DYNAMICS OF AGRICULTURAL SOIL FUNGI: IMPACT OF AGRICULTURAL LAND MANAGEMENT ON SOIL MICROBIAL BIOMASS, FUNGAL TAXONOMIC RICHNESS AND LIGNINOLYTIC GENE DISTRIBUTION

By

Christopher Allison Wright

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ABSTRACT

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Understanding the impact of human activity on environmental microbiota and their ecosystem processes is essential to formulating and employing a sustainable approach to agriculture. To that end, we examined how eight different agronomic land management strategies impacted the overall biomass of the microbiota inhabiting Midwestern United States soils, comparing this data with the microbial biomass of a nearby mid-successional field and two nearby forested sites. We further examined how agricultural land management practices impacted the taxonomic structure and genetic potential for lignolytic activity of the fungi residing in the soils of two 3-crop agricultural rotations, one under conventional till and the other under no till management. We compared the taxonomic structures and ligninolytic potentials of the agricultural treatments to each other and to those of soils of a nearby, deciduous forest, the native terrestrial ecosystem from which the agronomic treatments originated, and an early-successional treatment that was historically under agronomic management but abandoned from agriculture 22 years prior. We found that the particular agricultural land management employed had a significant impact on the microbial biomass of the soils from each treatment. Further, we found that although land management did not significantly affect the Operation Taxonomic Unit (OTU) richness of soil fungi in the till and no-till treatments, when compared to the fungal communities of the early-successional and native

forest soil fungal communities, it did strongly impact how the fungal communities in each treatment were structured in terms of OTU membership. Lastly, although land management practices did not make a statistically significant difference in the total number of lignolytic genes detected in the fungal community from each treatment, it did make a significant difference in the ligninolytic gene structure of each treatment.

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KEY TO SYMBOLS AND ABBREVIATIONS

AMF: Arbuscular Mycorrhizal Fungi

C: Carbon

CO_2: Carbon Dioxide

DOM: Dissolved Organic Matter

ECM: Ectomycorrhizal Fungi

PLFA Phospholipid Fatty Acid

N: Nitrogen

P: Phosphorus

LiP: Lignin Peroxidase

MCSE Main Cropping System Experiment

MnP: Manganese Dependant Peroxidase

VP: Versatile Peroxidase

LDE: Lignin Degrading Enzyme

AMF: Arbuscular Mycorrhizal Fungi

KBS: Michigan State University's W.K Kellogg Biological Station

LTER: Long Term Ecological Research

SOC: Soil Organic Content

SOM: Soil Organic Matter

I. LITERATURE REVIEW

Introduction

Human activity has had a significant impact on the structure and functions of microbial communities on Earth (Joergensen and Emmerling, 2006; Hunter, 2007). However, we are just beginning to understand how human-mediated activities manifest differences in microbial communities and consortia. A key topic in contemporary ecology is whether changes in microbial processes are occurring through a shift in community species membership, a shift in the relative abundance or activity of organisms actively mediating biogeochemical processes, or both.

Among the many ecosystem services provided by soil microbes in terrestrial ecosystems, lignin degradation stands out as crucial and highly significant. The majority of energy and nutrients necessary for all primary and, therefore, downstream processes in soils are mostly delivered as plant biomass. Lignin degradation unlocks energy and nutrients bound in plant biomass, making them available for biological processes. Without lignin degradation, plant biomass, and the nutrients therein, would quickly accumulate rather than being recycled, and the flow of nutrients for primary production would cease. Consequently, lignin degradation is an essential process in the intra-ecosystem biogeochemical cycling of energy, minerals and nutrients.

Soil and the microbial community

Soil is a complex, heterogeneous and dynamic environment. Physically, soil consists of approximately 45% mineral matter, 25% gasses, 25% water, and 5% organic matter by volume (Birkeland, 1999). Biologically, there is an incredible diversity of organisms living in the soil

environment. These organisms range in size from the tiniest, one-celled microbes, to multi-cellular fungi, nematodes and micro-arthropods, to the visible earthworms, insects, small vertebrates, and plants (Paul, 2007). Soil microbes vary in size from approximately 0.2 um to 200 um, although the fruiting bodies of many fungi can be larger. This group includes the bacteria, archea, fungi, viruses, protozoa, stramenopiles and other microeukaryotes.

The density of microbes in soil can be substantial; a single gram of soil may house up to 10 billion microorganisms, encompassing thousands of distinct species (Torsvik and Ovreas, 2002). This can include up to 6,000-50,000 species of bacteria (Curtis *et al*., 2002), and 200 m of fungal hyphae (Leake *et al*., 2004). Although largely unseen, these organisms provide a myriad of crucial and essential ecosystem services. Indeed, the functional stability of a soil is dependent on its indigenous microbiota.

Ecosystem services provided by soil microbiota

Microbial soil communities are dynamic assemblages of taxonomic and functional groups who synergistically maintain ecosystem dynamics, provisions and processes. Microbial-mediated processes are essential to maintaining soil fertility and health, and contribute to soil stress resistance and resiliency. Ecosystem services and processes provided by microorganisms include the following:

1. The fixation of nitrogen (N). Diazotrophic bacteria (e.g. *Frankia* and *Rhizobia* species) fix atmospheric N_2 and convert it into NH_3^+, a form of N readily utilizable by plants (Alexander, 1977).

2. The decomposition and turnover of organic matter, which releases mineral nutrients such as N, P, and K to the soil, making them available for plants, thereby influencing the long-term, primary productivity of terrestrial ecosystems (Swift *et al*., 1979).
3. Providing plants access to nutrients, including N and P, and water via belowground fungal root symbionts called mycorrhizae, thus influencing long-term, primary productivity (Smith and Read, 1997).
4. The production of glycoproteins, such as glomulin, that enhance soil aggregation, structure, tilth and porosity. The presence of these glycoproteins results in an increase in water infiltration, a reduction in runoff, and an increased potential for C sequestration. (Singh *et al*., 2013, Treseder and Holden, 2013).
5. The biogeochemical cycling of elements. 80–90 % of the chemical processes in soil are reactions mediated by microbes (Coleman and Crossley, 1996).
6. Soil genesis through the mineral weathering of parent material (Anderson, 1988).
7. The protection of plants from root pathogens (Elad, 2000).
8. Food for other organisms (Ngosong *et al.,* 2014).

Accordingly, maintaining microbial diversity is acutely necessary for the continuing sustainability of soil. An assessment of the soil microbial community can provide a consolidated snapshot of the soil's health and potential for sustainable production (Brussard *et al.,* 2007). Further, as the microbiological properties of soil are a dynamic indicator of perturbation and land-use change, a quantitative description of the soil's microbial community structure is a major indicator of the soil's overall quality (Zelles, 1999).

Fungi in soil

Fungi are not only abundant in soil, but they are a key, functional component of the microbial soil community. Their ranks include saprotrophs, predators, ectosymbionts, endosymbionts, biotrophs and pathogens, with some of these categories overlapping. Soil fungi live in both moderate and harsh environments, degrade almost all organic residues, and outrank all other soil microbiota, including bacteria, in both richness of species and biomass (Hawksworth, 1993; Hawksworth, 2001). Soil fungi are non-photosynthetic chemoheterotrophs, and have an osmotrophic mode of nutrition whereby they secrete enzymes into their immediate environment to break down larger compounds into smaller compounds, which are then absorbed across the surface of their hyphae. To this end, fungal genomes contain extensive sets of genes encoding substrate-specific and non-specific secreted enzymes geared towards decomposing plant litter (Sinsabaugh, 2010; Paul, 2007). Some soil fungi possess genes that encode for highly non-specific, hydrolytic and oxidative enzymes that confer a broad spectrum of degradative capabilities. Most notable are the oxidative enzymes that allow certain fungi the ability to deconstruct lignin, an intractable yet key component of lignocelluloses. Lignocelluloses, macromolecular complexes of lignin, cellulose and hemicellulose, are the dominant components of vascular plant tissues, and with an estimated mass of 350 to 500 pentagrams, constitute the largest renewable carbonaceous biomass on earth (Paul, 2007).

Fungi are uniquely equipped to seek out and metabolize lignin, lignocellulose, as well as many other organic substances in soils for several reasons. First, the filamentous nature of their growth generates enormous mechanical pressure that allows them to invade and

penetrate both soil and substrates. Second, they are also one of the few groups of organisms that produce lignin-degrading enzymes (LDEs), which they secrete into their surrounding environment. Secretion enables the lignolytic activity of fungal LDEs to permeate regions of the soil matrix that would otherwise be inaccessible. In addition to decomposing lignocellulose LDEs also enhance the overall quality of soils by mineralizing (to CO_2) a variety of environmental pollutants such as polychlorinated biphenyls (PCBs), dioxanes, dioxins, chlorobenzenes and petroleum hydrocarbons, all of which share structural similarity to lignin (Reddy, 1995; Crawford, 1995; Barr and Aust, 1994). Fungi can also utilize substrates with a broad range of C/N ratios, and are able to grow across a broad range of pH. Finally, they are able to intercellularly translocate nutrients from one soil zone to another throughout their mycelial thallus, thus enabling them to grow through nutritionally deficient zones in order to seek out and utilize suitable substrates.

