases by 1.2% for 1% of mispairing (Bautz and Bautz, 1964; Ullman and McCarthy, 1973). Additionally, the Ad Hoc Committee recommends that organisms that share 5 °C or less thermal stability difference are considered as the same species (Wayne et al., 1987; Stackebrandt and Goebel, 1994). The approach of using the entire genome in DDH analysis as the basis of speciation is convincing because it empowers higher resolution in taxonomy. Limitations of
DDH approach are also noticeable: 1) it is a laborious technique and 2) it requires to compare genomes against known species.

In 1998, Maiden and colleagues developed a method named multilocus sequence typing (MLST) (Maiden et al., 1998). They employed 11 housekeeping genes to classify species within 107 meningococcal strains. The housekeeping gene in this approach was defined as gene encoded proteins with conserved functions. They found that six out of eleven genes provided sufficient resolution at species level. As the conclusion, Maiden et al. suggested that MLST should be performed for species delineation on the basis of 6-10 housekeeping genes. The intention of MLST was to define genetic types and complexes within species (Glaeser and Kampfer, 2015). Vanlaere et al. used MLST analysis to revise the taxonomic position of taxon K within the *Burkholderia cepacia* complex (Bcc) (Vanlaere et al., 2009). They indicated that a 3% difference in concatenated genes were equivalent to 70% DHH and 95% whole-genome average nucleotide identity (ANI) for species delineation. Multilocus sequence analysis (MLSA), originated and alternated from MLST, is now widely used to elucidate relationships within a genus (Maiden et al., 1998; Godoy et al., 2003; Gevers et al., 2005). MLSA is a method to classify prokaryotes on the basis of several (usually seven, at least five) housekeeping genes (Gevers et al., 2005). The selected genes should be protein-coding, single copy, scatter at different chromosomes, and ubiquitously exist in taxa of interest (Stackebrandt et al., 2002; Gevers et al., 2005; Glaeser and Kampfer, 2015). Unlike 16S, protein-coding genes are less conserved and evolve constantly at a slow rate. Higher genetic diversity permits higher phylogenetic resolution. Furthermore, classification on the basis of multiple gene sequences circumvents the bias introduced by single genetic fragment. Altogether, Gevers and colleagues proposed a combination of rRNA sequencing and MLSA for prokaryotic classification. They
recommended first to narrow unknown organisms down to family or genus level using rRNA sequencing, then use MLSA for species assignment. Schleifer further reviewed MLSA as a valuable additional approach for hierarchical classification and recommended that at least 12 concatenated housekeeping genes should be used for phylogenetic tree construction (Schleifer, 2009). The lack of detailed guidelines, such as selection of gene, gene size, and primer design, however, hinders the proper application of MLSA. Claeser and Kampfer continued with the discussion of MLSA application and proposed detailed criteria for proper MLSA procedure (Glaeser and Kampfer, 2015). They listed the most common issues one might encounter for carrying MLSA and offered solutions to potential problems, such as gene selection, primer design, sequence quality control, and sequence alignment. They not only emphasized the value of MLSA as a phylogenetic tool with high resolution at species level, but also advocated the possibility of substituting DHH with MLSA.

Microorganisms are the major components in the Tree of Life. The number of named microbial species, however, is incongruously low compared to animals and plants. Reasons underlie such counterintuitive phenomenon as 1) the concept of prokaryotic species has not reached a consensus yet, 2) difficulties in describing prokaryotic phenotypes, 3) techniques cannot provide sufficient resolution on highly related microbial taxonomy, and 4) vast existences of HGT and genetic recombination obscure speciation results. Furthermore, microbiologists concede that the historical definition of species is anthropocentric and mostly for clinical practice purposes (Gevers et al., 2005). Despite that the facts that traditional naming system is self-explanatory and informative for pathogens (e.g., *Mycobacterium tuberculosis*, *Bacillus anthracis*, or *Neisseria gonorrhoeae*), it provides little information regarding to evolutilional or ecological roles of prokaryotes. Gevers and colleagues proposed to define prokaryotes with the
incorporation of complete genomes, MLSA results, and ecological roles in order to make prokaryotic taxonomy meaningful. Many microbiologists reach into the congruence that microbial systematics and taxonomy will be, if not have been, on the basis of genomic sequencing data with little inference from anthropocentricity (Gevers et al., 2005; Sentausa and Fournier, 2013; Ramasamy et al., 2014; Glaeser and Kampfer, 2015; Balint et al., 2016). Even though 16S sequencing cannot support the speciation of all the bacteria, it is the pioneer approach when it comes to unknown and unculturable ones. The combination of 16S and MLSA sequencing covers deficiencies for each other and poses as a promising strategy for prokaryotic taxonomy.

1.10. NGS and metagenomics

The term “metagenomics” refers to the study of microbial community on the basis of genomes from all the species existing in an environment. There are two main approaches one may study metagenomics by employing NGS, namely amplicon sequencing and shotgun metagenomics analysis (Sharpton, 2014; Tonge et al., 2014). NGS technologies such as Illumina, Ion Torrent, and PacBio are major sequencing platforms. The amplicon sequencing analysis is better referred as “metaprofiling” owing to the fact that it assesses microbial community on the basis of one genetic marker. (Escobar-Zepeda et al., 2015). The choices of a single marker include 16S, internal transcribed spacer (ITS), large subunit (LSU), etc. On the other hand, shotgun metagenomics analysis, is to study microbial community on the basis of the total genomes in an environment. The total genomes data is obtained via sequencing the whole shotgun libraries (Sharpton, 2014). In 2014, Tonge and colleagues used multiple amplicons (ITS1, ITS2, and LSU) fingerprinting to analyze two mock fungal communities containing 8 and
20 known identity and concentration, respectively. They obtained an average of 700,000 raw reads via Ion Torrent platform. USEARCH and nucleotide blast (BLASTn) were used for data mining and OTUs classification. They demonstrated that the community composition was faithfully represented via amplicons-based survey, the absolute numbers of species, however, were obscured on the basis of OTU classification. Furthermore, they stated that no single genetic marker was able to classify all the 28 species in the study, emphasizing the necessity of employing multiple markers to avoid distorting results. The regional-bias is one of the limitations of 16S sequencing survey, others including existing of multiple copy numbers, generation of chimeras, and lacks of information on microbial functions and metabolic interactions. As a result, shotgun metagenomics sequencing is an approach to circumvent shortcomings of 16S survey (Sharpton, 2014). Briefly, DNA is first isolated from an environment. The genomic DNA is then sheared into small fragments for sequencing. Sequencing reads are placed in their corresponding genomic locations. Genomic loci such as 16S are used for taxonomy, while functional loci such as protein coding sequences are used for functional and metabolic analysis (Escobar-Zepeda et al., 2015). Therefore, the metagenomic study answers not only the question “who are all there”, but also “what are they all doing” (Kunin et al., 2008; Meyer et al., 2008; Fuhrman, 2012). The microbial taxonomy can be determined in three ways in the metagenomic study, namely, marker genes analysis, grouping reads binning, and distinct genomes assembling (Fuhrman, 2012; Hanemaaijer et al., 2015). These three classification methods are not exclusive to each other, they are rather complementary approaches that can be used synergistically.

As for understanding microbial functions on the basis of genomic sequencing, protein-coding sequences are first aligned against database of genes, proteins, and metabolic pathways. The functional and metabolic information are then determined based on the comparison results to
the database (Consortium, 2012). There are generally two ways for functional annotation: using sequencing reads directly or assembling metagenome first. The metagenomic assembly is followed by gene prediction and annotation, which reveal microbial functions (Qin et al., 2010; Yandell and Ence, 2012). In 2010, Qin and colleagues performed metagenomic sequencing from faecal samples of 124 healthy European individuals to describe gut microbial communities. They obtained 576.7 gigabases of sequencing reads and assembled reads into 3.3 million non-redundant microbial genes. They reported that over 99% of genes in this study originated from bacteria and 1,000 to 1,150 prevalent bacteria found in the cohort. Consistent with previous studies, they found that the amount of Bacteroidetes and Firmicutes were the highest in communities (Wang et al., 2003; Eckburg et al., 2005). Moreover, they annotated the predicted genes by using databases including the non-redundant protein sequences in NCBI, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups (COG). Although they did not describe microbial functions in detail, they reported two types of functions on the basis of metagenomic data, namely, housekeeping functions that required by all the bacteria and functions that particularly needed by living in the GI tract. More importantly, they determined the minimal gut metagenome and gut bacterial genome by investigating functions among all the individuals and most of the bacteria, respectively. Besides genome reconstruction and functional annotation, metagenomics also provides pathway reconstruction, which leads to the understanding of metabolic interactions in microbial community (De Filippo et al., 2012).

In summary, microbiology embraces a new era under the influences of the “Woeseian revolution” and phylogenetics. NGS technologies and metagenomics analysis provide higher resolution and broader horizon for microbiologists to look into microbial world in a new perspective.
2.1. Methods of studying soil microbial community

Soil is one of the largest reservoirs on earth containing massive amount of biomass. Soil microbiome are arguably the group with the largest amount and variations; for instance, Faegri and colleagues found over $10^{10}$ bacterial cells in one gram of soil (Faegri et al., 1977). Similar to any other environmental microhabitats, most of soil microbes are difficult to cultivate, characterize, and classify in traditional ways; less than 1% of microorganisms observed under the microscope can be isolated (Torsvik and Ovreas, 2002). The microbial diversity and richness in soil, is vastly unknown. In 1980, Torsvik observed $1.1 \times 10^{10}$ and $2.2 \times 10^{10}$ cells/g of bacteria and isolated 90 and 187 µg/g bacterial DNA in two soil samples, respectively (Torsvik, 1980). The direct DNA isolation and purification from soil lead to soil molecular microbial ecology study, which is to investigate soil microbial community on the basis of DNA (Ogram, 2000).

Soil microbial ecology studies the richness, evenness, genetic variability, and functions of microbiome in soil (Torsvik and Ovreas, 2002). Microbial diversity describes the complexity of community composition, interactions among microbes, and trophic/metabolic pathways (Kozdroj and van Elsas, 2001). The diversity of soil bacterial community used to be evaluated either at the process level or on the basis of phenotypic description of isolated strains (Torsvik et al., 1990; Holben et al., 1998). The unavailability of culturing or identifying the most of soil bacteria (99.5-99.9%) clearly hinders bacterial ecology study. DNA isolated from soil, on the other hand, contains ca. 50-80% of bacterial genetic materials, making the study of microbial ecology via DNA a promising alternative (Faegri et al., 1977; Steffan and Atlas, 1988). In 1990, Torsvik and colleagues determined soil bacterial community structure on the basis of DNA heterogeneity (Torsvik et al., 1990). They first isolated and purified bacterial DNAs from soil. DNA
heterogeneity was measured via single-stranded DNA association using spectrophotometer. They found that bacterial DNAs in soil were mostly heterogeneous with over 4,000 completely different genomes. They concluded that the bacterial community in a deciduous-forest soil was highly diverse. More importantly, they developed a method to estimate bacterial community diversity by using DNA re-association.

2.1.1. GC content analysis

Besides DNA re-association, other genetic-based technologies are broadly used to describe soil bacterial community. The succession of guanine plus cytosine (GC) content, for instance, is applied as an indicator for community composition shifting (Holben and Harris, 1995). Holben and Harris developed an approach to study bacterial community composition by analyzing GC content of total bacterial DNA isolated from soil (Holben and Harris, 1995). They fractionated total bacterial DNA and measured the change of buoyant density in responding to the alteration of GC content. The density change was measured by the binding of bisbenzimidazole to DNA. The rationales for profiling bacterial community on the basis of GC content are first, although the general GC content in a bacterial community varies from 24% to 76%, the GC content within a taxonomic group differs less than 3-5% (Tiedje et al., 1999). Second, bisbenzimidazole is a non-intercalating DNA binding dye that preferentially binds to AT rich regions (Weisblum and Haenssler, 1974; Comings, 1975). Finally, the variations of DNA-dye binding result in distinguishable buoyant density changes (Jorgenson et al., 1978).

Altogether, the variations of GC content in chromosomal DNA correlate with different bacterial taxonomic groups; therefore, analyzing variations of GC content provides a coarse scale of resolution in determining bacterial community composition (Holben and Tiedje, 1988;
Nusslein and Tiedje, 1998). The advantages of GC analysis are 1) it uses total bacterial DNA without PCR amplification, thereby avoiding biases caused by PCR, 2) it provides quantitative bacterial community survey, and 3) it reveals groups in low abundance in the community. The disadvantages of GC analysis, however, also exist because 1) it requires a large amount of DNA (e.g., 50 µg), 2) it requires the instrument for ultra-centrifuging, and 3) this approach cannot profile bacterial community in a high resolution (i.e., at species or sub-species level) (Tiedje et al., 1999).

2.1.2. PCR-based methods

PCR-based community composition surveys, on the other hand, provide a higher resolution in profiling bacterial community. Techniques such as terminal restriction fragment length polymorphism (T-RFLP), denaturing and thermal gradient gel electrophoresis (DGGE and TGGE), and amplified ribosomal DNA restriction analysis (ARDRA) are all PCR-based methods for microbial community analysis on the basis of 16S rRNA amplicons (Holben et al., 1992; Muyzer et al., 1993; Moyer et al., 1994; Amann et al., 1995; Moyer et al., 1996). One of the common processes of techniques listed above is the amplification of 16S rRNA. The usage of 16S amplicons and rationales for each technique, however, are different. For instance, DGGE and TGGE survey the bacterial community on the basis of helix stability by using thermal gradient gel (Muyzer et al., 1993). DGGE and TGGE approaches are sensitive to structural variations, even to the alternation of single base. The disadvantages of these methods, however, are low in resolution. Furthermore, there is no database for global comparison of sequences to reveal relationships between thermal stability and bacterial taxonomy (Kowalchuk et al., 1998). Techniques like RFLP and ARDRA distinguish the polymorphisms of 16S length in the
community by using restriction digesting method (Pace et al., 1986; Vaneechoutte et al., 1992; Yang et al., 1997). RFLP and ARDRA are useful to sense changes occurring in the community, yet they provide little quantitate information when it comes to community survey (Smit et al., 1997). T-RFLP alternates the RFLP approach by fluorescently labeling the 5’ terminus and sequencing only the terminal fragments (Liu et al., 1997). Sequencing terminal fragments alone not only reduces the complexity for data processing, it also increases the sensitivity of base change detection (Osborn et al., 2000). More importantly, T-RFLP surveys bacterial community quantitatively and generates phylogenetic comparative results (Marsh, 1999). In 1997, Liu and colleagues were the first developing the T-RFLP protocol (Liu et al., 1997). Briefly, 16S amplicons were amplified from the total DNA. The 5’ terminus was fluorescently labeled by one of the two primers. PCR products were purified and then digested by at least two restriction enzymes generating 4 bp recognition sites. The digests were sequenced via ABI automated sequencer. Multiple restriction enzyme digestions ensures the complete digestion and increases the peak resolution of sequencing (Dunbar et al., 2001). Liu et al. used T-RFLP to differentiate all the bacterial strains in a mock bacterial community with expected community composition. They also used T-RFLP to survey bacterial community in activated sludge, bioreactor sludge, aquifer sand, and termite guts to present the broad application of T-RFLP among ecosystems. The advantages of T-RFLP are 1) it can process large amount of samples, 2) it produces high-throughput dataset, and 3) it rapidly generates results with comprehensive and comparable microbial community structures and elucidates phylogenetic relatedness within and between groups. The disadvantages of T-RFLP include 1) the inherent biases and limitations as a PCR-based fingerprinting approach, 2) the obscured results caused by 16S copy number variations,
and 3) limited detecting resolution; it cannot detect bacteria that are lower than 1% of the population (Polz and Cavanaugh, 1998; Prakash et al., 2014).