There are two major types of fungal, extracellular enzymes; hydrolytic enzymes and oxidative enzymes. Hydrolytic enzymes cleave residues through the addition of water, where the water and reactant exchange functional groups ($A–B + H_2O \rightarrow A–OH + B–H$). Some of the key hydrolytic enzymes employed by fungi are those used in the degradation of non-lignified carbohydrates, such cellulose and hemicellulose. These include cellobiohydrolases, endocellulases, β-glucosidases, β-glucanases, and xylanases (Baldrian, 2008). Oxidative enzymes cleave residues through the abstraction of electrons from their substrates, rendering the substrates unstable and reactive (Sinsabaugh, 2010). All ligninolytic enzymes employed by fungi are mechanistically oxidative.

Soil fungi and plant litter decomposition

Plant litter decomposition is essential to the maintenance of soil fertility, as the availability of nutrients in soil is directly tied to the dynamics of plant litter decomposition (Berg and McClaugherty, 2008). During decomposition, nutrients are liberated from organic matter, making them available for primary production and incorporation into microbial biomass, which retains nutrients in the ecosystem that might otherwise be leached (Paul, 2007). Without this service, soil fertility would be unsustainable.

Plant litter decomposition is also critical to the global biogeochemical cycling of carbon (C), in which lignin decomposition is the rate-limiting step. Furthermore, the dynamics of litter decomposition in soils determine whether plant litter C is fluxed to the atmosphere or sequestered in the soil (Paul, 2007). Therefore, an understanding of how land management may affect ligninolytic gene distribution of soil metagenomes is essential to developing sustainable land management practices that allow maximum production while minimizing environmental impact.

The suite of genes used in plant biomass degradation may differ among fungal species, with environmental variables selecting for specific combinations of cellulases, phosphatases and hydrolases (Brink and Vries, 2011). However, although enzymes involved in the transformation of organic matter in soils have been the subjects of extensive study, there has been a paucity of studies investigating fungal ligninolytic enzymes in soils (Sinsabaugh, 2010).

Succession of enzymes important in plant litter decomposition

When plant litter first enters the soil, soluble and low-molecular weight compounds including simple sugars, proteins, and low-molecular-weight phenols are rapidly metabolized during the initial stages of decomposition. Degradation of polymer carbohydrates such as cellulose and hemicellulose follows, leaving behind lower quality, recalcitrant materials such as lignocellulose, which persist (Berg and McClaugherty, 2008). Snajdr *et al*. (2011) observed that hydrolytic cellulases were the most active enzymes during the early 4-month stage of decomposition, corresponding with the largest loss of litter mass in comparison to later stages. Xylanase activity peaked after 4 months, with a slight decrease in net mass substrate loss. After 12 months, ligninolytic enzymes were the most active enzymes, although mass loss was less than noted during earlier stages of decomposition. Soil fungi primarily mediate the later stage of lignin decomposition (Paul, 2007; Kubicek *et al*. 2010), although fungal enzymes are important to the whole process of plant litter decomposition (Snajdr *et al*., 2011).

Plant polymers in soil

Lignin is the second most abundant organic polymer in the biosphere. It is a stereochemically complex, aromatic and heterogeneous biopolymer (Figure 1) that comprises 20-30% of the dry mass of woody plants (Boominathan and Reddy, 1992). Lignin imparts

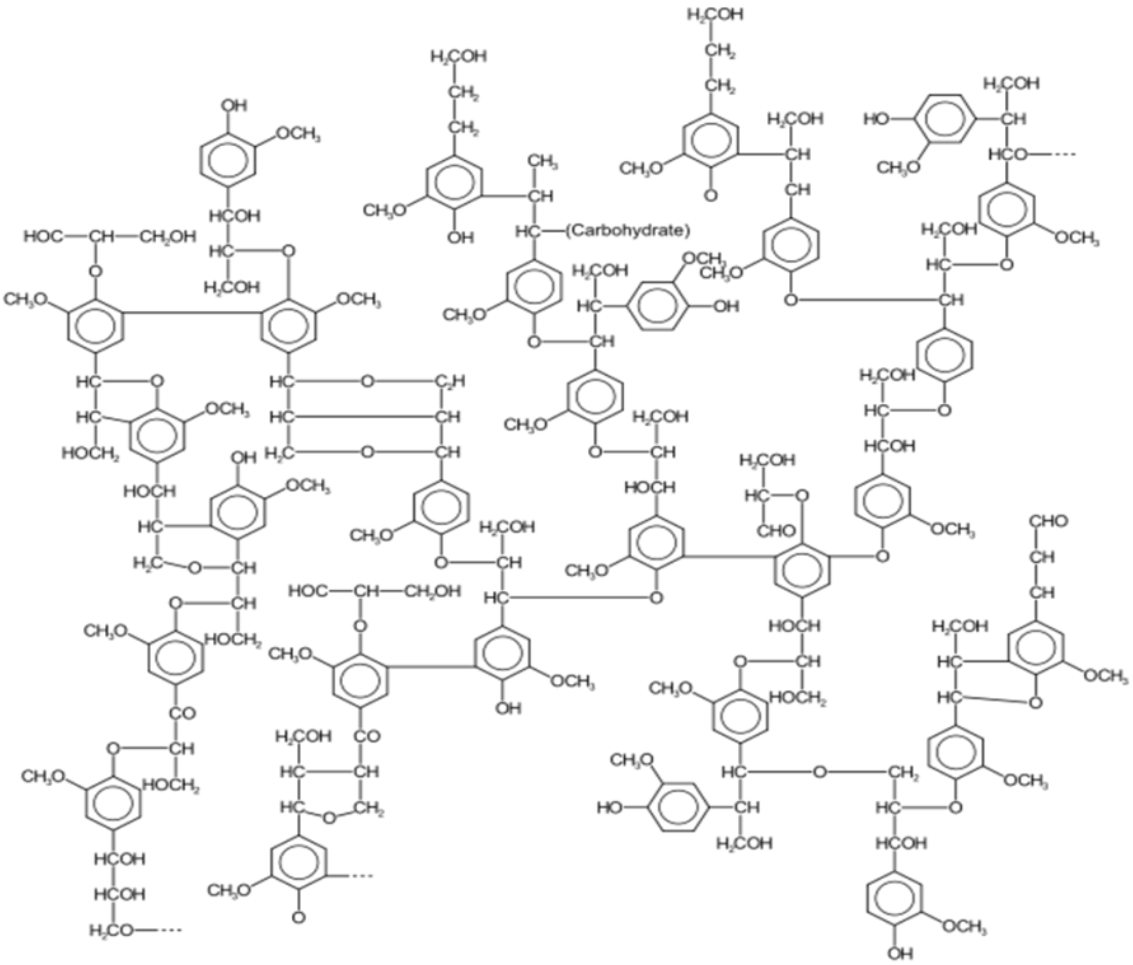

Figure 1. The structure of lignin (reprinted with permission from sustainable-energ.lsu.edu).

structural rigidity to woody plant tissue, protects cellulose and hemicellulose from enzymatic attack, aids in the transport of water, and is the major precursor of coal (Robinson, 1990).

Lignocellulose is comprised of the carbohydrate polymers cellulose and hemicellulose, as well as lignin. It has evolved to be resistant to degradation, thereby conferring both hydrolytic stability as well as structural robustness to the cell walls of plants. This resistance is primarily due to the recalcitrance of its lignin component (Kirk and Farrell, 1987), in conjunction with the cross-linking between its polysaccharide constituents, cellulose, hemicellulose, other plant cell wall polymers and lignin via ester and ether linkages. Ester linkages arise between

oxidized sugars, uronic acids, and the phenolic and phenylpropanic subunits of the lignin. Since lignin occurs in close physical and chemical association with the cellulose and hemicellulose components of lignocellulose, it limits the access of polysaccharide digesting enzymes (i.e. cellulases and hemicellulases) to these polymers (Kirk and Farrell, 1987). Thus, the deconstruction of lignin, which constitutes the rate-limiting step in the global C cycle, is essential to the efficient recycling of nutrients bound up in an ecosystem's plant litter inputs.

Types of ligninolytic enzymes

Ligninolytic enzymes employed by soil fungi form two major classes: one that uses oxygen as its terminal electron acceptor [phenol oxygenases, including laccase (LAC)], and the other that uses hydrogen peroxidase as its terminal electron acceptor [class II peroxidases, including lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP)] (Sinsabaugh, 2010). Class II peroxidases or "heme proteins" generally have much higher redox potentials than phenol oxygenases. The Enzyme Commission of the International Union of Pure and Applied Chemistry (EC) classifies phenol oxygenases into taxa EC 1.10.3 and class II peroxidases into taxa EC 1.11.1.

LACs

Extracellularly secreted LACs (EC 1.10.3.2) are glycosylated oxidoreductases that couple the oxidation of phenolic and other aromatic and non-aromatic substrates with the reduction of molecular oxygen (O_2) to water (H_20). The free-radical products of this oxidation are unstable and may undergo further enzymatic or non-enzymatic reactions. The average size of a LAC enzyme is 66 kDa, and the average optimal temperature is approximately 55^{o} C, but can vary

greatly (Baldrain, 2006). LACs were first found in the Japanese lacquer tree *Tunus vernicefera* (Yoshida, 1884), but have since been found in numerous fungi, including *Lentinus tigrinus* (Ferrarone et al, 2007), *Pleurotus ostreatus* (Pozdniakova et al, 2006), *Gaeumannomyces graminis, Magnaporthe grisea, Neurospora crassa, , Xylaria polymorpha, Pycnoporus cinnabarinus, Gloeophyllum trabeum, Laetiporus sulphureus* and many other Ascomycota and Basidiomycota fungi that have different ecological roles including white-rot, brown-rot, soft-rot and ectomycorrhizal taxa (Baldrain, 2006).