Studying microbial community at genetic level not only provides a comprehensive perspective of microorganisms in the environment regardless of cultivability, it also reveals interactions between community structure and function in microbial ecosystem (Gray and Head, 2001; Wu et al., 2001). In 2014, Krebs suggested to use alpha diversity to describe species diversity within a community; beta diversity to measure diversity between communities; and gamma diversity for total regional diversity (Krebs et al., 2014; Escobar-Zepeda et al., 2015). The full purposes of microbial community studies are to understand 1) the complete population census of existing species (diversity), 2) the total number of cells within each species (evenness), and 3) the physiological and functional interactions among microbes (Amann et al., 1995; Holben et al., 1998; Oliver et al., 2015). Furthermore, the concept of metagenomics study is to survey a microbial community on the basis of all the genomes collected in an environment (Bernstein et al., 1998). In addition to allowing a better understanding on microbial community structure, the application of metagenomics analysis leads a more comprehensive definition of species diversity (Escobar-Zepeda et al., 2015).

Most of the techniques discussed above take advantages of the Sanger sequencing. Sanger sequencing has been one of the greatest techniques that brought revolutions in many biological fields. NGS, by comparing, has even more attractive platforms that are low-cost, rapid, and high-throughput. Sanger sequencing generates longer read length (ca. 1000 bp) with higher read accuracy, yet lower in sequencing depth (i.e., insufficient amount of sequencing data). NGS, on the contrary, generates shorter reads with lower base calling accuracy, but
produces sufficient sequencing depth in a short amount of time to comprehensively survey the microbial community.

2.2. NGS and soil metagenomics

The term “soil metagenome” refers to all the microbial genomic DNA isolated from soil (Handelsman et al., 1998). In 1998, Handelsman and colleagues were the first to describe a method to clone bacterial DNA isolated from soil in bacterial artificial chromosome (BAC) and introduced the term “metagenomics”. Soil metagenomics focus on understanding microbial community composition, functions, and metabolic pathways on the basis of metagenomic sequences (Daniel, 2005). The metagenomic data used to be obtained via sequencing a vast number of soil DNA libraries, which are constructed via inserting soil metagenomic DNA fragments into vectors such as BAC, plasmid, and cosmid (Bernstein et al., 1998; Henne et al., 1999; Rondon et al., 2000). With the wide applications of NGS, metagenomic method evolves from cloning to direct high-throughput sequencing technologies. The direct high-throughput sequencing increases sequencing coverage of microbial community survey, reduces labors, and bypasses potential cloning-based biases. Metagenomics achieved by high-throughput sequencing are also referred as megagenomics to emphasize the considerably comprehensive genomic coverage (Handelsman, 2005). The advantages of metagenomics not only lie in the fact that it surveys the entire microbial community in an environment, more importantly, metagenomic approach circumvents biases introduced via PCR.

In 2005, Tringe and colleagues were the first to describe habitat-specific fingerprints by comparing metagenomes isolated from terrestrial and marine microbial communities (Tringe et al., 2005). In the study, they compared microbial (Bacteria, Archaea, and Eukaryota) community
compositions in one rich agricultural soil and three deep-sea “whale fall” carcasses. They found that microbial communities in soil were more diverse than the ones in the ocean. Furthermore, they predicted metabolic functions on the basis of metagenomes by using KEGG database. They found that ca. 50% of predicted proteins in all the samples were homologous to orthologous groups. They also assessed the habitat-specificities by grouping predicted proteins into four bins, namely orthologous group, operons that had correlations with metabolic pathways, higher order cellular processes obtained from KEGG, and broad functional predicted proteins from COG. They found features of environmental-specific genes were either related to functions (i.e., “life-style”) or interactions with surroundings. Tringe and colleagues made the first attempt to clone and sequence soil metagenomics in order to answer not only “Who is there?”, but also “What can it do?”.

2.3. NGS links phylogenetic groups to their functions

One of the challenges of microbial ecology is to understand the microbial community structure and its concomitant functions. Although metagenomics reveals capacities of metabolic and functional activities, genomic information does not distinguish expressed genes from unexpressed ones (Sorek and Cossart, 2010). The integration of metagenomics with other “omic” methods, such as metatranscriptomics, metaproteomics and meta-metabolomics, might shed the light on “Who is there?” and “What is it doing?”. (Tyson et al., 2004; Tringe et al., 2005; Morales and Holben, 2011). In 2006, Leininger and colleagues were the first sequencing cDNA directly by taking the advantage of NGS technologies (Leininger et al., 2006b). They obtained metatranscriptomic data via pyrosequencing from 12 soil samples collected in Europe. The abundance and copy number of the gene encoding ammonia monooxygenase (amoA) were
quantified in both archaeal and bacterial community. They found that \textit{amoA} was 3,000-fold more abundant in archaeal community than that in bacterial community; indicating that archaea, more specially crenarchaeota, posed to be the main microorganisms with ammonia-oxidizing activity in soil.

In 2004, Wilmes and Bond proposed the term “metaproteomics” referring to the entire protein complement extracted and purified from microbes in an environment in a snapshot (Wilmes and Bond, 2004). They used 2D polyacrylamide gel electrophoresis (2D-PAGE) and quadrupole time-of-flight mass spectrometry (Q-ToF MS) to characterize proteins isolated from microbial community in an active sludge. They identified proteins such as porin, acetyl coenzyme A acetyltransferase, and a protein belonged to ABC-type branched-chain amino acid transporter, were highly active in the community. Their study was the first presented proteomics in a complex microbial community. Metaproteomics is the approach to assess the real-time catalytic potentials of a microbial community.

2.4. Effects of soil structures, properties, and environmental conditions on microbial diversity

The composition of microbial community is subject to change under influences of soil structures, soil physicochemical properties, and other environmental conditions (Borneman and Triplett, 1997; Ranjard and Richaume, 2001; Sessitsch et al., 2001; Torsvik and Ovreas, 2002).

2.4.1. Soil structure

Soil structure refers to the arrangement of soil solid particles and their pore spaces into soil aggregates (Dumanski et al., 1979; Tisdall and Oades, 1982). Aggregates are often distinguished on the basis of size, namely macro- and micro-aggregates (> 250 µm and < 250
µm, respectively) (Edwards and Bremner, 1967). Although soil aggregation is affected by numerous biotic and abiotic factors, the focus of this review is mainly on the interactions between bacterial activity and aggregation. Bacteria preferably habitat in microaggregates, especially in fractions < 50 µm (Tisdall and Oades, 1982; Ranjard and Richaume, 2001). Microaggregates ranging from 2.5 µm to 9µm harbor the most abundant bacteria (Hattori, 1988). The microbial diversity and evenness, however, are lower in finer soil compared to those in coarser soil (Ranjard et al., 2000; Sessitsch et al., 2001; Treves et al., 2003). The lower diversity and evenness found in finer soil might because finer soil has higher homogeneity and pore connectivity as a microhabitat; therefore finer soil harbors more bacteria yet with lower diversity (Carson et al., 2010; Constancias et al., 2015). The coarser soil, on the other hand, contents larger pore spaces, thereby providing relatively insulated microhabitat for diverse yet minor bacteria to colonize (Chau et al., 2011).

In particular, Constancias et al. investigated succession of soil bacterial communities in response to geomorphologic, soil managements, and soil characteristic variables at 278 agricultural sites (Constancias et al., 2015). They surveyed bacterial community via 16S rRNA gene pyrosequencing method. They found that soil pH and texture were two of the most important factors affecting bacterial community, in terms of microbial biomass and richness. Specifically, the coarse scale mainly affected microbial richness and diversity; whereas fine scale affected the community evenness. That is, soil with finer texture harbored higher microbial abundance yet with lower diversity. Their results were consistent with previous studies (Chenu et al., 2001; Treves et al., 2003; Hanson et al., 2012).
2.4.2. Soil nutrient contents

Smit and colleagues drew a similar conclusion indicating correlations between soil nutrient contents and bacterial community composition (Smit et al., 2001). They collected four soil samples in Sep., Jan., May, and July to investigate seasonal succession of bacterial community. They used DGGE analysis to determine soil bacterial community composition. They found that the diversity was higher in Sep. and May, which might correlate with higher nutrient content. Bacterial community is subject to seasonal successions due to the fluctuations of temperature, water and nutrient availability, and vegetation community (Lauber et al., 2008b; Zolla et al., 2013; Zhang et al., 2016). Furthermore, they state soil with high nutrient content had r-selection effects on bacterial community, which is in favor of bacteria with high growth rates, such as α- and γ-proteobacteria. Whereas soil with low nutrient content had k-selection, in which Acidobacterium predominated. They also suggested that the ratio of Proteobacteria and Acidobacterium might serve as an indicator of the trophic level of soil. The ratio was low in soil with low SOM, and vice versa (McCaig et al., 2001; Smit et al., 2001). Smit et al. combined molecule- and cultivation-based approaches to survey bacterial community, making results more convincing. One of the shortcomings of their study, however, was the fact that they had only one soil sample to represent each season. The lack of replications hinders comprehensive assessment of microbial community changing patterns. Furthermore, limited statistical analysis methods could be performed due to no replication.

In 2017, Li and colleagues investigated fluvo-aquic soil treated with either no-, inorganic-, and organic plus inorganic (manure and straw)-fertilizer for 24 years (Li et al., 2017). They collected DNA from 33 soil samples and surveyed soil bacterial community on the basis of 16S
V4 region via Illumina MiSeq. One of the purposes in this study was to determine effects of fertilizers on soil properties and bacterial community. They found that the combination of inorganic- and organic-fertilizer significantly increased soil organic carbon (SOC) and total N (TN) contents compared to other fertilizers. As for soil bacterial community composition, they found that the community structures (measured by both weighted and unweighted UniFrac distance matrixes) were distinguishingly different under influences of different types of fertilizers. Overall, soil with higher SOC (organic plus inorganic treated soil) had higher richness in the bacterial community. This is because SOC serves as a selective filter enriching bacteria with high decomposition functions. Specifically, SOC promoted the predominance of both oligotrophic taxa (slow-growing bacteria) and copiotrophic taxa (fast-growing bacteria). Their results were in the agreement with previous report on soil bacterial ecological classification (Fierer et al., 2007). Furthermore, many of the taxa enriched by SOC have proficient organic decomposition capacities (Bauer et al., 2006; Rawat et al., 2012; Ichikawa et al., 2015).

2.4.3. Soil pH

Overall, soil pH is one of the most important determinants shaping microbiome, especially bacterial community. In 2009, Lauber and colleagues surveyed bacterial communities in 88 soil samples collected in North and South America via pyrosequencing (Lauber et al., 2009). They included a large amount of soil samples in order to provide a robust assessment of bacterial community structures and driving factors biogeographically. They found that the overall bacterial community structure was significantly affected by soil pH ($r = 0.79$). Bacteria such as Acidobacteria, Actinobacteria, and Bacteroidetes were sensitive to soil pH gradients. A number of studies indicated that soil pH had a strong association with microbial community
Soil pH has both direct and indirect effects on microbial community structure (Smit et al., 2001; Sefrova et al., 2010). Soil pH directly affects microbial community structure because of various pH sensitivities of microorganisms (Rosso et al., 1995). Most of bacteria have relatively narrow range of optimal pH (Fernandez-Calvino and Baath, 2010; Rousk et al., 2010b). The change of pH breaks the equilibrium between bacteria; the growths of some bacteria are surprised, while those of others are promoted. The indirect influences of soil pH refer to integrating relationships of soil to other soil properties such as SOM, water content, and salinity (Buckman and Brady, 1961). Soil pH might not shape bacterial community directly, but it determines community composition synergistically with other soil conditions (Fernandez-Calvino et al., 2011; Rousk et al., 2011). The direct and indirect effects of soil pH on bacteria explain different bacterial community compositions across soil pH gradients. Furthermore, soil pH can be used as an indicator for changes of soil bacterial communities.

2.4.4. Vegetation

There are two main agricultural management practices, namely monocultures and crop rotation. Venter et al. conducted a meta-analysis including 40 publications to study impact of vegetation on microbiome community (Venter et al., 2016). They concluded that overall, crop rotation resulted in higher richness and diversity of soil microbiome community compared to those in monoculture soil. The soil microbiome community is enriched not only because direct effects of vegetation diversity on rhizosphere during crop rotation, but also due to changes in soil physicochemical properties caused by vegetation diversity (Dias et al., 2015). In particular, crop rotation increases SOM content comprised by various plant litters and enhances soil structure
(Calegari et al., 2013; Suwara et al., 2016). The improved soil physicochemical properties promote both rhizosphere and bulk soil microbiome (Kennedy, 1999b; Zak et al., 2003). Some negative effects of crop rotation on soil microbiome, however, were also reported. It was mainly because of the rise in soil pH caused by wheat in rotation with either soybean or field pea (Yin et al., 2010; Reardon et al., 2014).

Many studies report that plant types have impact on soil microbial communities both in rhizosphere and bulk soil (Kent and Triplett, 2002; Costa et al., 2006). Because of specific root exudates, plants are selective to certain microbes, thereby shaping particular microbial (Haichar et al., 2008). Wen et al. investigated the microbial specificity to wheat, maize, and sunflower monoculture by sequencing 16S V1-V3 region vis pyrosequencing (Wen et al., 2016). They found that crop types had significant effects on bacterial community. Sunflower harbored bacterial community with the highest richness and diversity compared to that in maize and wheat. Crop types also significantly affected distributions of certain taxa, such as Bacteroidetes, Acidobacteria, Firmicutes, Planctomycetes, and Gemmatimonadetes. Specifically, Actinobacteria and Bacteroidetes predominated in wheat, Gammaproteobacteria and Pseudomonadales predominated in maize, and Alphaproteobacteria was the most abundant in sunflower.

A combination of large amount of environmental factors affect the heterogeneous distribution of bacteria in soils. Elucidating relationships between soil physicochemical properties and bacterial community composition is the first step to understanding soil microbial ecology. Understanding functions and effects of soil microbiome on soil quality and ecosystem is the next step.
3.1. Manure and agricultural soil

Animal manures are one of the major components of organic fertilizers. Other common organic fertilizers include composts and biosolids (Hole et al., 2005). Organic fertilizers serve as external low-inputs in agricultural soil aiming to increase soil productivity yet to decrease negative impacts in ecosystem (Gomiero et al., 2011). External low-input farming, opposing to intensive farming, features with the usage of large amount of manures, organic wastes, and crop residues; and restricts from the applications synthetic products, such as chemical fertilizers and pesticides (Heckman, 2006). Organic farming poses as a promising method of maximizing economic returns and ensuring the sustainability of environments (Liebman and Davis, 2000; Mäder et al., 2002). The general conclusion is that long-term (i.e. many years) organic fertilizers increase the soil carbon content, biomass, and biodiversity (Mader et al., 2002; Hole et al., 2005; Bunemann et al., 2006; Birkhofer et al., 2008). Although advantageous, the comprehensive understanding of organic fertilizers impacts, in terms of benefits, limitations, and challenges, are unknown for proper applications (Ebeling et al., 2012). Furthermore, effects of organic fertilizers on soil microbiome have not yet reached a consensus. It is because impacts of soil managements on microbiome are complex and case-, season-, and condition-dependent (Bunemann et al., 2006; Jangid et al., 2008; Lauber et al., 2008a).

3.2. Effects of manure on soil quality and microbiome

Soil microbiome are one of the key players to decompose soil organic matter (SOM); thereby possessing nutrient (C, N, P, and S) immobilization and mineralization functions in soil (Bunemann et al., 2006; Paul, 2014). Soil managements, in turn, vastly impact soil microbial diversity, evenness, and composition (Esperschuetz et al., 2007; Jangid et al., 2008; Chaudhry et
Moreover, because microbiome react to environmental and management conditions more rapidly and sensitively than soil properties do, microbial community composition better serves as an indicator of soil health, fertility, and plant health (Powlson, 1994). In 2003, Parham and colleagues evaluated soil microbial biomass and community composition under long-term cattle manure treatment (Parham et al., 2003). They collected soil samples from a long-term continuous winter wheat experimental plots in central Oklahoma, US. The plot was treated with cattle manure every four years since 1899. The last manure treatment was applied in 1999. They surveyed the culturable microbial community on the basis of total culturable microbial populations and r/K bacterial strategists. They found that the overall trend of the biomass of culturable bacteria in manure treated soil was 16 times higher than the one in control plot. The fungal population, however, was not significantly affected by manure. This might due to the fact that manure treatment did not dramatically alternate soil pH value. Additionally, fungi have a relatively broader living spectrum of soil pH (Frostegard et al., 1993; Arao, 1999; Rousk et al., 2010a). Furthermore, they reported that manure treatment proliferated both r- and K-strategists, which were fast- and slow- growing bacteria, respectively. The promoting effects of manure were due to large amounts of accessible organic C and other nutrients, and the enhancement of soil structure and aggregates stability. They also performed ARDRA using the almost full-length 16S sequences to analyze microbial community structure culture-independently. The DNA fingerprinting results further confirmed that long-term cattle manure application increased microbial richness and evenness in soil.

In 2008, Birkhofer and colleagues studied impacts of long-term organic farming on soil quality, productivity, and biomass in wheat plots (Birkhofer et al., 2008). They collected soil samples from two organic- and two inorganic-fertilizers treated plots for 30 years. They
conducted PLFA analysis to estimate the abundances of bacteria and fungi in soil. They measured soil microbial biomass C ($C_{mic}$) and N ($N_{mic}$) via chloroform-fumigation-extraction (CFE). Their results indicated that microbial abundances in organic treated soil were higher than those in inorganic treated plots. The amounts of $C_{mic}$ and $N_{mic}$ in organic systems pronouncedly exceeded those in inorganic treatments with 2.0- and 2.7-fold increases, respectively. It is established that long-term organic fertilizers application increases soil carbon content and promotes bacterial activities (Fließbach and Mäder, 2000; Fließbach et al., 2007). Indeed, similar results were found in Birkhofer’s study; soil organic carbon ($C_{org}$) and total soil nitrogen content ($N_{tot}$) were 18-19% higher in organic treatments than those in inorganic farming systems. Furthermore, they indicated that microbial biomass was 17-18% higher in organic system than that in inorganic treatments. They also found a positive association between mineral fertilizers and soil acidification. Acidified soil has a lower pH value and less of $C_{org}$ content (Mikha and Rice, 2004). Another possible explanation of lower $C_{org}$ found in inorganic treatment is that the long-term external mineral in-put decreases soil aggregate stability. The higher aggregate stability, particular in microaggregates, correlates to the higher $C_{org}$ content (Mader et al., 2002).

Birkhofer et al. concluded that long-term application of organic fertilizers improved soil quality, fostered microbial biomass and activity, and reversed soil nutrient.

Although impacts of soil management on microbial biomass are well established, the influences on soil microbial community at higher resolution are vastly unknown. With the advent of NGS, one can survey soil microbial community composition, identify key microbes, and better understand influences of various soil input managements (e.g., conventional-, organic-, and other farming system) on soil microbiome. In 2012, Chaundhry and colleagues compared bacterial community compositions under influences of inorganic- and organic- fertilizers by
using pyrosequencing technique (Chaudhry et al., 2012). They obtained soil samples in plots treated with inorganic- and organic- fertilizers over 16 years. DNA samples were isolated from soil, and V1-V2 region was amplified and sequenced. They found that total organic C content and microbial biomass in organic treated soil were significantly higher than those in inorganic treatment (2.7 times and 1.5 times higher, respectively). 16S sequencing results indicated that Proteobacteria, Bacteroidetes, and Gemmatimonadetes predominated in organic treated plot; whereas Actinobacteria were significantly abundant in inorganic treatment. Consistent with previous studies, they stated that the manure amendments increased total organic carbon content and promoted bacterial activities and diversity in soil.

In 2015, Hartmann and colleagues determined soil microbial diversity and microbiome community compositions impacted by different fertilizers over 21 years. They collected soil samples from low-input system, plots treated with either no- or organic- fertilizers (including rotted and composted); and high-input system, soil treated with conventional- (stacked manure and chemical fertilizer) and mineral-treatment (chemical fertilizer). Two different crop rotated in the study, namely winter wheat and grass-clover. They amplified and sequenced V1-V3 regions to survey bacterial community and ITS2 for fungal community. They found that farming systems and crop types affected microbial β-diversity significantly. Furthermore, crop types had larger impacts on fungal community than those on bacterial community. The microbial α-diversity in low-input systems was higher than those in high-input systems. They stated that all the five farming systems contained distinctly different microbial community structures. Manure fertilizers posed as the key driving factor by increasing richness, reducing dispersion, and shaping microbial community. Noticeably, the authors demonstrated a direct effect of manure amendments on soil microbiome. Because despite the large differences in nutrient inputs via
fertilizers, soil chemical properties did not differ distinguishingly. Soil microbial community compositions, on the other hand, were highly sensitive to soil managements. More specifically, Acidobacteria had the strongest responses to soil management; slow growing taxa belonging to Acidobacteria predominated in low accessible nutrient soil (treated with no- and inorganic-fertilizers). This result was consistent with previous studies indicating that Acidobacteria adapt well in less fertile or higher acid soil (Jones et al., 2009; Ward et al., 2009). Manure enriched taxa such as Bacillus, Clostridium, Epulopiscium, Paenibacillus, Solibacillus, Symbiobacterium, Tepidimicrobium, Thermobacillus and Ureibacillus that belong to Firmicutes. Many of these genera are highly efficient at decomposing organic matters from manure (Ryckeboer et al., 2003).

It is worth mentioning that identifying taxa responded significantly to a treatment is only the first step of understanding microbial ecology in agricultural management. The microbial ecological activities, lifestyles, and functions in farming systems are vastly unknown. The remaining challenges of elaborating microbial ecological roles might be addressed via metagenomic and metatranscriptomic approaches. Furthermore, it remains to be answered how manure shapes soil microbial community; either via introducing novel taxa and novel genetic materials, or serving as selective matrix for certain microbes. Additionally, little is known on how different types of manure amendments specifically shape microbial community.

3.3. Effects of various types of manure on soil microbiome

In 2006, Larkin and colleagues measured soil microbial activities in responding to dairy and swine manure amended soil in order to understand how different manures affected soil microbial community (Larkin et al., 2006). They collected soil samples treated with no-, dairy-,
and swine amendments in three different soil types. Soil microbial populations of bacteria and fungi were measured by soil dilution plating on agar media. The microbial utilization of sole carbon was determined by using BIOLOG GN2 plates. The substrate utilization (SU) of carbohydrates, carboxylic acids, amines and amides, amino acids, polymers, and miscellaneous compounds and community fatty acid profiles were measured for microbial activities. Soil bacterial community profiles were performed via whole-soil extractions of fatty acid methyl ester (FAME). They found that both soil type and manure amendment significantly affected bacterial and fungal population, composition, and SU patterns. Furthermore, they reported strong correlations between soil properties (e.g. texture, pH, nutrient, and cation exchange capacity) and microbial characteristics. For instance, bacterial population and activity were highly correlated with soil C content. The application of manure increases organic C input in soil, thereby promoting microbial activities in general (Gregorich et al., 1998). Not only Larkin et al. reported that manure amendments promoted population of gram-negative bacteria in soil, they further determined specific effects of dairy- and swine-manure on microbes by stating that dairy manure had higher impacts on SU and FAME parameters compared to swine manure. One of the possible explanations was that dairy manure contented higher C compared to swine manure (2.24 g/kg and 0.71 g/kg, respectively). The N level in both dairy- and swine-manure were the same. Moreover, dairy manure amendment associated with higher fungal/bacterial ratio by increasing unsaturated fatty acids in soil, which was consistent with previous studies (Bossio et al., 1998; Yao et al., 2000). Unsaturated fatty acids such as 16:1 ω5c, 17:1 ω7c, and 18:1 ω9c are essential components of arbuscular mycorrhizal (AM) and other soil fungi (Graham et al., 1995; Peacock et al., 2001).
In summary, besides confirming previous conclusion that manure amendments fostered soil microbial populations and activities, Larkin and colleagues determined specific effects of different manure types on soil microbes due to different nutrient contents and effects on soil properties. This information helps to explain and predict soil microbial community composition and potential functions in responding to different manure amendments. Although Larkin et al. provided useful information, other technologies are needed to survey microbial community in a more comprehensive way.

In 2009, Suzuki and colleagues used DGGE to profile soil microbial communities under influences of different types of manure amendments (Suzuki et al., 2009). They isolated DNA from soil samples treated with chemical-, rice husk and cow-, and swine-manure for eight years. 16S and 18S were amplified and used to survey bacterial and fungal community via DGGE analysis. They found that swine manure increased exchangeable Mg and Ca, soil pH, and P. Furthermore, they reported that bacterial community was sensitive to soil type rather than fertilizers; fungal community, on the other hand, was significantly under the influences of fertilizers. More specifically, swine manure promoted fungal population more than rice husk and cow manure. Four groups were identified as synergetic decomposers of external organic inputs. They suggested that organic amendments and outcomes could be predicted and monitored via fungal community composition. The fingerprinting technique they used for bacterial community profiling, however, underestimated microbial diversity due to existence of identical banding from closely related yet not the same taxa (Hamm et al., 2016b).

In 2016, Hamm and colleagues surveyed soil bacterial communities in no-, swine-, dairy-, and chemical-fertilizer treated soil via pyrosequencing, profiling microbial community in a
more accurate way with higher solution (Hamm et al., 2016b). They isolated DNA from soil samples, which were treated with granular urea N fertilizer, soil swine-, and dairy-manure. V1-V3 regions were amplified and sequenced for bacterial community survey. The goal of this study was to assess and compare bacterial richness, diversity, relative abundance, and community composition in responding to different types of external N inputs. Furthermore, they surveyed bacterial communities in manures to determine whether some bacteria transformed from manure to soil. They found that swine manure treated soil had the highest bacterial diversity, followed by dairy manure. Swine manure enriched Firmicutes; whereas dairy manure promoted populations of Bacteroidetes and Gemmatimonadetes. They found that Proteobacteria predominated in both swine- and dairy-manure, such results were consistent with previous study (Maeda et al., 2010). The abundances of Proteobacteria, however, were low (<0.4%) in chemical treated soils. Furthermore, genera Psychrobacter, which predominated in manures, absented in manure treated soils. The lack of taxa overlapping in manures and soils suggested that manure amendments serve as external inputs rather than bacterial inoculum. One of the possible reasons for limiting bacterial transformation from manures to soils is that manure originated bacteria were outnumbered by soil microbiome (Saison et al., 2006). Hamm and colleagues confirmed the conclusion that manure amendments elevated soil bacterial activities and populations compared to no- or chemical-fertilizer. They also specified impacts of swine- and dairy-manure on bacterial population. Furthermore, they demonstrated that manure amendments fostered soil bacterial activities and population by providing highly accessible nutrient, rather than importing manure bacteria to soil.
3.4. Manure effects N related soil microbiome

3.4.1. N fixing

Bacteria and Archaea that use N gas as the sole source of N are referred as diazotrophs (Bilal et al., 1990; Hurek et al., 1993). Most of N fixing microbiota have nitrogenase reductase (\textit{nifH}) (Kondorosi et al., 1973; Hageman and Burris, 1978; Mevarech et al., 1980). N fixing is an energy costing process, requiring microbiome to obtain energy via phototrophs, lithotrophs, and heterotrophs. N fixing diazotrophic prokaryotes are classified as free living-, associative-, and symbiotic-N fixing ones. Because of the extensive energy cost of N fixing, the free living phototrophic microbiome, such as Cyanobacteria have the strongest N fixing potentials. Additionally, Eckford et al. found that some free living heterotrophic N fixing microbiome had particular hydrocarbon degrading functions in fuel spill contaminated soil (Eckford et al., 2002).

Associative N fixing prokaryotes such as \textit{Azospirillum, Gluconacetobacter, and Burkholderia} obtain energy from plants, especially roots. Those diazotrophs, in turn, provide various N sources to plants and soil. The N fixing symbioses have two components: N fixing bacteria and photosynthetic host. The N fixing ability of symbioses are relatively high if no other limitations (Masson-Boivin et al., 2009). Many rhizobia belong to Alpha- and Beta-Proteobacteria (Sy et al., 2001). Rhizobiaceaea which belong to Alphaproteobacteria have five major genera that involve with N fixing, namely \textit{Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium,} and \textit{Ensifer}. Other genera such as \textit{Burkholderia} and \textit{Cupriavidus} that belong to Betaproteobacteria and \textit{Frankia} that belongs to Actinobacteria (Rivas et al., 2002; Chen et al., 2003; Elliott et al., 2007).
3.4.2. Nitrification

Soil microbiome are important players in the process of nitrification. Many studies reported the nitrification abilities of both Bacteria and Archaea in soil (Leininger et al., 2006a; Di et al., 2010; Verhamme et al., 2011b). Bacterial and archaeal nitrifiers are the ones with ammonia monooxygenase (amoA) (Könneke et al., 2005; Treusch et al., 2005). Leininger and colleagues first reported in 2006 that Archaea had competitive capacities of nitrification, even stronger than Bacteria specifically in niches with low ammonia substrate concentration (Leininger et al., 2006a). Later on, researchers demonstrated that ammonia-oxidizing bacteria (AOB) preferred to colonizing in niche with high ammonia substrate, while ammonia-oxidizing archaea (AOA) preferred to low ammonia substrate (Di et al., 2010). This is also partially explained by Verhamme et al. reporting that AOB community was more sensitive to ammonia concentration than AOA community (Verhamme et al., 2011a). The terrestrial AOB belong to Betaproteobacteria, with major genera including *Nitrosomonas* and *Nitrosospira* (Norton, 2011). Prosser and colleagues found that *Nitrosospira* and archaeal phylum Thaumarchaeota predominated in most soils (Prosser, 2011). Later in a review, Prosser et al. summarized that besides different ammonia affinity, soil pH and physicochemical properties also affected activities of bacteria and archaea, thereby impacting N cycling in soil (Prosser and Nicol, 2012).

3.4.3. Denitrification

Microbiome that have nitrogenase reductase (*NifH*), nitrous oxide reductase (*NosZ*), cytochrome cd₁-containing nitrite reductase (*NirS*), and Cu-containing nitrite reductase (*NirK*) involve in denitrification. Rosch et al. identified many taxa that belonged to Acidobacteria and Proteobacteria had denitrification activities in soil (Rösch et al., 2002). Furthermore, Yoshida et
al. reported that Burkholderiales and Rhodocyclales, which belonged to Betaproteobacteria, had essential denitrification functions (Yoshida et al., 2010). Furthermore, they indicated that denitrifiers with nirK are more efficient than those with nirS gene in rice paddy soil. Katsuyama et al. investigated denitrification activities in both wet and dry soil (Katsuyama et al., 2008). They found that although both nirK and nirS genes were detected in wet soil, only nirK was found in dry soil, suggesting water content was one of the driving factors of denitrification.

Other important factors impacting nitrification include soil pH, temperature, electrical conductivity, and other physicochemical properties (Stres et al., 2008; Attard et al., 2011; Dörsch et al., 2012; Herold et al., 2012). Soil conditions are found playing even more critical roles on controlling denitrification than gene abundance (Attard et al., 2011). For instance, Herold et al. found that pH affected potential denitrification rates of soil microbiome via alternating bacteria/fungi rates (Herold et al., 2012). Bacterial community and their denitrification activities were significantly affected by soil pH gradient, whereas fungi were not. As the results, changing of soil pH impacts denitrification rate in soil.