LAC lignin-depolymerization reactions are one-electron oxidations of substrates that generate a free radical (Figure 2). Four single-electron oxidations are required in order to reduce oxygen to water. LACs have four copper ions that mediate redox reactions in their catalytic, reaction center. These atoms are classified into three different types, T1, T2 and T3, based on their paramagnetic resonance spectrum (Kunamneni et al., 2007). T1 copper ion clusters are involved in substrate oxidation, after which the abstracted electrons are transferred to the T2/T3 copper cluster, which is involved in the reduction of O_2 to H_20 (Figure 10). Initially, a T1 Cu(II) is reduced to Cu(I). That electron is then transferred to theT2/T3 complex. Once four single-electron oxidations have occurred, and electrons have been transferred, the T2/T3 complex reduces O_2 to H_20 via the formation of a peroxide intermediate that contains a dioxygen anion. The T2/T3 complex coppers are then concurrently oxidized back to Cu(II), completing the reactive cycle.

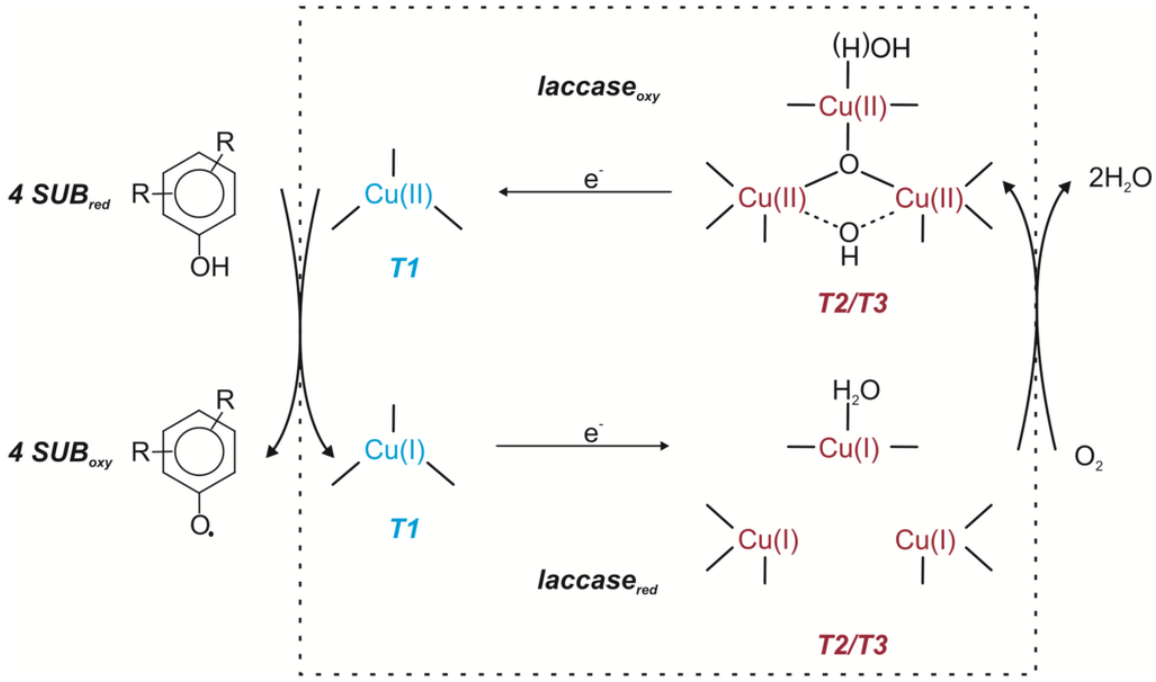

Figure 2. The catalytic cycle of laccase (reprinted with permission from Guzik *et al*., 2014). SUB represents the phenolic substrate being oxidized.

LACs generally have lower redox potentials (450 to 800 mV) than class II peroxidases (>1000mV), but some of the reductants that are produced may, themselves, oxidize other compounds that require a higher redox potential than that of LAC (Leonowicz *et al*., 2001). These reductants include reactive quinines, semiquinones as well as phenoxy and cation radicals. LACs can also catalyze the oxidation of a myriad of non-specific reactants, which can then further react with a broad spectrum of other phenolic compounds (Sinsabaugh, 2010).

Class II peroxidases

Class II peroxidases are secretory monomeric glycoproteins that catalyze oxidative reactions using hydrogen peroxidase as the terminal electron acceptor. They were first discovered in the white-rot fungus *Phanerochaete chrysosporium* (Tien and Kirk, 1983). Class II

peroxidases are heme enzymes that have an Fe atom covalently bound to the center of a porphyrin ring. Both the iron, as well as the porphyrin ring, can serve as a source or sink of electrons during electron transfer, depending on the oxidation state of the enzyme. Class II peroxidases are classified into three major groups: LiPs (EC 1.11.1.14), MnPs (EC 1.11.1.13) and VPs (E.C. 1.11.1.16).

LiPs

LiPs are glycosylated heme-containing proteins, with a MW range of 37-50 kDa (Dashtban *et al.*, 2010). LiPs have a tryptophan residue on the surface of the enzyme that enables the direct oxidation of phenolic and non-phenolic compounds via long-range, multi-step, electron transfer. The products of this oxidation are intermediate radicals, such as phenoxy radicals and veratryl alcohol radical cations, which can then undergo non-enzymatic reactions including side-chain cleavage and demethylation. Due to their high redox potential of >1.4 V, LiPs are able to directly oxidize non-phenolic aromatic substances, and do not require intermediate mediators.

The native, ferric LiP enzyme (FeIII) is first oxidized by H_2O_2 to produce the oxidized, two-electron intermediate Compound 1, which is then able to oxidize lignin (AH) (Figure 3). This forms the oxidized, one-electron intermediate, Compound II, which can then oxidize a second substrate molecule. In an excess of H_2O_2, Compound II can be transformed to Compound III, which is then inactive (Tien, 1987; Gold *et al*., 1989).

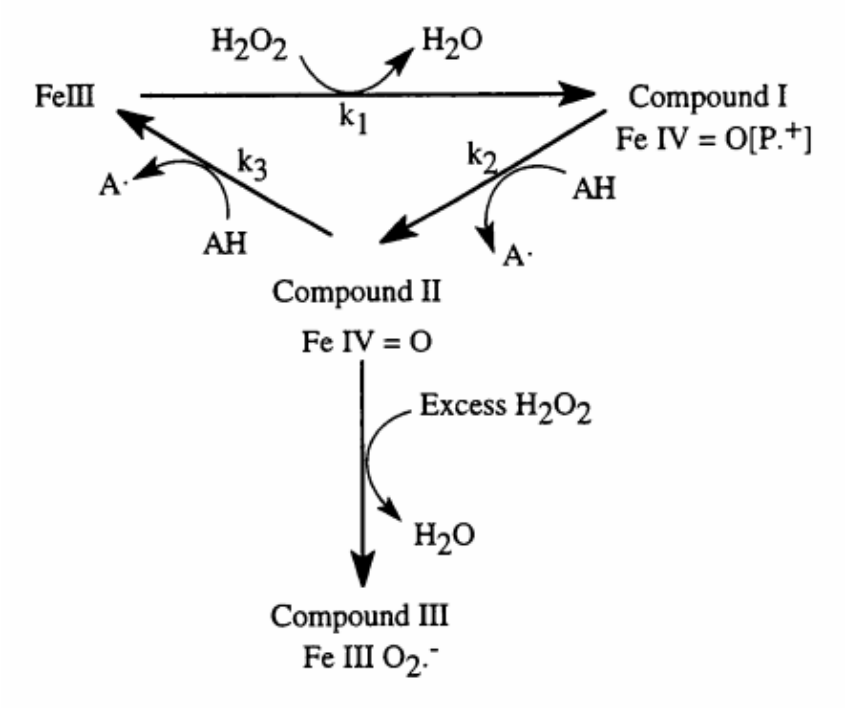

Figure 3. The catalytic cycle of lignin peroxidase (reprinted with permission from Gold et al. 1989).

MnPs

MnPs are the most common class II peroxidases produced by saprotrophic fungi (Hofrichter, 2002) and possess two to three residues that bind and oxidize Mn^{2+} to Mn^{3+}. They have a molecular weight of 38 to 62.5 kDa, with an average of 45 kDa (Dashtban *et al.*, 2010). MnPs were first discovered in the mid 1980s (Kuwahara et al, 1984; Glenn and Gold, 1985; Paszcynski *et al*, 1985) and can appear in several different isoforms in the same strain of fungus (Hatakka, 1994). This nonspecific oxidoreductase is believed to be a very important player in lignin deconstruction and widely occurs in several taxonomic groups of basidiomycotous and other fungi.

The native ferric MnP enzyme is initially oxidized by reducing H_2O_2 to H_2O in an iron-peroxide complex (Figure 4). The resulting Fe^{4+}-oxo-porphyrin-radical complex (Compound 1) can then be reduced by Mn^{2+}, which is commonly found in lignocellulose and soil, to Compound II. Mn^{2+} is concurrently oxidized to Mn^{3+}. A second Mn^{2+} can then further reduce the radical complex, producing a second, reactive Mn^{3+} ion and returning the MnP enzyme to its native, resting state.

The reactive Mn^{3+} ions are chelated with carboxylic acids such as oxalate (as well as malonate, malate, tartrate and lactate), and act as diffusible redox mediators that can effect a one-electron oxidation of various phenolic and amino-aromatic substrates. These oxidized substrates, now phenoxyl and amino radicals, may further react with other substrates, or undergo oxidative cleaving reactions with lignin. Chelated Mn^{3+} ions may also react with other substrates and with themselves to create reactive, radical intermediates, which may then undergo further spontaneous, oxidative reactions.

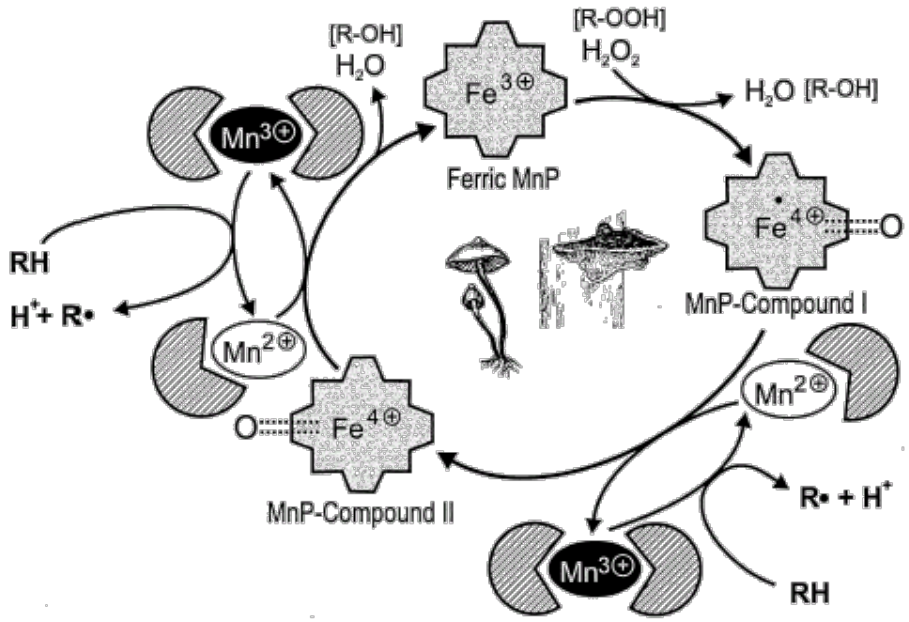

Figure 4. The catalytic cycle of manganese peroxidase (reprinted with permission from Hofrichter, 2002).

VPs

Versatile peroxidases (VPs) are hybrid enzymes that possess both the tryptophan residue found in LiPs as well as Mn-binding sites found in MnPs. They may oxidize both phenolic and non-phenolic compounds, as well as Mn^{2+}. They have been described in several fungi, including *Pleurotus eryngii* and multiple *Bjerkandera* species (Heinfling *et al*., 1998).

Methodology

Historic surveys and studies on the temporal and spatial distribution of soil fungi and the enzymes they produce have mostly relied on the isolation of culturable fungi and the visual detection of those that produce macroscopic fruiting bodies (Clark *et al*., 1980; Gourley, 1983). However, not all fungi are culturable, and for those that are culture-based techniques heavily

bias towards faster-growing saprotrophic species. Further, the appearance of most fungal fruiting bodies is ephemeral, restricted to narrow ranges of temporal and environmental conditions. Many fungal fruiting bodies are also inconspicuous or hypogeous and are consequently overlooked. Accordingly, although historically invaluable for the initial investigations of soil fungi, the current utility of traditional culture and forage-based surveys is limited as these methods are incapable of completely characterizing the full complexity of fungal soil communities.

Recent advances in methodology and instrumentation such as high throughput, massively parallel, next generation sequencing platforms and advanced DNA microarray chips have provided new tools for assessing the membership, structure and functional potential of fungal soil communities (Orgiazzi *et al*., 2013). These new, culture-independent methods based on the characterization of DNA extracts from soil allow a more thorough and encompassing approach to assess microbiotic diversity, and have resulted in the discovery of novel lineages of non-culturable bacteria (Delong and Pace, 2001).

In the present study, the 454 pyrosequencing platform (Roche 454, Life Sciences, Basel, Switzerland) was employed to assess species richness of fungal soil communities. FungChip (Tu et al, 2014), a newly-developed DNA microarray that includes oligonucleotide probes for genes involved in the biogeochemical cycling of C, was employed to detect, identify and quantify the metagenomic potential for lignolytic function of the fungal soil community.

Relevance to current topics in global ecology

Agriculture, human's largest engineered ecosystem, presently covers approximately one third of the global land mass (FAOSTAT, 1999, Antle and Capalbo, 2002), and it is predicted that during the next half-century the global human population will increase 50% to approximately 9 billion—implicitly necessitating the conversion of even more terrestrial ecosystems to agricultural use. Therefore, resulting impacts on the biogeochemical cycling of C and the responses of soil microbes to environmental and human-mediated changes in terrestrial ecosystems are likely to have global consequences. Accordingly, it is imperative to understand how differing land management practices, including the conversion of native ecosystems to agricultural use, as well as subsequent abandonment and succession to the native state, will affect the biology and ecology of those ecosystems, their respective biogeochemical processes, and the overall impact on the global climate. Further, because microbial-mediated transformations of organic materials (e.g. organic inputs of C and N) are major determinants of both crop productivity and climate change, understanding what parameters may affect these transformations and the microbes who mediate them is tantamount to our ability to maximize agricultural yields while simultaneously minimizing environmental damage.

Related areas in soil ecology needing further investigation

There are several topics in soil ecology regarding ligninolytic soil fungi that merit further investigation and development. For example, due to the recent recognition of the widespread presence of ligninolytic genes in ectomycorrhizal fungi, there is currently a debate regarding the relative roles of saprotrophic and ectomycorrhizal fungi in the decomposition of recalcitrant soil

organic matter (Baldrian, 2009; Cullings and Courty, 2009; Talbot *et al*., 2008; Fernandez and Kennedy, 2015). Talbot *et al.* (2013) recently proposed independent roles for saprotrophic and ectomycorrhizal species, finding no correlation between the diversity of saprotrophic fungi in soils and peroxidase activity, hypothesizing that ectomycorrhizal fungi were more important than saprotrophs in degrading recalcitrant, N-rich compounds such as lignocellulose. They posited that saprotrophs were more genetically equipped to degrade large C-rich compounds such as cellulose and pectin, as saprotrophic fungal genomes encode more for labile-C-targeting hydrolases. However, all the ecosystems investigated in their study were forests, and therefore this study cannot explain the roles of various guilds of decomposer fungi in non-forested ecosystems such as grasslands and agricultural fields. It is more probable that different assemblages of fungi, selected by environmental parameters such as the plant community composition within each ecosystem, are responsible for lignin decomposition within those ecosystems.

Fernandez and Kennedy (2015) recently addressed this topic in an article reviewing the "Gadgil effect," whereby saprotrophic and mycorrhizal fungi synergistically provide the system of lignin decomposition in different ecosystems (Gadgil and Gadgil, 1971). According to the Gadgil effect, the competition between ectomycorrhizal and saprotrophic fungi for limiting resources can suppress decomposition rates in ecosystems where both guilds are present. This is due to ectomycorrhizal fungi primarily mining N from organic matter, as these fungi obtain their C from their autotrophic, photobiont partners. This results in a higher C:N ratio in the organic matter of the ecosystem, which causes slower decomposition rates due to the N limitation this places on saprotrophic fungi. However, It is debated whether the two functional

guilds interact antagonistically through competition and niche exclusion, or synergistically through niche differentiation. Because the two different guilds of fungi often cohabitate in the same ecosystems, there is a need to study both their inter- and intra-guild processes and interactions to gain a more comprehensive understanding of what cues and environmental parameters are mediating decomposition processes of the two guilds in differing ecosystems.

The differential decomposition rates explained by the Gadgil effect touches on another related area in soil ecology that bears further investigation, the mechanisms that control C flux versus C sequestration in soils. Current interest in the increase of atmospheric greenhouse gasses, including CO_2, corresponding changes in the global climate and the manner in which human-induced changes may affect the emission of greenhouse gasses, have all led to an increased focus on organic matter decomposition in soils (Averill *et al*.,2014). Soil contains three to four as much C than the atmosphere (Paul *et al*., 2015) and the fungal decomposition of lignocellulose and other organic matter is a key mechanism in the partitioning of C between the soil and the atmosphere. Soil C can either be fluxed as CO_2 through decomposition, or sequestered in the soil. The Gadgil effect predicts that in soils where ectomycorrhizal fungi dominate and mine recalcitrant organic matter for N, C flux will be decreased and organic matter will accumulate due to N limitations on saprotrophic fungi, while in ecosystems where saprotrophic fungi dominate, C flux will occur at a much higher rate. This process, along with the integration of plant-derived C into the rhizosphere, where microbes including saprotrophic and biotrophic fungi assimilate it, and the sequestering of C in fungal exudates and biomass of recalcitrant, legacy hyphae, which may persist in soils for decades (Clemmenson *et al.*, 2013), drives the dynamics of soil C flux and sequestration. Accordingly, maintaining the delicate

balance between C being sequestered or being effluxed as CO_2 (the source or sink dynamics of soil C) is a major concern to those scientists studying the biogeochemical component of global greenhouse effects. Since fungi are at a critical step in the partitioning of C between the soil and atmosphere, and because that partitioning may be altered in response to changes in the community structure of soil fungi, understanding the relationship between land management practices and changes in the fungal community that mediate C partitioning is of particular interest to those studying the flux of greenhouse gasses from terrestrial environments. Understanding the environmental mechanisms that control the dynamics of soil C efflux and sequestration is also essential to accurately predict and model soil ecosystem C dynamics (Talbot *et al*.,2008; Treseder and Holden, 2013) .