Changing the microbiome community composition has large impacts on N turnover rate in soil and around the global (Schimel and Weintraub, 2003; Ollivier et al., 2011). Bacterial community carries higher volumes of activities in N cycling compared to other microorganisms. For instance, bacteria had up to 54% of potential denitrification potential, whereas fungi had as high as 18% (Herold et al., 2012). It is well established that bacterial community is highly sensitive to pH gradient (Lauber et al., 2009; Rousk et al., 2009; Fernandez-Calvino et al., 2011). Changing in soil pH, therefore, has large impacts on soil N cycling via alternating bacterial community (Bolan et al., 1991; Nicol et al., 2008). Other changes of soil environments have various effects among bacterial, fungal, and archaeal community. For instance, SOM has
significant effects on fungal community, while soil temperature has large impacts on archaeal community (Cusack et al., 2011; Zinger et al., 2011). All the alternations among soil microbiome community result in substantial impacts in the N cycling and N balance globally (Ollivier et al., 2011; Philippot et al., 2013; Nunan et al., 2017). Specifically, Jung et al. used metagenomic and functional analyses methods to determine impacts of changes in soil bacterial community on soil ecosystem (Jung et al., 2016). They reported that as the diversity of microbial community decreased, a structure shift occurred as well. Actinobacteria predominated in the less diverse community, whereas Proteobacteria claimed the dominance in more diverse community. They further pinpointed that taxa belonged to Actinobacteria carried the most narG gene related to nitrate-reducing activities. The numbers of genes which were responsible for nutrient cycling declined along with reduced community structure. Specifically, they found genes (e.g. napA, nirK, norB, and nosZ) that involved with denitrification was significantly decreased in community with less diversity. The denitrification, therefore, was hampered in less diverse community. Altogether, they demonstrated that changes in community composition and certain key microbial players had large impacts on nutrient cycling in soil.

3.5. Phosphorus and soil microbiome

Phosphorus (P) is essential for lives in the forms of phosphate ions. Naturally, P is enriched in rocks and ocean sediments. P is released via rock weathering into soil. Anthropogenically, P enters soil and water via fertilizer inputs. The inorganic P in soil is taken up by plants and other soil fauna. The organic P, on the other hand, has to be first degraded by soil microbes. Soil microbiome mineralize P mainly in two ways: decomposing organic matter to release many nutrient including P and breaking down P using phosphatase enzyme (Uroz et al.,
Bunemann et al. demonstrated that hydrolyzing P directly was the main method of providing available P in soil (Bünemann et al., 2011).

Bacteria such as Proteobacteria, Firmicutes, and Planctomycetes carry important functions in P cycling (Moreno-Letelier et al., 2011; Lepleux et al., 2012; Pollet et al., 2014). One of the best studied bacteria as P solubilizers is *Bacillus megaterium* (phylum Firmicutes) (Wu et al., 2005). *B. megaterium* is commercially available applying in biofertilizers (Yao et al., 2006). Tan et al. studied relationship between microbiome with long-term (42 years) P treated soil (Tan et al., 2013). They also assessed the distribution of alkaline phosphomonoesterase (ALP) coding genes, namely *PhoA*, *PhoD*, and *PhoX* in soil bacterial community. They found that *PhoD* was the most distributed gene in soils. The ALP genes and the richness and diversity of microbial community increased along with higher P input in soil. They also found that *Pseudomonas fluorescens* was highly sensitive to P concentration in soil. They therefore concluded that external P input affected significantly on soil microbial community structure. The soil microbiome, in turn, had large impacts on P cycling. Mander et al. found that high P concentration in soil was selective to phosphate-solubilizing bacteria (PSB) such as Actinobacteria and Proteobacteria (e.g. Pseudomonadaceae and Moraxellaceae) (Mander et al., 2012). They identified a core community of PSB among three long-term fertilized soils. The core community contented 11 commonly shared phyla, which contributed to approximately half of the richness in the communities. The top four phyla of the core community included Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes. They family Pseudomonadaceae (Gamma-proteobacteria) predominated in P rich soil. Their results were consistent with previous study (Browne et al., 2009). Altogether, studies indicate the importance of certain bacteria involve with P and nutrient cycling and supplying for soil fauna.
Similar to N cycling, changes in microbiome and certain core microbes affect P cycling as well. Loss the core microbiome and particular microbes closely related to P cycling will result in P deficiency in soil, thereby impairing physiological activities in soil fauna. Wang et al. demonstrated the imbalanced microbiome not only disturbed P equilibrium, the homeostasis of C cycle was also broken (Wang et al., 2014). On the other hand, Anderson et al. reported that increased soil microbiome abundance had positive correlations with C, N, and P cycling (Anderson et al., 2011). They suggested that higher microbiome diversity promote nutrient cycling potentials.

The microbiome, in turn, is subject to changes coping with various soil conditions, such as pH, temperature, C and other nutrient accessibility, water content, and structure variables (Bünemann et al., 2004; Oberson and Joner, 2005; Achat et al., 2010). Specifically, the turnover rate of P in microbiome community increased in responding to extra C input in soil (Oehl et al., 2001). This might suggest that soil microbiome potentially compete P with plants in the short term. In the long-term, however, microbiome immobilize and preserve P for other soil fauna due to the rapid turnover rate of P in the microbiome community (Olander and Vitousek, 2004). Achat et al. further verified the preserve P for plant concept (Achat et al., 2010). Not only had they found that the P pool in soil microbiome increased along with higher concentration of SOM, they also demonstrated that the microbiome community with largest P pool had the fastest turnover rate, therefore playing important role in P cycling and supplying in soil. Altogether, microbiome is closely involved in P cycling. Changing microbiome affects the turnover rate of P, as well as other nutrient such as N and C in soil.
It is worth mentioning that effects of manure on soil microbiome have not yet reached a consensus. It is because impacts of soil managements on microbiome are complex and case-, season-, and condition-dependent (Bunemann et al., 2006; Jangid et al., 2008; Lauber et al., 2008a). For instance, various types of soil managements vastly impact soil microbial diversity, evenness, and composition (Esperschuetz et al., 2007; Jangid et al., 2008; Chaudhry et al., 2012). One has to consider and compare specific conditions to draw a fairly comprehensive conclusion on effects of fertilizers on soil microbiome.

In summary, soil microbiome and their community composition respond rapidly to external soil inputs and managements. Furthermore, soil microbial diversity has direct impacts on plant growth, competition, and nutrient uptake (Kennedy, 1999a; Kennedy et al., 2004). Altogether, soil microbiome community has crucial roles in soil quality, productivity and plant health.
CHAPTER II

LONG-TERM CHANGE IN SOIL MICROBIOME. I. SHAPED BY EIGHT YEARS OF SOLID BEEF MANURE AND SWINE EFFLUENT

Abstract

Bovine and swine manure are two of the most common organic amendments used in low-input agricultural practice. Many studies reveal change in soil microbiome under long-term effect of organic and inorganic fertilizer; few studies, however, characterize soil microbiome shaped by manure with different phase (e.g. solid vs. liquid) and animal origin (e.g. bovine vs. swine). In this study, we characterize soil microbiome under long-term (eight years) influence of solid beef manure (BM) and swine effluent (SE). Illumina HiSeq was used to deeply sequence 16S hypervariable region 4 (V4) for the bacterial community survey. Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), were assessed for soil fertility. Mantel test and Pearson correlation coefficient were used to reveal the overall and specific correlation of soil properties with microbiome and phylum, respectively. Our results indicate that soil microbiome are different under long-term impact of BM and SE amendment. We find that TC is the key driving factor shaping microbiome in BM treated soils; whereas K is the main determinant on SE treated soil microbiome. Phyla
Actinobacteria and Firmicutes were enriched in BM; while Gemmatimonadetes and Nitrospirae were responsive to SE amendment. This study characterizes soil microbiome impacted by long-term BM and SE amendment. Our observations fill the gap of knowledge on impact of manure on soil microbiome.

Introduction

Low-input agricultural management poses as a promising approach meeting needs of modern agriculture, which are to maintain both high productivity and sustainability in soils (Reeves, 1997; Tilman et al., 2002b; Tilman et al., 2011). Utilizing manure as an organic fertilizer is part of the low-input agricultural practice (Clark et al., 1998). It is well-established that manure increases soil fertility, prevents soil deterioration and acidification, and enriches biodiversity in soils (Bationo and Mokwunye, 1991; Edmeades, 2003; Diacono and Montemurro, 2010). Organic amendment enhances soil fertility mainly via increasing soil nutrient contents, adjusting soil pH, stabilizing soil structure, and promoting soil microbiome activities (Gerzabek et al., 1997; Motavalli and Miles, 2002). Soil microbiome are sensitive to soil physicochemical properties (Marschner et al., 2003b; Lauber et al., 2008b). Soil microbiome changes in responsive to alteration of soil physicochemical properties, such as pH, nutrient contents, and soil structures (Esperschutz et al., 2007; Lauber et al., 2008b; Chaparro et al., 2012; Zhang et al., 2016). The microbial community, in turn, affects the fertility and stability of soils, playing an important role on the sustainable agricultural management (Konopka, 2009; Bakker et al., 2012; Sharma et al., 2013; Bender et al., 2016).
Many studies report that long-term inorganic-, organic-, and no-fertilizing results in different soil properties and microbial communities (Parham et al., 2003; Sun et al., 2004; Bunemann et al., 2006; Esperschutz et al., 2007; Gong et al., 2009; Francioli et al., 2016). Copiotrophic bacteria, such as Firmicutes, Proteobacteria, and Zygomycota are abundant in soils with organic fertilizer (Francioli et al., 2016). Bacteria with high chitinase and phosphatase activities are enriched by inorganic fertilizer (Olander and Vitousek, 2000). Oligotrophic taxa, such as Acidobacteria predominate in soils without fertilizer (Shah et al., 2011; Rime et al., 2015). It is determined that manure shapes soil microbiome mainly via altering soil properties rather than directly inputting animal originated microbiome (Hamm et al., 2016a). Hamm et al. surveyed bacterial communities in manures and soils before and after fertilization. They found that the differences of microbiome in manures and soils were not significantly assimilated after fertilizing. Moreover, genus (e.g. *Psychrobacter*) which predominated in manures was not found in fertilized soils. Their results agree with previous studies concluding that manure microbiome had little direct impact on soil microbiome, because manure microbiome is outnumbered and less competitive due to unfavorable environmental conditions in soils (Unc and Goss, 2004; Sun et al., 2015).

Impact of inorganic- and organic-fertilizer on soil properties and microbiome were revealed in previous studies (Smith et al., 1997; Lehmann et al., 2003; Marschner et al., 2003a; Bengtsson et al., 2005; Ding et al., 2016). Few studies, however, further investigate the change in soil microbiome influenced by manures from various animal sources. Manures from different animals (e.g. swine, cattle, and chicken) have various nutrient composition, therefore, might lead to different soil properties and microbiome. Previous studies reveal that overall, various nutrient compositions from animal manures result in different soil microbiome (Albiach et al., 2000;
Gomez et al., 2006). Furthermore, different phases of manure fertilizer (e.g. solid, slurry, and composted) have various effects on soil properties, consequentially on soil microbiome. For instance, solid cattle manure treated soils have significantly higher soil organic carbon (SOC) and water stable aggregates than those of in pig slurry treated soils (Domingo-Olivé et al., 2016). Altogether, fertilization results in changes in soil properties. Soil properties, such as soil pH, total organic carbon, soil organic matter (SOM), and other nutrients (e.g. NPK), could be key driving factors shaping soil microbiome (Motavalli and Miles, 2002; Esperschutz et al., 2007; Lauber et al., 2009; Rousk et al., 2009; Borjesson et al., 2012; Siciliano et al., 2014; Kim et al., 2016).

Bovine and swine manure are two of the most common organic amendments. Recent studies reveal that bovine and swine manure shape different soil microbiome (Hamm et al., 2016a; Das et al., 2017a). Hamm et al. surveyed soil microbiome under influence of solid pig and dairy manures. They find that compared to solid dairy manure, solid pig manure results in higher soil microbiome diversity (Hamm et al., 2016a). Das et al. compared effect of composted cattle and swine manure on soil microbiome in submerged rice paddy. They find that composted cattle manure results in higher microbial richness and more copiotrophic bacteria. They further conclude that composted cattle manure better promot soil fertility and crop yield (Das et al., 2017a). Their studies shed lights on different effects of cattle and swine manure on soil properties and microbiome. Little is known, however, on impact of manure with different phases and animal origins (e.g. soild beef manure (BM) and swine effluent (SE)) on soil microbiome. Although there are many variables in BM and SE, these two fertilizer have been widely used in practice. Impact of BM and SE on soil properties, profitablility, and sustainability was evaluated in previous studies (Park et al., 2010; Turner et al., 2010). Little is known, however, on the influence of BM and SE on soil microbiome.
In this study, we characterize and compare soil microbiome under long-term (eight years) influence of BM and SE. We hypothesized that manure with different phase (solid vs. liquid) and animal origin (bovine vs. swine) had different effect on soil nutrient composition, thereby cascadingly shaping different soil microbiome. Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), were measured to assess soil fertility. Our objectives are 1) to characterize and reveal difference of soil bacterial communities shaped by long-term BM and SE fertilizer using Illumina HiSeq sequencing platform and 2) to determine specific soil property as key driving factor determining soil bacterial community.

Methods and materials

Site description

The long-term experiment was conducted from 1995 to 2007 at the Oklahoma Panhandle Research and Extension Center (OPREC) in Goodwell, Oklahoma (36°35 N, 101°37 W). The mean annual precipitation varied 341 to 488 mm during the study period. Detailed descriptions of soil characteristics were reported in previous studies (Park et al., 2010; Turner et al., 2010). Briefly, the study was conducted on Gruver soil series, which are the soil series of fine, mixed, super-active, and mesic Aridic Paleustoll, on approximate of 0-2% slope. The Gruver soils are types of calcareous soils that are rich in calcium phosphate (> 15%). The calcareous soil is prone to loss N coupled with increasing soil pH (Chesworth et al., 2008). The concentration of P is often low in calcareous soil (Hedley et al., 1982). The soil in this study is composed of 21% sand, 47% silt, and 33% clay, with desirable gas and water permutations.
Experimental design

The plots (4.6 m × 9 m) were randomized with three replicates for each fertilizer, namely BM and SE manure. Both BM and SE were calculated and applied at nitrogen rate (NR) of 168 N ha⁻¹ yr⁻¹. The NR of 168 N ha⁻¹ yr⁻¹ is the recommended level with desirable yield in Oklahoma (Zhang and Raun, 2006). Wheat (Triticum spp.) had been grown in the plots for several years before this study. Corn (Zea mays) had been grown in these plots since 1995. The corn planting data, cultivar selection, seeding rate, and other information were reported in previous study (Park et al., 2010). Given that vegetation history and crop rotation might affect soil microbial composition (Yin et al., 2009; Nielsen et al., 2010); soil microbiome was surveyed starting in 1999 to avoid influence of previous planting on microbiome.

Macronutrients (P and K) and micronutrients (Mg, Ca, S, Fe, and Mn) either met or exceeded plant requirements prior to the experiment. The level of N in soil (141 kg ha⁻¹), however, was below the recommended level (190 kg ha⁻¹) (Zhang and Raun, 2006). Soil pH was not adjusted because 1) soil pH is one of the major driving factors shaping soil microbiome (Lauber et al., 2009), and 2) adjusting pH would mislead the result of change in soil pH due to fertilizing (Heinze et al., 2010; Aula et al., 2016).