Finally, our development of a deeper understanding of the roles of ligninolytic fungi in the environment necessitates developing new, applicable methods for future studies in soil fungal ecology. The combined use of high throughput applications in metagenomics, metatrascriptomics and metalipidomics along environmental gradients, will undoubtedly expand our understanding of how fungi carry out ecosystem processes, and what environmental cues and regulations mediate these processes.

In summary, the study of soil fungi is crucial to research in three priority areas of ecology: biodiversity, ecosystem function and global change. Despite their axiomatic importance, however, fungi have historically been largely excluded from diachronic studies of terrestrial microbiomes, although interest in the roles of fungi in these processes has been increasing over the last few years. Much remains to be learned about the diversity of soil fungi and the processes they are involved in, and the ecosystem controls and environmental factors

influence that their diversity, structure and function. Here, we address these issues in order to understand the impact of human activity on the fungi that provide essential ecosystem services to our Midwestern United States soils.

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II. MOLECULAR ANALYSIS OF FUNGI INHABITING MIDWEST AMERICAN AGRICULTURAL SOILS

ABSTRACT

We examined the taxonomic composition of fungal communities inhabiting the soils of a former oak-hickory forest in the Midwestern United States that has been converted to conventional 3-crop, rotational agriculture systems managed under conventional-till and no-till. The compositions of these communities were compared to each other, to those inhabiting the soils of an early-successional community that had been abandoned from agriculture 22 years prior and to an adjacent native deciduous forest, the original ecosystem from which of all the other treatments originated. We hypothesized that fungal Operation Taxonomic Unit (OTU) richness would mirror the richness of plant litter inputs coming into each system. Accordingly, the agricultural treatments will have less fungal OTU richness than the deciduous forest, and the successional field will have the highest fungal OTU richness. We further hypothesized that tillage would negatively impact fungal OTU richness and that early succession from agricultural land use would positively impact fungal OTU richness. The results show that fungal OTU richness did not statistically vary between any of the treatments we examined, although the composition of the soil fungal community in each treatment was remarkably distinct from that of the others, both in terms of taxonomic membership and dominant ecological niche. The Basidiomycota was found to be the most abundant fungal phylum detected in all treatments. The Zygomycota ranked second in all treatments, except the conventional-till treatment. The Chytridiomycota was the third-most abundant phylum in all treatments except in the conventional-till treatment, in which The Zygomycota was the third-most abundant phylum. The agricultural treatments and early-successional field had a much higher abundance of

Glomeromycota taxa than the deciduous forest. Ecologically, the agricultural treatments and early-successional field were all dominated by saprotrophic fungi, although the taxonomic composition of each treatment was distinct. The deciduous forest was dominated by ectomycorrhizal fungi, with saprotrophic fungi being second in abundance. Plant-root biotrophic fungi were the second-most abundant ecological type of fungi in the early-successional field and were low in abundance in the deciduous forest, but were not detected in either of the agronomic treatments. Interestingly, both of the agronomic treatments were found to have a higher relative abundance of ectomycorrhizal fungi than arbuscular-mycorrhizal fungi, despite a lack of woody photobionts, raising the need to further explore the ecological role of ectomycorrhizal fungi in soils. The differences we found in the fungal soil communities, both in terms of species composition and ecological niche, demonstrate the dynamic nature of fungal soil communities in response to disturbance and land management.

Introduction

Soil microbiota, including fungi, are responsible for most of the fundamental ecological processes that occur in terrestrial ecosystems (Reynolds *et al.*, 2003). However, due in part to human influence, these processes are being impacted and our planet has recently entered into an era of unprecedented species extinction, which is resulting in a reorganization of the Earth's biodiversity (Barnosky *et al*., 2011). A fundamental topic in modern soil ecology related to this reorganization is how human-induced environmental change is influencing the community composition and function of microbes living in terrestrial ecosystems, whether it is by changes in community composition, changes in relative abundance, activity, or both. This topic needs to be addressed in order to develop and maintain a sustainable plan to preserve Earth's natural resources, including its microbiota.

Agricultural land management, spanning over one-third of the Earth's landmass (FAOSTAT, 1999), stands out as humankind's largest engineered ecosystem. It is known that agricultural practices can affect the physical and chemical properties of soil, thereby affecting the activity and abundance of the microbes that live therein (Govaerts *et al*., 2007). However, there remains little information regarding the effect of agricultural land management on the taxonomic composition of fungal soil communities, and whether those communities may undergo further changes and succession when agricultural practices are abandoned. Given the extent of land use being currently devoted to agriculture, as well as that which will be necessarily devoted to agriculture in the future in order to feed an ever-increasing human population, it is critical to understand what changes in fungal and other microbial communities

are manifested by agronomic land management practices in order to develop a sustainable approach to agriculture.

Fungi constitute one of the most diverse groups of eukaryotes on Earth, and can be found in virtually all terrestrial ecosystems (Mueller and Schmidt, 2007). Fungal communities in soil are ecologically heterogeneous assemblages of essential, functional groups including saprotrophs, ecto-and-endo mycorrhizal mutualists, endosymbionts, biotrophs and pathogens (Dix, 1994). Saprotrophic fungi decompose organic inputs into soils such as plant and insect litter. Mycorrhizal symbionts form mutualistic associations with the roots of plants where the fungus scavenges minerals and water from the soil and delivers them to the plant in exchange for photosynthetically-fixed carbon, which the plant roots deliver to the fungus. Endosymbionts live within plants and impact herbivory, host competitiveness, seed germination success, resistance to water and drought stress and resistance to seed predators (de Sassi, *et al.,* 2006; Madej and Clay, 1991; Knoch *et al.*, 1993). Biotrophs establish a long-term feeding relationship with their floral hosts by forming structures called haustoria within plant cells that act as a nutrient drain on the plant (Halbwachs *et al.,* 2013). Pathogenic fungi cause disease in plants, insects and other organisms (Dix, 1994). Despite their noted importance and cosmopolitan presence, however, there is still much to learn regarding the environmental parameters that shape the structure of fungal communities in soils.

Most previous studies addressing the fungal communities of soils have relied on culture-dependent methods and visual censuses of macroscopic reproductive structures (Clark *et al*, 1980; Gourley, 1982). Such methods, however, are extremely biased towards saprotrophic and easily culturable fungi, are dependent on the seasonal production of sporocarps, and are

plagued with problems associated with distinguishing species based on morphological characteristics. Further, the appearances of most fungal fruiting bodies are ephemeral, being restricted to narrow ranges of temporal and environmental conditions. Many fungal fruiting bodies are also inconspicuous or hypogeous, and are consequently overlooked. In summary, culture-dependent and visual census-based methods do not give the depth of discovery necessary to reflect the true fungal diversity of an ecosystem.

With the advent of rapidly advancing molecular techniques, we are now able to obtain a more thorough census and documentation of fungal diversity using a non-culture based approach. To that end, we employed 454 next-generation pyrosequencing technology to examine the impact of converting a native, deciduous forest to agronomic management on the community composition of the resident, soil fungi, as well as the relative impact of till versus no-till management on the fungal community structure in agricultural soils, and what changes occur in early succession when an agricultural site is abandoned.

Two different agricultural land management practices were investigated in this study: a conventionally-tilled, corn/wheat/soybean treatment receiving conventional inorganic enrichment (T1) and a similar no-till corn/wheat/soybean treatment receiving conventional inorganic enrichment (T2) (Table 1). The fungal communities of these treatments were compared to each other and to those of an early successional community abandoned from agriculture 22 years prior to this study (T7), as well as the native, deciduous, oak-hickory forest from which the other treatments originated (DF). We hypothesized that agricultural land management practices such as tillage would negatively impact fungal species richness compared to the native, terrestrial ecosystem, and that succession from agriculture would

positively impact fungal species richness, as tillage and agricultural land management has been shown to negatively affect arbuscular mycorrhizal fungi (AMF) species richness (Oehi *et al*, 2003). Alternately, because above-ground plant species richness has been positively correlated with belowground species richness of bacteria (Eichorst *et al*., 2011), and because different fungi may be functionally capable of metabolizing different litter inputs, we hypothesized that the same would be true for the fungi. Accordingly, fungal OTU richness would be highest in T7>DF>T1=T2, as this corresponds to the recorded level of plant species richness in our test plots, where agronomic practices have lowered plant species richness in comparison to the native deciduous forest. However, early succession has led to an increase in plant species richness in comparison to both the agricultural and native forested treatments. We also hypothesized that edaphic factors including labile carbon (C), $N0_3^-$ and NH_4^+ would vary between treatments and have an impact in shaping fungal community structure in each treatment.

Materials and Methods

Field treatments

The Long Term Ecological Research (LTER) site of the Main Cropping Site Experiment (MCSE) at the W.K. Kellogg Biological Station (KBS) offers an opportune location to study the ecology and relationship between land management strategies and the resident, soil microbiota. There are a broad variety of agronomic treatments laid out on a contiguous landscape, each with its own agro-ecosystem. The physiography of the KBS site is characteristic of a mature, glacial outwash plain and moraine complex. Taxonomically, the soils of the

treatments are Alfisols (Gray Forest soils), formed under deciduous and oak-savanna vegetation, with the texture of a fine-to-course loamy, mixed, mesic, Typic Hapludalf (Paul, *et al.,* 2015). The LTER MCSE is comprised of four annual, rotational crop treatments (corn/soybean/wheat), two perennial systems (continuous alfalfa, *Medicago sativa*) and poplar (*Populus* sp.) clone, an early successional treatment abandoned from agriculture in 1989, and a never-tilled, annually mown perennial, grassland control site. There are also nearby successional fields, abandoned from agriculture for 40 to 60 years, and two different forested sites, one coniferous and one deciduous. The treatments examined in this study are described in Table 1.

Table 1. KBS-LTER treatments investigated in this study[1].

	Treatment	Inorganic N added	Tillage	Cover Crop	Plant Species Richness	Lignin Content	# Replicates
T1	Corn/Wheat/ Soybean Rotation	Yes	Yes	No	Low[2]	Low	6
T2	Corn/Wheat/ Soybean Rotation	Yes	No	No	Low[3]	Low	6
T7	Early Successional Field	No	N/A	N/A	High[4]	Moderate	6
DF	Deciduous Forest	No	N/A	N/A	Moderate[5]	High	3

[1] For further information see: http://lter.kbs.msu.edu/research/long-term-experiments/main-cropping-system-experiment/

[2] No more than 1 plant species per replicate other than crop reported.

[3] No more than 2 plant species per replicate other than crop.

[4] Average of 35.3 plant species per replicate plot.

[5] Average of 11.3 plant species per replicate plot. http://lter.kbs.msu.edu/datatables/40.csv, http://lter.kbs.msu.edu/datatables/182.csv.

Soil sampling

Samples from the November 2011, KBS LTER monthly soil sampling of the MCSE were examined in this study. We sampled after the growing season when the soil fungal communities were likely to be temporally stable, minimizing potential short-term effects associated with tillage (Calderon *et al*., 2000; Calderon *et al*., 2001). Sampling followed the normal, monthly KBS soil sampling protocol (http://lter.kbs.msu.edu/protocols/11). Briefly, two soil cores were extracted from each of five sampling stations per replicate plot. Soils were sampled to a depth of 25 cm using a 2.5 cm diameter soil corer. In the case of the agricultural treatments (T1 and T2), there were an equal number of cores taken within and between each row at 10 cm intervals. After sampling, the cores from each plot replicate were composited, sieved through a 4 mm screen to remove pebbles, and frozen at -80^0 C until downstream processing.

Soil DNA extraction

Total community DNA was extracted from 0.5 grams of each sample on the Eppendorf epMotion 5075 robot using the PowerMag® Soil DNA Isolation Kit (optimized for epMotion), (Mo Bio Laboratories, Carlsbad, CA), according to the manufacturer's instructions. Briefly, 0.5 g of each sample was placed into individual wells of a Eppendorf epMotion 5075 bead plate, to which 750 µl of PowerMag^{tm} Bead Solution and 60 µl PowerMag^{tm} Lysis Solution were added. The bead plate was placed on a 96-well plate shaker (Mixer Mill MM 400, Retsch Corporation, Haan, Germany) and shaken for 10 minutes at a vibrational frequency of 20 Hz (1200 min^{-1}),

rotated 180^0, and shaken for an additional 10 minutes at 20 Hz. The bead plate was then removed and centrifuged at room temperature for 6 minutes at 4,500 x g. The robot transferred the supernatant from each well to a new 96-well plate. 450 µl PowerMag^{tm} Inhibitor Removal Technology Solution was added to each well and the plate was horizontally vortexed for 5 sec, incubated at 4^0C for 10 minutes, and centrifuged at room temperature for 6 minutes at 4,500 x g. The robot transferred 850 µl supernatant to a new 96-well plate and added 850 µl ClearMag^{tm} Binding Solution/ClearMag^{tm} Beads to each well. The plate was then shaken for 10 min and incubated for 15 minutes on a magnet. The beads were washed twice with 500 µl ClearMag^{tm} Wash Solution, and DNA was eluted from the beads with 100 µl ClearMag^{tm} Elution Buffer. DNA was adjusted to 40 ng/µl and used as the template to generate amplicon libraries.

PCR amplification

Amplicon libraries were generated using fused primers that included the template-specific sequence, sample barcode and 454-adapter sequence. Each sample was amplified independently using the primer pair LR3 (***A*xxxxx**CCGTGTTTCAAGACGGG) and LR22 (***B***CCTCACGGTACTTGTTCGCT) (http://sites.biology.duke.edu/fungi/mycolab/primers.htm) where ***A*** is the adapter for the sequencing primer, ***B*** is the adapter for non-sequencing primer, and **xxxxx** is the sample-identifying index.

To account for heterogeneous amplification from the environmental template, and to minimize stochastic amplification biases, three separate 30 µl PCR reactions were performed for each sample and pooled. Each 30 µl PCR reaction contained a cocktail of 20 µl dH_2O, 6 µl 5x master buffer, 0.6 µl 10 mM dNTP mix, 1.1 µl 10 mM LR22 forward primer, 1.1 µl 10 mM LR3

reverse primer, 0.6 µl PHUSION® high-fidelity taq polymerase (NEB, Ipswich, MA, USA) and 0.6 µl of 40 ng/µl DNA template. PCR was performed using the following thermocyling conditions: an initial denaturation of 4 minutes at 94° C followed by 25 cycles at 94° C for 30 s, 60° C for 45 s, and 72° C for 90 s, with a final extension at 72° C for 10 minutes.

PCR products were purified using the AMPure SPRI magnetic PCR Purification system (AgenCourt, Beverly, MA, USA), and DNA content was quantified using a Quant-IT™ assay kit (Life Technologies, Carlsbad, CA, USA). Equimolar quantities of each PCR product (133 ng each) were pooled and submitted for pyrosequencing, which was performed on the 454 GS Junior sequencer (454 Life Sciences, Branford, CT, USA).

Sequencing and Bioinformatics

The samples were divided and sequenced on two 454 GS Junior (Roche 454, Life Sciences, Basel, Switzerland) runs. The data from the two runs was merged, sorted to sample by barcode and then trimmed using the parameters pdiffs=2, bdiffs=1, qwindowaverage=30, qwindowsize=50, maxhomop=8, minlength=200 in mothur v.1.28.0 (Schloss *et al*, 2009). Sequences were then aligned, screened, filtered and pre-clustered in mothur v.1.28.0 (Schloss *et al*, 2009). Chimeras were removed using UCHIME 4.2 (www.drive5.com/uchime), and the sequences were then classified. Taxonomic classification was executed in mothur v.1.28.0 (Schloss *et al*, 2009) using the SILVA LSU ref database release 119 (http://www.arb-silva.de/no_cache/download/archive/release_119/ARB_files/) as well as by submitting sequences to the RDP Classifier (https://rdp.cme.msu.edu/classifier/classifier.jsp). Non-fungal lineages were removed and the two classifications were hand-curated, side-by-side. Where

ambiguities between the two classifications arose, the fasta sequence in question was subjected to National Center for Biotechnology Information's Basic Local Alignment Search Tool's (BLAST's) nucleotide BLAST (http:blast.ncbi.nim.gov/blast/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_KOC=blasthome) to resolve the classification. For each such query, the 10 best-matching references were used to annotate the sequences as accurately as possible. OTUs were categorized to ecological niche based on current knowledge of the metabolic lifestyle of each individual taxa.

Rarefaction, alpha and beta analyses including the generation of a Venn diagram to visually depict beta diversity between the treatments were calculated and performed in mothur v.1.28.0 (Schloss *et al*, 2009). All other statistical analyses including non-parametric multivariate analysis of variance (NPMANOVA)(Anderson, 2001), used to test whether groups have similar distributions, analysis of similarities (ANOSIM)(Clark, 1993), used to test the significance of difference between one or more groups, and ordination analyses, including both principal components (PCoA)(Davis and Sampson 1986) and non-metric multidimensional scaling (NMDS)(Taguchi and Oono, 2005) with regressions to help visually demonstrate both similarity in membership of the communities and correlated edaphic factors were performed in PAST (Hammer et al., 2001). The Jaccard similarity index was used to calculate similarity of the sample sets for all ordination analyses (Jaccard, 1912).

Permanganate oxidizable carbon (POXC)

The POXC content of each sample was determined as previously described (Culman *et al*., 2012). Briefly, 2.5 g of air-dried soil of each sample was placed in 50 ml centrifuge tubes. Deionized water (18 ml) and $KMnO_4$ (2 ml, 0.2 M) were added, and the tubes were capped and

shaken by hand vigorously for 2 sec to assure soil dispersion. The tubes were then shaken for 2 minutes at 240 oscillations per minute. The tubes were subsequently allowed to settle for 10 minutes. Supernatant (0.5 ml) from each tube was transferred to centrifuge tubes containing 49.5 ml deionized water. Absorbance was measured at 550 nm.