Nutrient content in manure was calculated and adjusted to the same N level. Oklahoma State University Soil Testing Lab standard procedures were used for nutrient measuring (Turner, 2004). Detailed nutrient content in BM and SE was reported in previous study (Tanganelli, 2011). Overall, the salinity, Na, Ca, Mg, K, TN, TC, Mn, S, B, Fe, Zn, and Cu in BM were higher than those of in SE. Only ammonia in BM was lower than that of in SE. Swine effluent was collected from a commercial nursery lagoon; while beef manure was collected from a local
feedlot. Detailed descriptions of manure application was reported in previous study (Park et al., 2010). Briefly, SE was applied via irrigation between 20 April and 11 June each year, which were after the seedling emergence; whereas BM was directly applied on soil surface between 21 February and 8 April, which were before the seedling emergence. Regular irrigation was performed after SE application.

Soil sample collection

Soil samples were collected using autoclaved stainless steel hydraulic probe (4.2 cm × 120 cm) prior to manure application in spring every year. Soil samples from five layers (20 cm per layer, 0-100 cm deep in total) were collected in each plot. Collected soil samples were passed through an autoclaved 2 mm stainless steel sieve, then stored at ambient temperature. A set of subsamples (2 g) from each layer (total of 10 g per subsample) were mixed via Large Capacity Mixer at 1,600 rpm for 30 min (Glas-Col, Terre Haute, IN, USA). The subsampling was performed in order to comprehensively present soil microbiome.

Soil properties measurements

Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), were measured to assess soil fertility. Detailed descriptions of soil properties measurements were reported in previous study (Turner et al., 2010). Briefly, soil pH was measured by using 0.01 M Calcium chloride (CaCl₂) (Schofield and Taylor, 1955). Dumas dry-combustion method was used to determine TC and TN (Bremner, 1996; Nelson and Sommers, 1996). Soil K, P, Mg, and Ca were measured via 2 M potassium chloride (KCl) and Mehlich III extracts (Mehlich, 1984; Mulvaney, 1996).
**Soil DNA isolation**

Soil samples in year 1999, 2000, 2003, 2005, and 2007 were used to present change in soil microbiome. Soil DNA was isolated by using the MoBio PowerSoil DNA extraction kit, following manufacturer’s instructions with modifications (MoBio Laboratories, Carlsbad, CA, USA). An additional incubation step was added for 10 min at 65 °C, followed by beads beating for 2 min to avoid DNA shearing (Lauber et al., 2009). DNA samples were purified by using a 0.8% (wt/vol) low melting point agarose gel, followed by agarose gel DNA purification using the gel purification kit (TaKaRa Bio USA, Inc., Mountain View, CA, USA). Purified DNA samples were quantified by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA samples were stored at -20 °C.

**PCR amplification and 16S V4 region sequencing**

DNA samples were sent to Novogene Co., Ltd (CA, USA) for PCR amplification and sequencing. Briefly, an aliquot of 50 ng DNA was used as template for PCR amplification. The 16S V4 region was amplified using primer set: 515F (5’-GTGCCAGCMGCCGCGGTAA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) with barcodes. PCR amplification was performed by using Phusion® High-Fidelity PCR Mater Mix (New England Biolabs, Ipswich, MA, USA). PCR products quantification and qualification were performed by mixing the same amount of 1X loading buffer that contained SYBR green with PCR products. Samples were loaded on 2% electrophoresis agarose gel for detection. Gel purification was performed by using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).
The sequencing library was generated by using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following manufacturer’s recommendations. Index codes were then added. The library quality was verified using the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina HiSeq2500 platform (Illumina, Inc., San Diego, CA, USA), 250bp paired-end raw reads were generated.

**Bioinformatic Analysis**

Mothur (v. 1.39.5) was used for data mining following mothur MiSeq standard operating procedure (Schloss et al., 2009). Briefly, paired-end reads were assembled and assigned to each sample based on their unique barcode using ‘make.contigs’ command. The command ‘screen.seqs’ was used to trim the data with the criteria of ‘maxambig = 0, maxhomop = 8, and maxlength = 275’. Contigs that were longer than 275 bp, or contained longer than 8 homopolymers, or contained undermined base were excluded. The trimmed sequences were further denoised by using commands ‘trim.seqs’, ‘pre.cluster’, and ‘chimera.uchime’. Uchime was used to remove chimeric sequences (Edgar et al., 2011). Sequences were then classified using the Bayesian classifier with the ‘classif.seqs’ command. Silva non-redundant v123 database was used as the reference (Quast et al., 2013). Archaea, chloroplasts, and mitochondria were excluded by using command ‘remove.lineage’ with ‘taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota’. Command ‘cluster.split’ was used to assign qualified sequences were into operational taxonomic units (OTUs) based on at least 97% similarity (OTU_{0.03}). All the sequences of OTU_{0.03} were classified into taxonomic groups at the bootstrap threshold of 80% by using command ‘classify.otu’. Random subsampling using the least amount of sequences in all
the samples was performed in order to avoid sequencing bias. The beta-diversity was calculated by using mothur with the command ‘dist.seqs’, ‘clearcut’, and ‘dist.shared’. The similarities among communities based on communities’ membership and relative abundance were measured by using unifrac-based matrix generated with command ‘unifrac.weighted’. Raw sequencing data was submitted to Sequence Read Archive (SRA) in NCBI, access number SRP123111.

Statistical analysis

Two-way Analyses of variance (Two-way ANOVA), uncorrected Fisher’s Least Significant Difference (LSD) test, and Pearson correlation analyses were performed by using R 3.3.2 statistical software (Team, 2013). Two-way ANOVA were used to determine significant effect of treatments and time on soil properties. Soil properties were considered significantly different to one another if the $P$ value $< 0.05$. Variables were considered significantly correlated if the $P$ value $< 0.05$.

Distance-based redundancy analysis (db-RDA) was performed by using vegan package in R (Oksanen et al., 2014). The db-RDA was used to perform the direct gradient analysis, which constrains and accumulates explanatory variables separate on the ordination exes (RDA1 and RDA2). In this study, db-RDA was employed to present distribution patterns of microbiome and to measure the amount of variations explained by soil properties variables. The two ordinations axes (RDA1 and RDA2) were constructed by constraining an entire set of explanatory variables, seven soil properties in this study. Mantel test at 999 permutations was performed by using vegan package in R (Oksanen et al., 2014). Mantel test analysis was used to evaluate impact of soil properties on soil microbiome. Impact of soil properties on microbiome was considered significant if $P < 0.05$. 

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Linear discriminant analysis (LDA) Effect Size (LEfSe) was performed to determine significant change of relative abundance in soil microbiome in responsive to manure (Segata et al., 2011). More specifically, the non-parametric factorial Kruskal-Wallis (KW) test was first used to detect microbiome with significant abundant differences between fertilizers ($P < 0.05$). The unpaired Wilcoxon test was used to compare the significant abundant differences of each taxa under the influence of manure ($P < 0.05$). Linear Discriminant Analysis was applied to calculate the effective size of abundant difference. The LAD scores were normalized by log10.

**Results**

*Changes in soil properties*

Soil pH, P, K, Mg, Ca, TN, and TC were measured in BM and SE fertilized soils over years. Two-way ANOVA was used to determine significant changes in soil properties over treatment and time (Supplementary Table S2.1). Changes in soil properties are shown in Figure 2.1. Overall, soil P content was significantly affected by treatments and time; whereas pH, Mg, and Ca only significantly changed over time. Soil K, TN, and TC contents were not significantly affected by either treatment or time. Beef manure significantly increased soil P compared to that of in swine effluent in almost all the years, with an exception in year 2000. Soil P content in BM fertilized soil increased over time; whereas P content in SE fertilized soil slightly increased over time and had a sharp decline in 2007. Most of soil properties fluctuated over time without obvious trends, with an exception of Mg, which significantly decreased over time. Soil pH and Ca significantly changed over time, with the pH value found the highest in 2005; whereas Ca was the highest in 2003 among all the years. Specifically, soil pH significantly decreased in both BM and SE fertilized soils in 2007 compared those of in 1999. Both BM and SE fertilized soils
in 2003 had Ca content significantly higher than that of in the rest of time points. The overall trends of TN and TC were not significantly different by either treatments or time. Total nitrogen and total carbon contents in BM fertilized soils in 2007, however, were significantly higher than those of in the rest of samples.
FIGURE 2.1. Changes in soil properties that are fertilized by beef manure (BM) and swine effluent (SE) over time. Soil properties, pH, phosphorus (P), potassium (K), magnesium (Mg),
calcium (Ca), total nitrogen (TN), and total carbon (TC), are measured. Year 1999 is in color red; 2000 is in color yellow; 2003 is in color lavender; 2005 is in color cyan; and 2007 is in color pink.

**Soil microbiome**

A total of 30 soil samples representing two treatments, three replicates, and five time points (year 1999, 2000, 2003, 2005, and 2007) were sequenced for 16S V4 region. A total of 2,294,735 paired-end raw reads were generated using Illumina HiSeq. An average of 76,491 ± 15,173 paired-end raw reads were obtained in this study. A total of 1,905,950 qualified reads were obtained after data trimming and subsampling, with an average of 63,531 ± 12,659 reads per sample. A total of 29 phyla were classified with all the qualified reads. All the reads were further categorized at class, order, family, and genus levels. Overall, 57 classes, 136 orders, 280 families, and 816 genera were classified among all the samples.

The changes in top four phyla (Actinobacteria, Firmicutes, Alphaproteobacteria, and Acidobacteria) over treatments and years are shown in Figure 2.2. Two-way ANOVA was used to assess change in phylum over fertilizer and time (Supplementary Table S2.2). Overall, all the trends were not significantly different over time. Uncorrected Fisher’s LSD test was used to assess significant change in phylum. Phylum Actinobacteria was more abundant in SE fertilized soils in 2000 than that of in several treatments (e.g. BM 1999, BM 2003, BM 2007, SE 1999, and SE 2005); whereas Firmicutes were more abundant in BM fertilized soils in 2003 than that of in other treatments (e.g. BM 2000, BM 2005, SE 1999, SE 2000, SE 2003, SE 2005, and SE 2007).
FIGURE 2.2. Changes in top four phyla that are influenced by beef manure (BM) and swine effluent (SE) over time. Year 1999 is in color red; 2000 is in color yellow; 2003 is in color lavender; 2005 is in color cyan; and 2007 is in color pink.

The trends of top four genera, Gaiella, Solirubrobacter, Paenibacillus, and Nitrospira, are shown in Figure 2.3. Two-way ANOVA was used to assess change in genus over fertilizer and time (Supplementary Table S2.3). Results indicated that only Nitrospira significantly changed over treatment. Uncorrected Fisher’s LSD test was used to assess significant change in genus. Specifically, SE significantly increased the amount of Nitrospira compared to that of in BM, with an exception in 2005. The abundance of Gaiella in SE fertilized soils in 2000 was significantly higher than that of in other treatments. The abundance of Paenibacillus in BM fertilized soils in 2003 was significantly higher than that of in all the SE fertilized soils.
FIGURE 2.3. Trends of top four genera that are influenced by beef manure (BM) and swine effluent (SE) over time. Year 1999 is in color red; 2000 is in color yellow; 2003 is in color lavender; 2005 is in color cyan; and 2007 is in color pink.

**Soil microbiome**

Shannon and Ace indices were calculated to show diversity and richness of microbiome community using mothur (Figure 2.4). Two-way ANOVA was used to assess significant changes over fertilizer and time. Overall, the diversity and richness were not significantly altered by either treatment or time.
FIGURE 2.4. Trends of diversity and richness that are influenced by beef manure (BM) and swine effluent (SE) over time. Year 1999 is in color red; 2000 is in color yellow; 2003 is in color lavender; 2005 is in color cyan; and 2007 is in color pink.
Influence of soil property on microbiome

The db-RDA was used to reveal distribution of soil microbiome (Figure 2.5). The distribution of soil microbiome was calculated using unifrac-weighted matrix. Soils fertilized with BM and SE were separated along RDA1 and RDA2. Mantel test at 999 permutations was used to calculate impact of soil properties on microbial community (Table 2.1 and 2.2). Overall, TC ($r^2 = 0.16$) was the main driving factor shaping BM fertilized soil microbiome; whereas K ($r^2 = 0.29$) was the key factor determining SE fertilized soil microbiome.

**FIGURE 2.5.** The distance-based redundancy analysis (db-RDA) diagram shows the relationship of soil properties with microbiome shaped by beef manure (BM) and swine effluent (SE). Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), are labeled in color black. Beef manure
fertilized soil microbiome is shown in shape square and in color orange; whereas SE fertilized microbiome is shown in shape triangle and in color blue.

**TABLE 2.1.** Mantel test is used to determine relationship of soil properties and microbiome treated with beef manure.

<table>
<thead>
<tr>
<th>Soil variables</th>
<th>$R^2$</th>
<th>$F$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC</td>
<td>0.17</td>
<td>2.60</td>
<td>0.03*</td>
</tr>
<tr>
<td>pH</td>
<td>0.05</td>
<td>0.79</td>
<td>0.48</td>
</tr>
<tr>
<td>Mg</td>
<td>0.05</td>
<td>0.82</td>
<td>0.42</td>
</tr>
<tr>
<td>TN</td>
<td>0.05</td>
<td>0.74</td>
<td>0.51</td>
</tr>
<tr>
<td>Ca</td>
<td>0.05</td>
<td>0.72</td>
<td>0.47</td>
</tr>
<tr>
<td>K</td>
<td>0.04</td>
<td>0.50</td>
<td>0.63</td>
</tr>
<tr>
<td>P</td>
<td>0.03</td>
<td>0.39</td>
<td>0.71</td>
</tr>
</tbody>
</table>

* , $P < 0.05$. Total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca).
**TABLE 2.2** Mantel test is used to determine relationship of soil properties and microbiome treated with swine effluent.

<table>
<thead>
<tr>
<th>Soil variables</th>
<th>$R^2$</th>
<th>F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>0.19</td>
<td>3.04</td>
<td>0.03*</td>
</tr>
<tr>
<td>Ca</td>
<td>0.10</td>
<td>1.69</td>
<td>0.18</td>
</tr>
<tr>
<td>Mg</td>
<td>0.07</td>
<td>1.20</td>
<td>0.30</td>
</tr>
<tr>
<td>$p$</td>
<td>0.06</td>
<td>0.94</td>
<td>0.30</td>
</tr>
<tr>
<td>TC</td>
<td>0.07</td>
<td>1.19</td>
<td>0.39</td>
</tr>
<tr>
<td>pH</td>
<td>0.02</td>
<td>0.36</td>
<td>0.86</td>
</tr>
<tr>
<td>TN</td>
<td>0.02</td>
<td>0.24</td>
<td>0.93</td>
</tr>
</tbody>
</table>

*, $P < 0.05$. Total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca).

Pearson correlation analysis was used to determine correlation between soil properties and top four phyla (Table 3). Phylum Actinobacteria in BM fertilized soils was significantly enriched by TC ($r^2 = 0.57$); whereas Actinobacteria in SE fertilized soils were negatively impacted by pH ($r^2 = -0.59$). Firmicutes were negatively correlated with TC ($r^2 = -0.44$) in BM fertilized soils; whereas Firmicutes were positively correlated with K in SE fertilized soils ($r^2 = 0.55$). Acidobacteria and Gammaproteobacteria in SE fertilized soils were significantly correlated with Mg ($r^2 = -0.49$), K ($r^2 = 0.45$), and Mg ($r^2 = 0.62$).
**TABLE 2.3.** Pearson Correlation analysis is used to determine relationship of soil property and phylum treated with beef manure (BM) and swine effluent (SE).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Treatment</th>
<th>pH</th>
<th>P</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
<th>TN</th>
<th>TC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actinobacteria</strong></td>
<td>BM</td>
<td>0.28</td>
<td>0.17</td>
<td>0.41</td>
<td>0.13</td>
<td>0.073</td>
<td>0.41</td>
<td>0.57*</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>-0.59*</td>
<td>-0.35</td>
<td>-0.34</td>
<td>0.11</td>
<td>0.2</td>
<td>-0.11</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>Firmicutes</strong></td>
<td>BM</td>
<td>-0.29</td>
<td>-0.13</td>
<td>-0.26</td>
<td>-0.084</td>
<td>0.06</td>
<td>-0.33</td>
<td>0.44*</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>-0.092</td>
<td>-0.13</td>
<td>0.55*</td>
<td>0.38</td>
<td>0.19</td>
<td>0.014</td>
<td>-0.0048</td>
</tr>
<tr>
<td><strong>Acidobacteria</strong></td>
<td>BM</td>
<td>0.048</td>
<td>0.35</td>
<td>0.13</td>
<td>-0.17</td>
<td>0.0084</td>
<td>0.3</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.11</td>
<td>0.36</td>
<td>-0.21</td>
<td>-0.49*</td>
<td>-0.32</td>
<td>0.39</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>Gammaproteobacteria</strong></td>
<td>BM</td>
<td>0.084</td>
<td>-0.42</td>
<td>-0.015</td>
<td>0.27</td>
<td>-0.12</td>
<td>-0.0019</td>
<td>-0.0003</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>0.37</td>
<td>0.092</td>
<td>0.45*</td>
<td>0.62**</td>
<td>0.079</td>
<td>-0.19</td>
<td>-0.13</td>
</tr>
</tbody>
</table>

*, $P < 0.05$; **, $P < 0.01$. Total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca).
The Lefse analysis was used to determine phyla that significantly responded to manure over time (Figure 2.6). Phyla Gemmatimonadetes and Nitrospirae significantly responded to SE. Genera selected by BM and SE over time are shown in Figure 2.7. Overall, BM enriched genera such as *Turicibacter* (Firmicutes), *Panacagrimonas* (Gammaproteobacteria), and *Acidicaldus* (Alphaproteobacteria) (in 1999); *Actinocorallia* (Actinobacteria) and *Paucisalibacillus* (Firmicutes) (in 2003); *Chryseolinea* (Bacteroidetes), *Gracilibacillus* (Firmicutes), *Flavobacterium* (Alphaproteobacteria), and *Tepidimicrobium* (Firmicutes) (in 2005); and *Melghirimyces* (Firmicutes), *Lentibacillus* (Firmicutes), and *Oceanobacillus* (Firmicutes) (in 2007). Swine effluent enriched *Kribbella* (Actinobacteria) and *Phenylobacterium* (Alphaproteobacteria) in 1999 and 2000, respectively.
The Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis identifies phyla significantly responding to beef manure (BM) and swine effluent (SE). Response is considered significant when $P < 0.05$, logarithmic LDA score $\geq 2.0$. 

**FIGURE 2.6.**
FIGURE 2.7. The Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis determines genera significantly responding to beef manure (BM) and swine effluent (SE). Response is considered significant when $P < 0.05$, logarithmic LDA score $≥ 2.0$. Swine effluent in year 1999 is in color brown; SE in year 2000 is in color red; BM in year 1999 is in color cyan; BM in year 2003 is in color green; BM in year 2005 is in color blue; and BM in year 2007 is in color purple.

Discussion

Beef manure and swine effluent have been widely used as organic fertilizer (Ye et al., 2005). Nutrient content in BM and SE is vastly different (Tanganelli, 2011). Previous studies focus on understanding impact of BM and SE on soil properties, nutrient availability, and productivity (Dayton et al., 2003; Park et al., 2007; Richards et al., 2011). Few study, however, aims to reveal effect of BM and SE on soil microbiome. This
study provides information to fill the gap of knowledge on effect of long-term solid beef manure and swine effluent on soil microbiome. This study also reports the impact of soil properties on soil microbiome and bacterial taxa, respectively.

In this study, we address the first aim by characterizing soil microbiome under influence of BM and SE fertilizer. To address the second aim, we find that TC is the main driving factor shaping soil microbiome in BM fertilized soils; whereas K is the key driving factor determining soil microbiome in SE fertilized soils. In BM fertilized soils, TC significantly enriched Actinobacteria and Firmicutes. In SE fertilized soils, K had significant correlation with Firmicutes and Gammaproteobacteria; whereas Mg had significant correlation with Acidobacteria and Gammaproteobacteria.

Influences of beef manure and swine effluent on soil property

Both BM and SE effectively decreased soil pH over time. Change in soil pH impacted by BM and SE, however, were not significantly different. Our results agree with previous studies concluding that manure fertilizer generally prevents the acidification and corrects pH in alkaline soils (Hue, 1992; Whalen et al., 2000; Hansen et al., 2004; Pizzeghello et al., 2011). Total carbon and total nitrogen contents significantly increased in BM fertilized soils in 2007, suggesting an enriched and accumulative effects of BM on nutrients over time. Total carbon in SE fertilized soils, however, had almost no change over time; whereas TN showed a slightly decreasing trend over time. These results indicate that manure is an ideal type of fertilizer in calcareous soil because organic acids in manure reversely chelate to ions, slowly release nutrients, and maintain a steady nutrient level in soils (Mathers and Stewart, 1984; Chang et al., 1991).
In this study, soil P showed the most distinguishable difference caused by BM and SE. Soil P in BM fertilized soils significantly increased over time; whereas soil P in SE significantly declined in 2007. The increased soil P content is not only due to the direct input of P from manure to soil; it is also because manure fertilized soils have less affinity to P; therefore, more P is available in soils. The decreased affinity of soil to P is caused by Al and Fe exchanges and competition of organic acids from manure (Iyamuremye et al., 1996; Haynes and Mokolobate, 2001). Furthermore, manure increases soil P availability by slowly releasing the inorganic P and consequentially reducing concentration of P in runoff (Mohanty et al., 2006; Halajnia et al., 2009). Although manure has some favorable features of enhancing P accessibility in soils, some disadvantages of manure application are also worth discussing. Specifically, higher content of water-extractable P (WEP) and inorganic P in manure might induce higher dissolved P in runoff (Pote et al., 1996; Kleinman et al., 2005; Vadas et al., 2005). High P in runoff potentially causes P pollution in water, resulting in eutrophication, harmful algal blooms, water contamination, and other environmental concerns (Sharpley et al., 1994).

Manure amendment in the form of slurry and effluent has higher nutrient runoff potential compared to that of in solid manure (Withers et al., 2001; Jarvie et al., 2006). Swine manure has higher WEP than there is in beef manure. Swine manure in effluent form that used in this study, might result in higher P runoff. The high levels P in SE and high P in runoff might explain the dramatic decline of soil P in SE fertilized soils in 2007. Overall, BM in solid phase poses to be a better organic fertilizer in this study.

Even though many studies indicate that soil pH is one of the most important determinants on soil microbiome, soil pH in this study did not show a significant
influence on soil microbiome. This could be due to previous studies utilizing samples derived from large geographical distribution and with significant pH variation (Lauber et al., 2009; Rousk et al., 2009). Soil pH, therefore, was considered the most important driving factor shaping soil microbiome in previous studies. In this study, however, soil pH changed in a relatively narrowed range and was not significantly affected by manure. On the contrary, other nutrients, such as P, TN, and TC had wide changing ranges and were significantly altered by manure. This might explain certain soil nutrients, rather than soil pH, had large impact on soil microbiome in this study.

_Total carbon is the main factor shaping beef manure fertilized soil microbiome_

Phyla Actinobacteria and Firmicutes were two of the most abundant taxa in manure fertilized soils. Mantel test indicated that TC was the main driving factor shaping BM fertilized soil microbiome. Correlation coefficient analysis further revealed a positive relationship between TC and Actinobacteria/Firmicutes in BM fertilized soils. Results suggested that BM increased TC in soil and consequently enriched Actinobacteria and Firmicutes. Our results are consistent with numerous previous studies revealing that copiotrophs, such as many taxa that belong to Actinobacteria and Firmicutes predominate in nutrient rich environments, with high enzyme activities of degrading complex carbon materials (Cleveland et al., 2007; Fierer et al., 2007; Větrovský et al., 2014; Ventorino et al., 2016). The correlation between TC and Actinobacteria ($r^2 = 0.57$) suggests a contribution of Actinobacteria to nutrient availability in soils. Indeed, many taxa that belong to Actinobacteria have high cellulose, lignin, and other polysaccharides.
decomposition potentials (Větrovský et al., 2014; Lladó et al., 2016; Taschen et al., 2017).

Seven genera (*Turicibacter, Paucisalibacillus, Gracilibacillus, Tepidimicrobium, Melghirimyces, Lentibacillus, and Oceanobacillus*) that belong to phylum Firmicutes were significantly enriched in BM fertilized soils over time. Interestingly, previous studies also report that genera *Turicibacter* and *Melghirimyces* are abundant in cattle manure fertilized soils, where TC content is generally high, suggesting high microbiome respiration and soil nutrient decomposition rates (Cleveland et al., 2007; Chroňáková et al., 2015). Genera *Gracilibacillus, Oceanobacillus, and Lentibacillus* have some species (e.g. *Gracilibacillus ureilyticus* sp., *Gracilibacillus halophilus* sp., *Gracilibacillus boraciitolerans* sp., *Oceanobacillus damuensis* sp., *Oceanobacillus rekensis* sp., and *Lentibacillus salis* sp.), which are halotolerant bacteria isolated from saline-alkaline soils (Ahmed et al., 2007; Chen et al., 2008; Huo et al., 2010; Long et al., 2015). Although soil salinity was not reported in this study, it is revealed in a previous study with the same experimental design, that long-term cattle manure application has cumulative effect on increasing soil salinity (Turner et al., 2010). The predominance of taxa discussed above might be due to the high level of TC and salinity caused by long-term BM fertilization.

Soil potassium mainly shapes SE fertilized soil microbiome

Mantel test indicated that soil K was the key driving factor shaping SE fertilized soil microbiome. Potassium is the third most essential macronutrient in soils. Although K content was not significantly affected by either manure or time in this study, the concentrations of soluble K in soils are generally low (Parmar and Sindhu, 2013).
Correlation analysis indicated that K had positive relationships with Firmicutes and Gammaproteobacteria. Several genera that belong to Firmicutes and Gammaproteobacteria, such as *Bacillus* and *Azotobacter* are potassium solubilizing bacteria (KSB), which have high K solubilizing abilities and predominate in K rich environments (Sugumaran and Janarthanam, 2007; Basak and Biswas, 2010). Our results suggest that phyla Firmicutes and Gammaproteobacteria might contribute to releasing and maintaining stable K level in soils.

Moreover, the negative correlation of Mg with Acidobacteria found in this study is consistent to previous reports (Pessoa-Filho et al., 2015; Chrungu et al., 2017). The negative correlation to Mg also exists in Acidobacteria subdivision 1; whereas subdivision 4 and 6 have strong positive response to Mg (Pessoa-Filho et al., 2015; Kielak et al., 2016). Magnesium is broadly needed by microbiome for essential activities such as enzymes, DNA, and APT synthesizing (Navarrete et al., 2013). Magnesium is also required in trehalase activity in microorganisms. Trehalase is an enzyme, which requires both inorganic P and Mg to hydrolyze trehalose to glucose (Chrungu et al., 2017). Trehalose hydrolysation is an important physiological function in microorganisms involving with germination and resumption of growing in microbiome (Gélinas et al., 1989). Moreover, recent studies reveal that trehalase are involved in microbial protection mechanisms from heat, toxin, and other stresses (Benaroudj et al., 2001; Jain and Roy, 2009). Although Mg is an essential element, it cannot be directly up-taken by microbiome due to the impermeability of cell membrane. The Mg transporter is required to transfer Mg from soil to microbiome (Beyenbach, 1990). Species in Acidobacteria group, such as *Acidobacterium capsulatum*, can produce trehalase and obtains Mg
transporter (Inagaki et al., 2001; Shin et al., 2014). The correlation between Mg and Acidobacteria found in this and other studies might indicate some fundamental functions and effects Mg has in soil microbiome. More information is needed to shed light on how Mg shapes soil microbiome, especially on Acidobacteria.

In this study, we find that Gemmatimonadetes and Nitrospirae have significant response to SE fertilization. Our result confirms previous studies indicating a positive selection of swine manure on Gemmatimonadetes and Nitrospirae (Das et al., 2017b). Previous studies report that both Gemmatimonadetes and Nitrospirae have correlations with SOM and N contents in soils (Nemergut et al., 2008; Chen et al., 2016). Gemmatimonadetes are abundant in soils, physiological functions of Gemmatimonadetes, however, are not well-understood. Studies indicat that Gemmatimonadetes are sensitive to soil moisture and N-amendment (Nemergut et al., 2008; Cederlund et al., 2014). Nitrospirae are more abundant in fertilized soils than those of in unfertilized soils (Galantini and Rosell, 2006; Carbonetto et al., 2014). The relatively large amount of Nitrospirae found in SE fertilized soils suggests that SE enriches copiotrophic microorganisms, which have high potentials of degrading organic matter and processing nitrogen (Maixner et al., 2006; Attard et al., 2010). Genus Nitrospira was also significantly enriched in SE treated soils. Nitrospira are nitrite-oxidizing bacteria (NOB) that play important role in nitrification and N cycling. The other important bacteria in the same phylum are Nitrobacter-like NOB (Wagner et al., 1996). Study reveals that Nitrospira-like NOB are generally more abundant than Nitrobacter-like NOB and the former had higher substrate affinity (Koops and Pommerening-Röser, 2001). Furthermore, a recent study suggests that unlike Nitrobacter-like NOB, Nitrospira-like
NOB are more active when nitrite oxidation activity is low in soils (Attard et al., 2010). The high abundance and substrate affinity of *Nitrospira* might explain the significant positive selection of SE on *Nitrospira*.

**Conclusion**

This study characterizes soil microbiome influences of BM and SE fertilizer. Our results indicate that TC is the major driving factor shaping BM fertilized soil microbiome; whereas K is the key determinant on SE fertilized soil microbiome. Some other soil properties, such as Mg, K, and pH have correlations with specific phyla in soils. Actinobacteria and Firmicutes show predominance in BM fertilized soils; whereas Gemmatimonadetes and Nitrospirae are significantly enriched in SE fertilized soil. Genus *Nitrospira* is significantly responsive to SE, suggesting higher nitrification activities in SE fertilized soils. This study fills the gap of knowledge on soil microbiome shaped by manure amendment with different phase and animal origin, providing information on effect of manure application on soil microbiome.
CHAPTER III

LONG-TERM CHANGE IN SOIL MICROBIOME. II: AFFECTED BY EIGHT YEARS OF CONTINUOUS CULTIVATION

Abstract

Long-term intensive cropping without soil amendment tends to exploit soil nutrients, worsen soil quality, and disturb ecosystem. Many studies establish impact of intensive cultivation on soil physicochemical properties and productivity; few studies, however, focus on understanding effect of continuous farming on soil microbiome. Microbiome ubiquitously exist in soils and vastly affect soil fertility. This study reveals change in soil microbiome under influence of eight years intensive corn (*Zea mays*) farming with no fertilizer. Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), were measured for soil fertility. Illumina HiSeq platform was used to characterize soil bacterial community by deeply sequencing 16S hypervariable region 4 (V4). Mantel test was used to determine soil property as the main driving factor in soil microbiome. We find that soil nutrient content slightly decline over time. There is no obvious temporal change observed in soil microbiome. Soil K is the key driving factor shaping microbiome. A core microbiome of Actinobacteria, Proteobacteria, Firmicutes, Acidobacteria, Chloroflexi, Gemmatimonadetes, and Planctomycetes, is identified in this study. Many of the core microbiome (e.g. Actinobacteria, Proteobacteria, Firmicutes, and Chloroflexi) are
copiotrophic bacteria playing important roles in nutrients cycling in soils. Overall, this study suggests that the core microbiome remains stable in eight years intensively cultivated soil. Even though soil nutrients slightly decreased, soil productivity and microbiome were not significantly affected by long-term intensive farming. Our results indicate essential nutrients cycling activities and potential contribution to soil nutrients from microbiome. This study fills the gap of knowledge on impact of intensive cropping without nutrient replenishing on soil microbiome.