To calculate the POXY content of each sample we employed the following formula:

POXC (mg kg^{-1} soil) = [0.02 mol/ L - (a + b × Abs)] × (9000 mg C/ mol) × (0.02 L solution/ Wt)

Where: 0.02 mol/L = initial solution concentration

a = intercept of the standard curve

b = slope of the standard curve

Abs = absorbance of unknown

9000 = milligrams of carbon oxidized by 1 mole of MnO4 changing from Mn^{7+} → Mn^{4+}

0.02 L = volume of stock solution reacted

Wt = weight of air-dried soil sample in kg

Methodological considerations

The 5' end of the 28S rDNA gene was chosen here for the assessment of taxonomic diversity as it has highly conserved regions that can be used for primer selection, has hypervariable domains (e.g. D2) flanked by the highly conserved regions that allow taxonomic resolution of most sequences to the level of genus, and does not have the degree of intra-species sequence hypervariabilty associated with the ITS region. Here, a section of the 5' end of the 28S rDNA gene containing the D2 hypervariable region was targeted for amplification using the primer set LR22 and LR3 (http://sites.biology.duke.edu/fungi/mycolab/primers.htm).

Although similar surveys of bacteria usually employ the small-subunit (SSU) rDNA cistron for taxonomic assessment, this gene does not evolve rapidly enough in eukaryotes to be reliably used, except for higher-level taxonomic assessment (Vandenkoornhuyse *et. al*, 2002). Further, the ITS region, which has been proposed by some to be the "fungal barcode" (Schoch *et. al*, 2012), has recently been demonstrated to have intraspecies hypervariabilty, due in part to the polycistronic nature of fungal rDNA operons, and this hypervariability may be more widespread then presently realized (Li *et al*., 2014).

Differential DNA extraction efficiencies, due in part to differences in cell-wall composition, as well as the binding of DNA to clay particles and SOM, may influence the efficiency of DNA extraction from soil (Martin-Laurent et al, 2000). The choice of primers and gene (or segment thereof) used for taxonomic classification can also introduce bias (Bellemain *et al.*, 2010), as well as the parameters used for PCR (Feinstein et al, 2009). Finally, the downstream processing of data, depending on what software and what taxonomic reference database is used (and misannotation therein) can also introduce bias, as can the choice of values for parameters in bioinformatics processing.

Results

Bioinformatics

Merging the samples from the two 454 GS junior runs resulted in a combined total of 161,204 sequences. Trimming resulted in 90,290 total sequences, 58,743 of which were unique Alignment, screening and filtering further reduced the dataset to 70,570 total sequences and 41,916 unique sequences. Preclustering reduced the number of unique sequences to 35,237. Removal of chimeric sequences resulted in 70,334 total sequences and 35,046 unique

sequences. Removal of non-fungal lineages gave a final total of 41,547 total sequences, 16,738 of which were unique.

Operational taxonomic units (OTUs) and rarefaction analysis

OTUs were assigned at a 97% sequence similarity level. This level was optimal based on the rate of taxon accumulation as a function of sequence similarity (Figure 5). OTU richness had the highest stability in this area, reaching an increase or decrease of near-exponential growth just above or below this level, respectively.

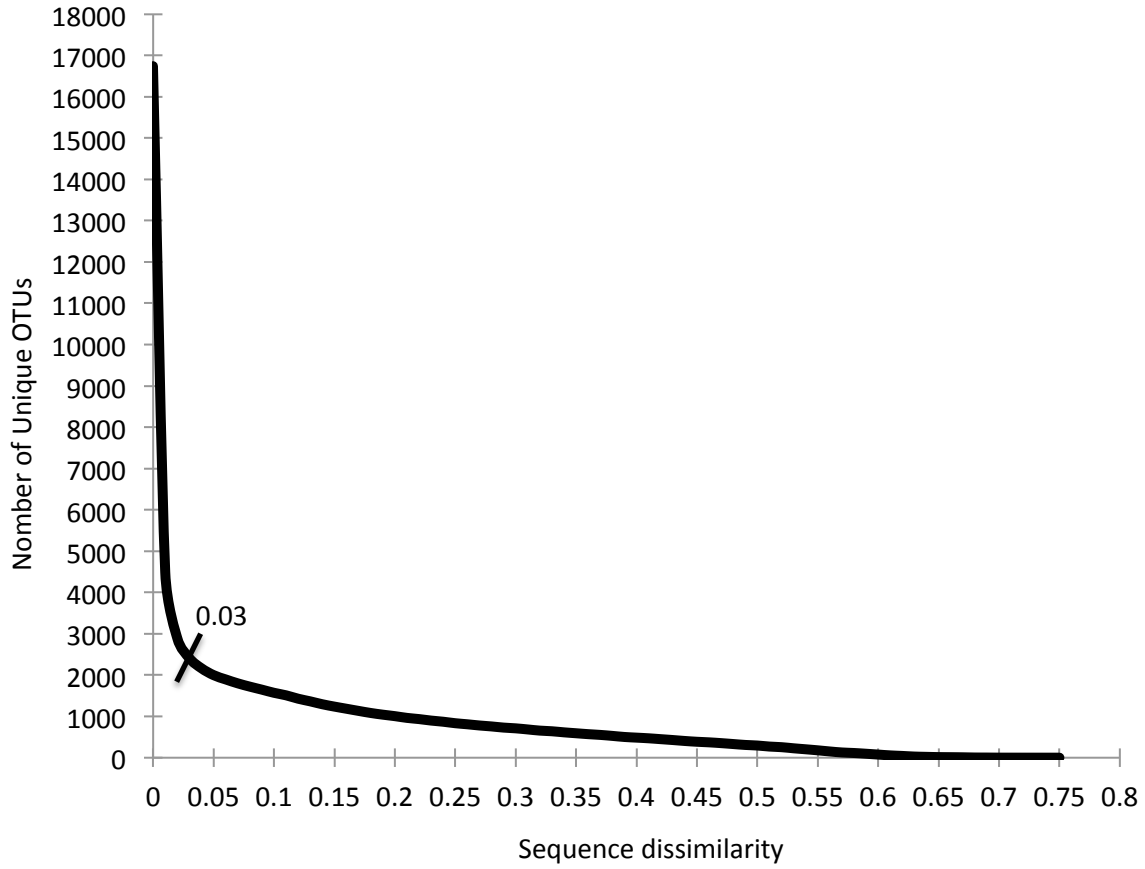

Figure 5. Operational Taxonomic Unit (OTU) richness as a function of sequence dissimilarity. A criterion of 0.03 sequence dissimilarity for assigning OTUs is shown.

There were 2407 fungal OTUs detected over all treatments including singletons and doubletons. Removing singletons resulted in 1305 OTUs, and removing doubletons decreased the count to 945 OTUS. Rarefaction analysis of the OTUs assigned at 97% similarity showed fungal richness from each treatment approaching a plateau, but not completely saturating (Figure 6).