**Introduction**

One of the fundamental goals in ecology is to understand spatiotemporal changes in communities (Preston, 1960). Studying changes in communities contribute to better understand functions and interactions of communities with environments. Furthermore, characterizing communities under various external influences reveal how communities respond to disturbances (Fraterrigo and Rusak, 2008). This information might be used to predict community compositions in the future. The spatiotemporal dynamics in macro-organisms, such as animal and plant communities are relatively well-studied (Stone and Ezrati, 1996; Pyšek and Hulme, 2009; Pinter-Wollman et al., 2013). Temporal change in territorial microbiome, however, remains to be discovered.

Although microorganisms widely exist, constantly interact with other organisms, and play important roles in the ecosystem; microbes have been historically referred to “the unseen majority” due to paucity of methods to study microbiome (Van Der Heijden et al., 2008). Consequentially, little is known about temporal change in microbiome compared to macro-organism communities. With advances of phylogenetic research and high-
throughput parallel sequencing platforms, numerous territorial microbiome have been studied (Rappé and Giovannoni, 2003; Wardle et al., 2004; Roesch et al., 2007). Impact of soil microbiome on soil fertility, plant health, and global ecosystem have been widely recognized (Karlen et al., 1997; Chaparro et al., 2012). An increasing number of studies reveal long-term change in soil microbiome impacted by external input in agricultural soils, such as inorganic, organic, and other amendments (Chinnadurai et al., 2014; Hartmann et al., 2015; Crews et al., 2016; Zhao et al., 2016). Overall, soil microbiome is more diverse and enriched in amended soils. Many copiotrophic bacteria that belong to Actinobacteria, Firmicutes, and Proteobacteria are sensitive to fertilizers; whereas oligotrophic taxa that belong to Acidobacteria are likely to be enriched in nutrients limiting soils (Kielak et al., 2009; Ding et al., 2013; Lladó and Baldrian, 2017).

Many studies focus on characterizing and comparing temporal change in soil microbiome shaped by various types of fertilizer (e.g. inorganic- and organic-fertilizer); few studies, however, reveal changes in soil microbiome resulted from long-term intensive cropping without soil amendment. Intensive cropping tends to exploit soil nutrients and decrease soil stably (Haynes and Naidu, 1998; Havlin et al., 2005). As a result, soil microbiome might change in responsive to intensive farming. It is important to know impact of long-term continuous farming on soil microbiome. The information might help to better understand influence of intensive cropping on the ecosystem.

In this study, we characterize long-term (eight years) change in soil microbiome under influence of continuous corn cropping with no amendment in agricultural soils. Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K),
phosphorus (P), magnesium (Mg), and calcium (Ca), were measured for soil fertility. Assuming nutrients availabilities would decline over time when no amendment was applied in soils, we hypothesized that soil microbiome changed in responsive to decreased soil nutrients. Our goals were to 1) characterize temporal change in soil microbiome shaped by annual cropping without fertilizer for eight years; 2) to identify core microbiome along temporal gradient; and 3) to determine soil property as the key driving factor shaping soil microbiome.

**Methods and materials**

*Site description*

The long-term experiment was conducted from 1995 to 2007 at the Oklahoma Panhandle Research and Extension Center (OPREC) in Goodwell, Oklahoma (36º35 N, 101º37 W). The mean annual precipitation varied 341 to 488 mm during the study period. Detailed experimental design was described in previous studies (Park et al., 2010; Turner et al., 2010). Briefly, the soil type in this study was Gruver soil, which is classified as soil with fine, mixed, super-active, and mesic Aridic Paleustoll, on approximate of 0-2% slope. The Gruver soil is a type of calcareous soil that is rich in calcium phosphate (> 15%). The calcareous soil is prone to loss N coupled with increasing soil pH (Chesworth et al., 2008). The concentration of P is often low in calcareous soil (Hedley et al., 1982). The soil is composed of 21% sand, 47% silt, and 33% clay, with desirable gas and water permutations.
*Experimental design*

The plots (4.6 m × 9 m) were randomized with three replicates without soil amendment. Wheat (*Triticum* spp.) had been grown in the plots for several years prior to this study. Corn (*Zea mays*) had been grown in these plots since 1995. The corn planting data, cultivar selection, seeding rate, and other information were previously reported (Park et al., 2010). Given that the vegetation history and crop rotation might affect soil microbiome (Yin et al., 2009; Nielsen et al., 2010); soil microbiome were surveyed starting in 1999 to avoid influence of previous planting on soil microbiome.

Macronutrients (P and K) and micronutrients (Mg, Ca, S, Fe, and Mn) either met or exceeded plant requirements prior to the experiment. The level of N in soil (141 kg ha\(^{-1}\)), however, was below the recommended level (190 kg ha\(^{-1}\)) (Zhang and Raun, 2006). Soil pH was not adjusted because soil pH might affect soil microbiome (Lauber et al., 2009).

*Soil sample collection*

Soil samples were collected using an autoclaved stainless steel hydraulic probe (4.2 cm × 120 cm) in spring every year. Soil samples from five layers (20 cm per layer) were collected in each plot. Collected soil samples were passed through an autoclaved 2 mm stainless steel sieve, then stored at ambient temperature. A set of subsamples (2 g) from each layer (total of 10 g per subsample) were mixed via Large Capacity Mixer at 1,600 rpm for 30 min (Glas-Col, Terre Haute, IN, USA). The subsampling was performed in order to comprehensively present soil microbiome.
Soil properties measurements

Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), were measured to assess soil fertility. Detailed descriptions of soil properties measurements were reported in previous study (Turner et al., 2010). Briefly, soil pH was measured by using 0.01 M Calcium chloride (CaCl₂) (Schofield and Taylor, 1955). Dumas dry-combustion method was used to determine TC and TN (Bremner, 1996; Nelson and Sommers, 1996). Soil K, P, Mg, and Ca were measured via 2 M potassium chloride (KCl) and Mehlich III extracts (Mehlich, 1984; Mulvaney, 1996).

Soil DNA isolation

Soil samples in year 1999, 2000, 2003, 2005, and 2007 were used to present temporal change in soil microbiome. DNA was isolated by using the MoBio PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA), following manufacturer’s instructions with modifications. An additional incubation step was added for 10 min at 65 °C, followed by bead beating for 2 min to avoid DNA shearing (Lauber et al., 2009). DNA samples were purified by using a 0.8% (wt/vol) low melting point agarose gel, followed by agarose gel DNA purification using the gel purification kit (TaKaRa Bio USA, Inc., Mountain View, CA, USA). Purified DNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). DNA samples were stored at -20 °C.
**PCR amplification and 16S V4 region sequencing**

DNA samples were sent to Novogene Co., Ltd (CA, USA) for PCR amplification and sequencing. Briefly, an aliquot of 50 ng DNA was used as template for PCR amplification. The 16S V4 region was amplified using primer set: 515F (5’-GTGCCAGCMGCGCGGTAA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’) with barcodes. PCR amplification was performed by using Phusion® High-Fidelity PCR Mater Mix (New England Biolabs, Ipswich, MA, USA). PCR products quantification and qualification were performed by mixing the same amount of 1X loading buffer that contained SYBR green with PCR products. Samples were loaded on 2% electrophoresis agarose gel for detection. Gel purification was performed by using Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany).

The sequencing library was generated by using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following manufacturer's recommendations. Index codes were then added. The library quality was verified using the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina HiSeq2500 platform (Illumina, Inc., San Diego, CA, USA), 250bp paired-end raw reads were generated.

**Bioinformatic Analysis**

Mothur (v. 1.39.5) was used for data mining following mothur MiSeq standard operating procedure (Schloss et al., 2009). Briefly, paired-end reads were assembled and assigned to each sample based on their unique barcode using ‘make.contigs’ command.
Sequences of each samples were then truncated by removing the barcode and primer sequences during the process. The command ‘screen.seqs’ was used to trim the data with the criteria of ‘maxambig = 0, maxhomop = 8, and maxlength = 275’. Contigs that were longer than 275 bp, or contained longer than 8 homopolymers, or contained undermined base were excluded. The trimmed sequences were further denoised by using commands ‘trim.seqs’, ‘pre.cluster’, and ‘chimera.uchime’. Uchime was used to remove chimeric sequences (Edgar et al., 2011). Sequences were then classified using the Bayesian classifier with the ‘classif.seqs’ command. Silva non-redundant v123 database was used as the reference (Quast et al., 2013). Archaea, chloroplasts, and mitochondria were excluded by using command ‘remove.lineage’ with criteria of ‘taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota’. Command ‘cluster.split’ was used to assign qualified sequences were into operational taxonomic units (OTUs) based on at least 97% similarity (OTU0.03). All the sequences of OTU0.03 were classified into taxonomic groups at the bootstrap threshold of 80% by using command ‘classify.otu’. Random subsampling using the least amount of sequences in all the samples was performed in order to avoid sequencing bias. The beta-diversity was calculated by using mothur with the command ‘dist.seqs’, ‘clearcut’, and ‘dist.shared’. The similarities among communities based on communities’ membership and relative abundance were measured by using unifrac-based matrix generated with command ‘unifrac.weighted’. Raw sequencing data was submitted to Sequence Read Archive (SRA) in NCBI, access number SRP123200.
Statistical analysis

One-way Analyses of variance (One-way ANOVA), uncorrected Fisher’s Least Significant Difference (LSD) test and Pearson correlation analyses were performed by using R 3.3.2 statistical software (Team, 2013). One-way ANOVA were used to determine significance of soil properties changed over time. The changes in soil properties were considered significantly different if the $P$ value $< 0.05$. Correlation of soil property with microbiome was analyzed using Pearson correlation analyses. Variables were considered significantly correlated if the $P$ value $< 0.05$.

Distance-based redundancy analysis (db-RDA) was performed by using vegan package in R (Oksanen et al., 2014). The db-RDA was used to perform the direct gradient analysis, which constrains and accumulates explanatory variables on two exes (RDA1 and RDA2). In this study, db-RDA was employed to present distribution patterns of microbiome and to measure the amount of variations explained by soil properties. The two ordinations axes (RDA1 and RDA2) were constructed by constraining an entire set of explanatory variables, seven soil properties in this study. Weighted unifrac distance was used to determine soil bacterial community structures. Mantel test at 999 permutations was used to assess impact of soil properties on soil microbiome. Impact of soil properties on microbiome was considered significant if $P < 0.05$. 
Results

Temporal changes in soil properties

One-way ANOVA was used to analyze temporal changes in soil properties without external amendment (Supplementary Table S3.1). Trends of soil properties are shown in Figure 1. Overall, soil P and TC significantly decreased since 2007 and 2003, respectively ($P < 0.05$). Although not significant, soil K and Mg had decreasing trends over time. The rest of soil properties, such as Ca, pH, and TN fluctuated over time with no significant trend.
FIGURE 3.1. Temporal change in soil properties. Soil properties, pH, phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), total nitrogen (TN), and total carbon
(TC), are measured. Year 1999 is in color red; 2000 is in color yellow; 2003 is in color lavender; 2005 is in color cyan; and 2007 is in color pink.

Soil microbiome

Total of 15 soil samples were sequenced, with three replicates in five time point (year 1999, 2000, 2003, 2005, and 2007) to represent temporal change in soil microbiome over eight years. A total of 1,247,858 paired-end raw reads were generated using Illumina HiSeq. An average of 83,190 ± 8,603 paired-end raw reads per sample were obtained in this study. A total of 1,036,401 qualified reads were obtained after data trimming and subsampling, with an average of 69,093 ± 9,527 reads per sample. A total of 27 phyla were classified with all the qualified reads. All the reads were further categorized at class, order, family, and genus level. Overall, 60 classes, 137 orders, 280 families, and 816 genera.

The top four most abundant phyla were Actinobacteria (37.63%), Firmicutes (16.87%), Alphaproteobacteria (11.20%), and Acidobacteria (9.14%) (Figure 3.2). One-way ANOVA was used to analyze temporal changes in top four phyla (Supplementary Table S3.2). There were no obvious trend found in these four phyla.

Core microbiome at phylum level was defined as phyla found in all the 15 samples and took up at least 1% of the abundance within a sample. Under this criteria, phyla Actinobacteria, Proteobacteria, Firmicutes, Acidobacteria, Chloroflexi, Gemmatimonadetes, and Planctomycetes were the core phyla. Core microbiome at genus level were genera found in more than 85% of all the samples with at least 1% of the
abundance within a sample. Genera *Gaiella, Solirubrobacter, Paenibacillus, Nitrospira, Skermanella, Sphingomonas,* and *Bryobacter* were the core genera in this study.

**FIGURE 3.2.** Temporal change in the top four phyla. Year 1999 is in color red; 2000 is in color yellow; 2003 is in color lavender; 2005 is in color cyan; and 2007 is in color pink.

The top ten genera that took up at least 5% of the total reads were *Gaiella* (27.65%), *Solirubrobacter* (12.9%), *Arthrobacter* (8.26%), *Skermanella* (7.27%), *Sphingomonas* (7.05%), *Blastococcus* (6.44%), *Nitrospira* (6.10%), *Enterobacteriaceae* (5.37%), *Paenibacillus* (5.25%), and *Streptococcus* (5.15%).

Shannon and Ace indices were analyzed to show diversity and richness of microbiome (Figure 3.3). Overall, the diversity and richness were not significantly alternated over time. The richness of communities slightly declined over time.
FIGURE 3.3. Temporal change in diversity (Shannon index) and richness (Ace index).

Year 1999 is in color red; 2000 is in color yellow; 2003 is in color lavender; 2005 is in color cyan; and 2007 is in color pink.

The db-RDA was used to represent distribution of soil microbiome (Figure 3.4).

No obvious separation was observed. Mantel test at 999 permutations was used to
calculate impact of soil properties on microbiome community (Table 3.1). Overall, K ($r^2 = 0.29, P = 0.001$) was the main driving factors shaping soil microbiome over time.

**FIGURE 3.4.** The distance-based redundancy analysis (db-RDA) diagram shows the relationship of soil properties with microbiome community compositions over time. Seven soil properties, pH, total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca), are labeled in color blue. Phyla are labeled in color red.
TABLE 3.1. Mantel test is used to determine relationship of soil properties and microbiome community.

<table>
<thead>
<tr>
<th>Soil variables</th>
<th>$R^2$</th>
<th>F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>0.29</td>
<td>5.06</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Ca</td>
<td>0.13</td>
<td>2.60</td>
<td>0.047</td>
</tr>
<tr>
<td>TN</td>
<td>0.11</td>
<td>2.53</td>
<td>0.045</td>
</tr>
<tr>
<td>pH</td>
<td>0.05</td>
<td>1.09</td>
<td>0.35</td>
</tr>
<tr>
<td>Mg</td>
<td>0.04</td>
<td>0.96</td>
<td>0.40</td>
</tr>
<tr>
<td>P</td>
<td>0.02</td>
<td>0.47</td>
<td>0.72</td>
</tr>
<tr>
<td>TC</td>
<td>0.02</td>
<td>0.42</td>
<td>0.74</td>
</tr>
</tbody>
</table>

***, $P < 0.01$. Total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca).
Pearson Correlation analysis was used to determine correlation between soil property and top four phylum (Table 2). Actinobacteria had negative correlations with K ($r^2 = -0.75$), Mg ($r^2 = -0.44$), and Ca ($r^2 = -0.67$). Firmicutes had positive relationship with K ($r^2 = 0.54$). Acidobacteria negatively responded to P ($r^2 = -0.48$) and Mg ($r^2 = -0.48$); whereas positively responded to TN ($r^2 = 0.66$) and TC ($r^2 = 0.64$). Alphaproteobacteria had positive correlation with Ca ($r^2 = 0.50$) and TN ($r^2 = 0.50$).
**TABLE 3.2.** Pearson Correlation analysis is used to determine relationship of soil property and phylum.

<table>
<thead>
<tr>
<th>phylum</th>
<th>pH</th>
<th>P</th>
<th>K</th>
<th>Mg</th>
<th>Ca</th>
<th>TN</th>
<th>TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>0.18</td>
<td>-0.07</td>
<td>-0.75***</td>
<td>-0.44*</td>
<td>-0.67**</td>
<td>-0.19</td>
<td>-0.09</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>-0.08</td>
<td>0.21</td>
<td>0.54*</td>
<td>0.4</td>
<td>0.09</td>
<td>-0.11</td>
<td>-0.16</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>-0.02</td>
<td>-0.48*</td>
<td>-0.32</td>
<td>-0.48*</td>
<td>-0.1</td>
<td><strong>0.66</strong></td>
<td><strong>0.64</strong></td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>-0.24</td>
<td>-0.02</td>
<td>0.04</td>
<td>-0.29</td>
<td>0.50*</td>
<td>0.50*</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* , $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$. Total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca).
Discussion

Long-term intensive cropping potentially worsens soil quality and stability (Tilman et al., 2002a). Many studies reveal influence of long-term intensive farming on soils and ecosystem. Few studies, however, characterize temporal change in soil microbiome affected by intensive cultivation (Robertson et al., 2000; Tilman et al., 2002a; Hamza and Anderson, 2005; Masto et al., 2008). To fill the gap of knowledge, this study reveals change in soil microbiome in response to eight years intensive cropping with no fertilizer. This study identifies the core microbiome (Actinobacteria, Proteobacteria, Firmicutes, Acidobacteria, Chloroflexi, Gemmatimonadetes, and Planctomycetes), which remains stable over time, suggesting potential contribution to soil nutrients. Furthermore, this study determines that soil K is the major driving factor shaping soil microbiome.

Temporal change in soil properties without amendment

In this study, we find that soil nutrients slightly decrease as the result of long-term extensive cropping. Total carbon slightly declined since 2003. Other nutrients, such as TN, P, K, and Mg, had overall decreasing trends. Soil cultivation involves with transferring nutrients from soils to crops (Clarke and Marshall, 1947). Long-term cropping without amendment, thereby, accelerates the loss of soil nutrients and worsens soil structures (Baver, 1968; Odell et al., 1984). Our result is consistent with previous studies suggesting that components of soil organic matter (SOM), such as TC and TN, are impacted by cultivation and amendment (Jenny and Raychaudhuri, 1969; Dalal and Mayer, 1986; Lal, 2002). It is well-established that long-term annual organic amendment
enhances soil quality, stability, and biodiversity (Diacono and Montemurro, 2010). Long-term intensive farming without exogenous nutrient replenishing, on the other hand, is considered as unfavorable soil management, which might cause soil erosion, loss of nutrients, and degradation of soil properties. There is no defined time frame on how long the intensive cropping leads to significant declines of soil fertility and stability. In this study, we find that although soil nutrients slightly decline over eight years of intensive cropping, the overall soil fertility is still adequate for cropping. Explanations could be 1) although soil has been exploited, SOM content still meets the minimum requirement of cropping and 2) the core soil microbiome found in this study are mostly copiotrophic taxa with many decomposition activities. Those bacteria might degrade SOM to release nutrients for cultivation. Soil organic matter is one of the important factors that maintains soil fertility and stability (Tiessen et al., 1994). Decomposition of SOM mainly occurs in heterotrophic microbiome impacted by several environmental factors, such as temperature, water content, and permutation of oxygen in soils. The physical soil properties and humus layer are not anthropically disturbed in this study. General soil conditions are favorable for microbiome activities. Overall, soil microbiome activities and consequential releasing of nutrients might explain the adequate soil fertility without amendment for eight years. It is worth mentioning that although no significant decrease of soil fertility is observed in this study, intensive farming is not a sustainable agricultural practice. Furthermore, a previous study suggests that soil quality significantly decreases due to loss of SOC when the intensive cropping with no fertilization lasts for over 50 years (Duff et al., 1995). Intensive farming is not recommended in modern agricultural management. It is partially because the soil quality and healthy soil microbiome
restoration are time-consuming and difficult processes (Chaparro et al., 2012; Huang et al., 2013; Lakshmanan et al., 2014).

*Temporal change in soil microbiome*

We find that the richness of soil microbiome slightly decreases over time, indicating an overall decline in microbiome abundance. The core microbiome remains stable over eight years. There is no distinguishable temporal change in microbiome observed in this study.

We find that phyla Actinobacteria, Proteobacteria, Firmicutes, Acidobacteria, Chloroflexi, Gemmatimonadetes, and Planctomycetes are the core microbiome in this study. Interestingly, the core microbiome contain many copiotrophic taxa, such as Actinobacteria, Proteobacteria, Firmicutes, and Chloroflexi, in this study. This result suggests that eight years cropping without fertilizer does not significantly impact the soil microbiome. Genera, *Gaiella*, *Solirubrobacter*, *Paenibacillus*, *Nitrospira*, *Skermanella*, *Sphingomonas*, and *Bryobacter*, are the core genera found in this study. *Gaiella* (Actinobacteria) is one of the most common and abundant genus in soils. Taxa that belong to *Gaiella* are strictly aerobic with oxidase and catalase activates (Albuquerque et al., 2011). Although ubiquitously existing, functions of *Gaiella* are not well-known (Albuquerque et al., 2011; Novello et al., 2017). *Solirubrobacter* (Actinobacteria) is the second most predominating genus in this study. Previous study reveals that *Solirubrobacter* is enriched by conventional farming method compared to that os in organic farming (Li et al., 2012). It might because *Solirubrobacter* has higher activities on degrading some herbicides and pesticides (Schrijver and Mot, 1999). Genus
*Paenibacillus* (Firmicutes) has species that contribute to nitrogen fixing, plant growth promotion, and plant protection from several pathogens (Timmusk et al., 2005; Berg, 2009; Xie et al., 2014). The predominance of *Paenibacillus* might improve soil fertility and plant health in the absence of fertilizer in this study. *Nitrospira* (Nitrospirae) contains many taxa that involve with the nitrification process, such as nitrite-and ammonia-oxidation activities (Krümmel and Harms, 1982; Koch et al., 2015). The predominance of both *Paenibacillus* and *Nitrospira* in this study might contribute on providing N in various forms in soils to support the biodiversity and cropping without the anthropogenic N input.

**Soil properties shape microbiome**

We find that soil K is the main driving factor shaping soil microbiome. As the third most important macronutrient in soils, K shows a declining trend over eight years intensive cropping in this study. Other macronutrients, TC and TN, fluctuate over time. The continuously decreasing of K might be one of reasons explaining K as the key determinant on soil microbiome. Because K is not only an essential element for microbiome, it also plays important roles on regulating osmotic stress, adjusting pH, and being the counter ion for glutamate (Epstein, 2003; Follmann et al., 2009). Virtually all the taxa in Actinobacteria have various K-channels and carriers facilitating K-uptake due to the ion impermeable of membrane (Ochrombel et al., 2011). Actinobacteria are sensitive to concentration of K in soils and obtain various K-transport systems to cope with K gradient (Follmann et al., 2009). K insufficiency might restrict bacterial growth (Stingl et al., 2007). We find a strong negative correlation between K and Actinobacteria in this study. A previous study also reports a strong negative correlation between K and
Actinobacteria (Pereira et al., 2014). The vast variabilities of K-transport system in Actinobacteria might explain their predominance in K limiting soils. Altogether, the prevalence of Actinobacteria and their close interaction with K might explain the large impact of soil K has on soil microbiome in this study.

The most of K is in the insoluble form existing in soils (Sparks, 1987; George and Michael, 2002). Besides, intensive farming results in soil K deficiency, which hampers physiological activities of soil fauna (Das and Pradhan, 2016). Potassium solubilizing bacteria (KSB) such as Actinobacteria, Firmicutes, and Proteobacteria, therefore, play important roles in releasing K in soils (Basak and Biswas, 2009; Maurya et al., 2014; Zorb et al., 2014; Ahmad et al., 2016). In this study, the availability of K in soil affects activities of many bacteria, thereby shaping microbiome. Specifically, we find that K has significant correlation with the top two most predominating phyla (Actinobacteria and Firmicutes). Many taxa that belong to Actinobacteria and Firmicutes are well-studied as KSB. Potassium solubilizing bacteria produ low molecular weight organic acids, which solubilize and mobilize K from minerals (Shanware et al., 2014). All currently defined KSB in Actinobacteria belong to families Microbacteriaceae and Micrococccaceae (Velázquez et al., 2016). Genus Arthrobacter (family Micrococccaceae) is the third most abundant genus found in this study. Genus Arthrobacter has been well-studied for their K-soluble activities (Diep and Hieu, 2013; Keshavarz Zarjani et al., 2013; Zhang and Kong, 2014). Arthrobacter produces organic acids and monohydroxamate siderophore to mobilize K in soils (Keshavarz Zarjani et al., 2013).

Genera Bacillus and Paenibacillus (Firmicutes) are also well-known for their K-soluble abilities. Specifically, some taxa (e.g. Bacillus mucilaginosus, B. edaphicus,
*Paenibacillus mucilaginosus,* and *P. frequentans*) produce polysaccharide and carboxylic acids such as, tartaric-and citric-acid, to increase K availability in soils (Richards and Bates, 1989; Hu et al., 2006; Sangeeth et al., 2012). Altogether, the predominance of *Arthrobacter* and *Paenibacillus* along with their K-solubilizing activities might explain impact of K on soil microbiome.

**Conclusion**

This study reveals that long-term intensive cropping without fertilizer slightly decreases soil fertility and richness in soil microbiome. Soil K consistently decreases over time due to the intensive cropping. This study establishes that soil K is the most important driving factor shaping soil microbiome. Furthermore, result shows that the core microbiome remains stable over time. More than half of core microbiome found in this study are copiotrophic bacteria, such as Actinobacteria, Proteobacteria, Firmicutes, and Chloroflexi, indicating adequate soil fertility and close interactions of microbiome with soil nutrients. Many taxa in the core microbiome have functions involved with N-cycling and K-solubilization, suggesting potential contribution of soil microbiome on providing nutrients in soils. This study fills the gap of knowledge on change in soil microbiome in responsive to long-term intensive cropping without fertilization.
REFERENCES


Gaiellaceae fam. nov. and Gaiellales ord. nov. *Systematic and applied microbiology* 34, 595-599.


bacterial communities with predictable responses of major bacterial phyla.

*Applied soil ecology* 84, 62-68.


denitrification: experiments with cells extracted from organic soils. *FEMS
microbiology ecology* 79, 530-541.

species. I. Conserved genetic and nucleic acid base sequence homologies.
*Proceedings of the National Academy of Sciences of the United States of America*
54, 491-49.

regions of the Pacific NW America.

Regional Land-Use Planning - Study of the Ottawa Urban Fringe. *Canadian

reproducibility and new method for analysis of terminal restriction fragment
profiles of 16S rRNA genes from bacterial communities. *Appl Environ Microbiol*
67, 190-197.

of experimental changes in plant diversity on cavity-nesting bees, wasps, and their


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Associations between Soil Nutrients and Specific Taxa Involved in Nutrient Transformations. *Frontiers in Microbiology* 8, 12.


Lladó, S., Žifčáková, L., Větrovský, T., Eichlerová, I., and Baldrian, P. (2016). Functional screening of abundant bacteria from acidic forest soil indicates the
metabolic potential of Acidobacteria subdivision 1 for polysaccharide decomposition. *Biology and fertility of soils* 52, 251-260.


Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T.,
Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao,
Batto, J.M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E.,
Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M.,
Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Dore,
J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Meta,

platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq
sequencers. BMC genomics 13, 341.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and
Glockner, F.O. (2013). The SILVA ribosomal RNA gene database project:
 improved data processing and web-based tools. Nucleic Acids Research 41, D590-
D596.

Ramasamy, D., Mishra, A.K., Lagier, J.C., Padhmanabhan, R., Rossi, M., Sentausa, E.,
genomic data for the taxonomic description of novel bacterial species.
International Journal of Systematic and Evolutionary Microbiology 64, 384-391.


16S-23S rRNA gene internal transcribed spacer sequences. *Journal of Clinical Microbiology* 36, 139-147.


N fertilization and pH in the 150-year 'Park Grass' UK grassland experiment.

_Fems Microbiology Ecology_ 76, 89-99.

Ryckeboer, J., Mergaert, J., Vaes, K., Klammer, S., De Clercq, D., Coosemans, J., Insam,

Saison, C., Degrange, V., Oliver, R., Millard, P., Commeaux, C., Montange, D., and Le
following compost amendment: effects of compost level and compost - borne
microbial community. _Environmental Microbiology_ 8, 247-257.

M.A., Miller, S.I., Hoogestraat, D.R., Cookson, B.T., Mccoy, C., Matsen, F.A.,
rRNA Next-Generation Sequencing of Polymicrobial Clinical Samples for
Diagnosis of Complex Bacterial Infections. _Plos One_ 8.

Sangeeth, K., Bhai, R.S., and Srinivasan, V. (2012). Paenibacillus glucanolyticus, a
promising potassium solubilizing bacterium isolated from black pepper (Piper


Methylobacterium bacteria nodulate and fix nitrogen in symbiosis with legumes. 

*Journal of bacteriology* 183, 214-220.


Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M.,
Community structure and metabolism through reconstruction of microbial

and thermal-stability of DNA duplexes. 2. Effects of deamination of cytosine.
*Biochimica Et Biophysica Acta* 294, 416-424.


majority: soil microbes as drivers of plant diversity and productivity in terrestrial


Zhang, H., and Raun, B. (2006). *Oklahoma soil fertility handbook*. Department of Plant and Soil Sciences, Oklahoma Agricultural Experiment Station, Oklahoma Cooperative Extention Service, Division of Agricultural Sciences and Natural Resources, Oklahoma State University.


**APPENDICES**

**Supplementary Table S 2.1.** Two-way ANOVA results of change in soil property affected by fertilizer and time.

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Two-way Analysis of variance (Two-way ANOVA), fertilizer (beef manure and swine effluent) is significant different if $P$ value < 0.05. $P$ value is in bold if the difference is significant. Total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca).
Supplementary Table S 2.2. Two-way ANOVA results of change in top four phylum affected by fertilizer and time.

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Two-way Analysis of variance (Two-way ANOVA), fertilizer (beef manure and swine effluent) is significant different if $P$ value $< 0.05$. $P$ value is in bold if the difference is significant.
Supplementary Table S 2.3. Two-way ANOVA results of change in top four genus affected by fertilizer and time.

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</table>

Two-way Analysis of variance (Two-way ANOVA), fertilizer (beef manure and swine effluent) is significant different if $P$ value < 0.05. $P$ value is in bold if the difference is significant.
**Supplementary Table S 3.1.** One-way ANOVA results of change in soil property over time.

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>df</th>
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<td>Time</td>
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</tr>
<tr>
<td>Ca</td>
<td>Time</td>
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One-way Analysis of variance (One-way ANOVA), treatment is significant different if P value < 0.05. P value is in bold if the difference is significant. Total nitrogen (TN), total carbon (TC), potassium (K), phosphorus (P), magnesium (Mg), and calcium (Ca).
**Supplementary Table S 3.2.** One-way ANOVA results of change in soil properties over time.

<table>
<thead>
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<th>P value</th>
</tr>
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<tbody>
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</table>

One-way Analysis of variance (One-way ANOVA), time is significant different if P value < 0.05. P value is in bold if the difference is significant.
VITA

XUWEN WIENEKE

Candidate for the Degree of

Doctor of Philosophy

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Education:

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