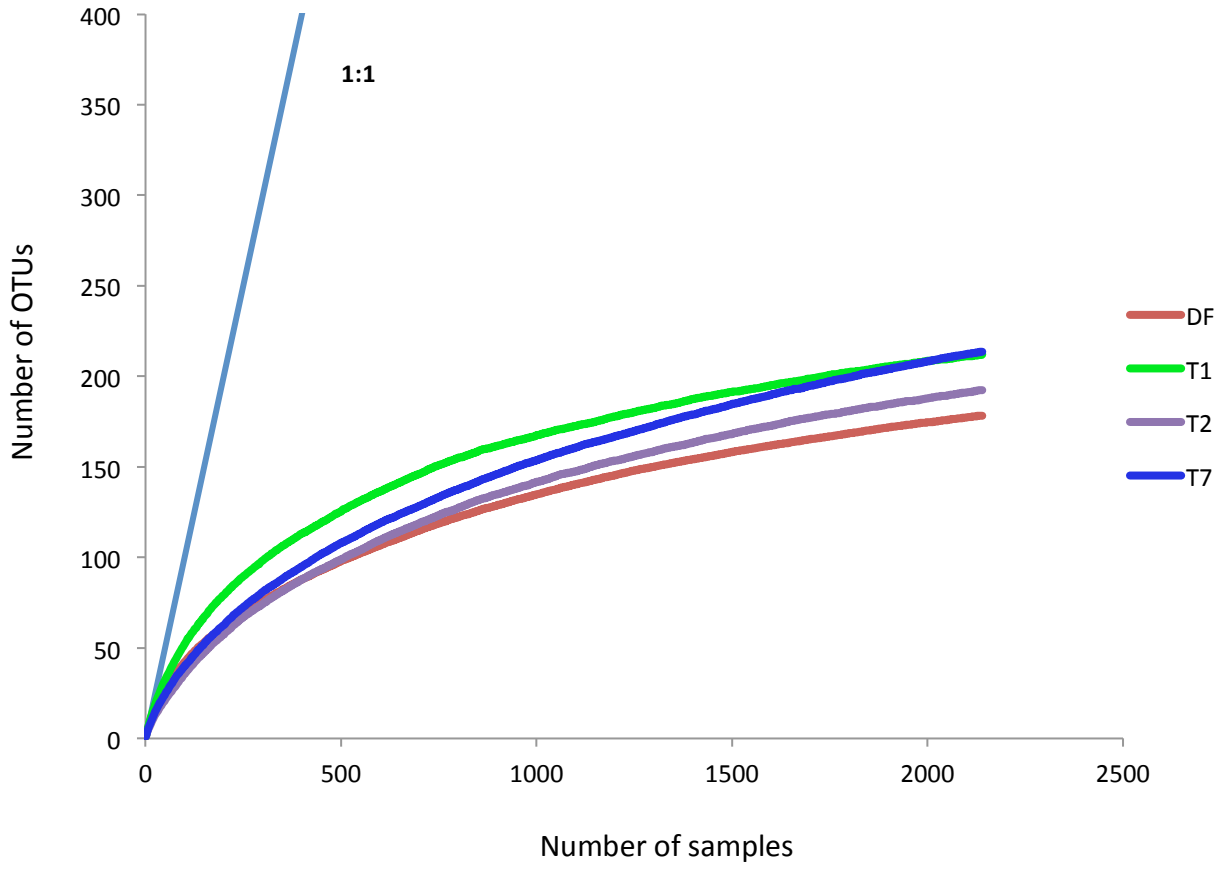

Figure 6. Fungal OTU rarefaction curves for all treatments. For interpretation of the references to color in this and all other Figures, the reader is referred to the electronic version of this dissertation.

Alpha diversity

OTUs in each treatment

There were an average of 196 ± 23 OTUs detected in each sample from T1, 167 ± 36 OTUs from T2, 179 ± 89 OTUs from T7, and 202 ± 14 OTUs from each sample of DF (Table 2). There was no statistically significant difference in the total number of OTUs detected in the samples across all treatments. Average estimated sample diversity was measured by the Chao 1 index, ACE and Jackknife, and the Simpson's Diversity Index was employed as a measurement of OTU evenness in the treatments.

Beta diversity

To understand how fungal OTUs were distributed among the treatments, we characterized the differences and similarities of fungal OTU membership between the treatments. We found 112 OTUs common to all treatments (see Table 3 and Figure 7). The agronomic treatments had 345 OTUs in common, making them the most similar treatments as far as species composition. Each had a similar number of unique OTUs, with T1 having 74 unique OTUs and T2 having 73. T7 had 129 unique OTUs, almost twice as many than either of the agricultural treatments. T2 shared a third more OTUs with T7 than T1. DF had more unique OTUs than either of the agricultural treatments. Because there were only three reps for DF, compared to six reps for T7, a comparison between these two treatments is not included here.

Table 2. Average OTUs detected, diversity and evenness indices. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatment	Average Detected OTUs Per Sample (>N=2)	Average Chao-1 Per Sample	Average ACE Per Sample	Average Jackknife Per Sample	Simpson's Diversity (1-D) Treatment Ave.
T1	T1-1 = 212 T1-2 = 230 T1-3 = 200 T1-4 = 169 T1-5 = 175 T1-6 = 190 Total Ave. = 196 ± 23	254 ± 24	262 ± 25	279 ± 32	0.035 ± 0.009
T2	T2-1 = 171 T2-2= 197 T2-3= 216 T2-4= 161 T2-5 = 139 T2-6 = 118 Total Ave. =167 ± 36	225 ± 45	241 ± 51	250 ± 54	0.055 ± 0.065
T7	T7-1 = 352 T7-2 = 160 T7-3 = 188 T7-4 = 97 T7-5 = 148 T7-6 = 131 Total Ave. = 179 ± 89	232 ± 43	232 ± 104	265 ± 136	0.129 ± 0.095
DF	DF-1 = 204 DF-2 = 187 DF-3 = 214 Total Ave. = 202 ± 14	236 ± 13	241 ± 15	252 ± 14	0.047 ± 0.008

Table 3. Unique and shared OTUs from combined replicates of each treatment. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatment	Unique OTUs	Treatments	Shared OTUs	Treatments	Shared OTUs
T1	74	T1, T2	345	T1, T2, T7	212
T2	73	T1, T7	186	T1, T2, DF	144
T7	129	T1, DF	193	T1, T7, DF	138
DF	116	T2. T7	279	T2, T7, DF	131
---	---	T2, DF	178	T1, T2, T7, DF	112
---	---	T7, DF	186	---	---

Taxonomy and relative abundance of fungal phyla in each treatment

Over all treatments, the Phylum Basidiomycota was the richest in terms of numbers of OTUs, with a total of 423 OTUs being detected, comprising 72.26% of all OTUs (Table 4). The Basidiomycota was also the most abundant phylum in all treatments in terms of numbers of sequence reads (Table 6 and Figure 8). Seven different phyla of fungi were represented in the OTUs detected over all treatments. 228 OTUs could not be classified below the kingdom level. T7 had the largest diversity of fungal phyla with 7 different phyla being present. Each of the other treatments had 6 different phyla present. Unclassified fungi accounted for 4.09% of all reads in T1, 3.86% in T2, 2.76% in T7 and 2.49% in DF (Table 6).

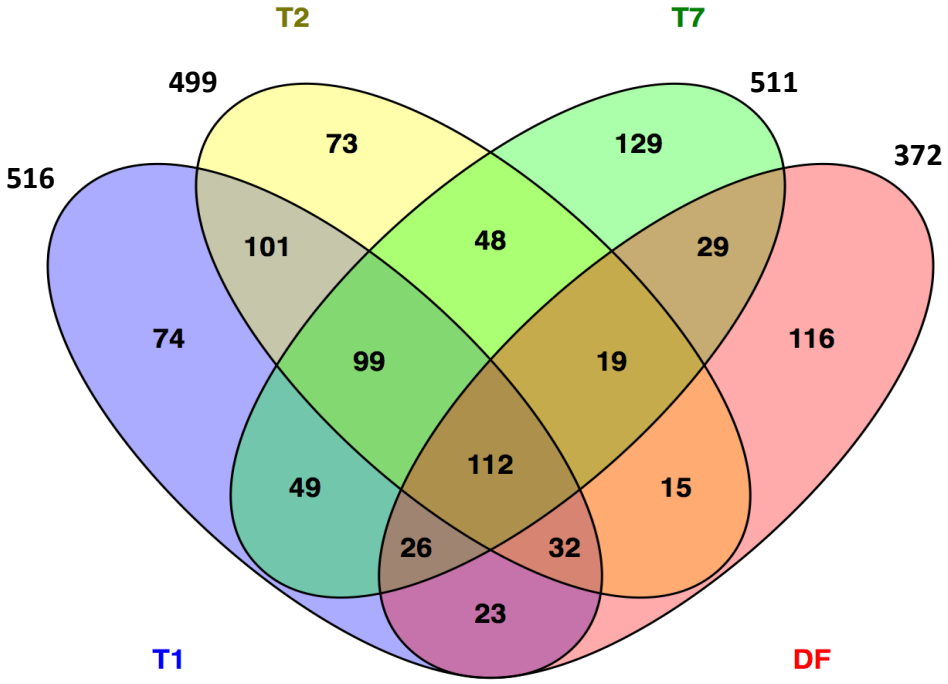

Figure 7. Venn diagram of shared and unique fungal OTUs across each treatment. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Table 4. Number of OTUs detected from each fungal phylum across all treatments.

# of OTUs	Phylum	Percentage of Total
423	Basidiomycota	72.26
143	Chytridiomycota	8,3
71	Zygomycota	7.5
56	Glomeromycota	2.21
21	Blastocladiomycota	1.8
4	Ascomycota	0.15
2	Entomophthoromycota	.03
1	Neocallimastigomycota	0.07
228	Unclassified	3.22

Table 5. Taxonomic assignment and ecological niche of the top 20 OTUs from each treatment. S = Saprotroph, BT = Biotroph, EM = Ectomycorrhizal, AMF = Arbuscular Mycorrhizal, P = Parasite/Pathogen, B = Basidiomycota, BL = Blastocladiomycota, C = Chytridiomycota G = Glomeromycota, Z = Zygomycota.

	T1				T2		
	OTU #	Niche	Genus (Phylum)		OTU #	Niche	Genus (Phyla)
1	Otu002	S	*Daedaleopsis* (B)	1	Otu004	BT	*Conocybe* (B)
2	Otu003	S	*Gamsiella* (B)	2	Otu002	S	*Daedaleopsis* (B)
3	Otu006	S	*Mycena* (B)	3	Otu003	S	*Gamsiella* (Z)
4	Otu013	S	*Lentinula* (B)	4	Otu028	BT	*Conocybe* (B)
5	Otu017	S	*Bjerkandera* (B)	5	Otu014	S	*Mortierella* (Z)
6	Otu030	S	*Trechispora*(B)	6	Otu006	S	*Mycena* (B)
7	Otu014	S	*Mortierella* (Z)	7	Otu013	S	*Lentinula* (B)
8	Otu031	S	*Pholiota*(B)	8	Otu010	S	*Mortierella* (Z)
9	Otu020	P	*Cryptococcus* (B)	9	Otu051	P	*Tremella* (B)
10	Otu007	S	*Mortierella* (Z)	10	Otu017	S	*Bjerkandera* (B)
11	Otu024	P	*Cryptococcus* (B)	11	Otu055	P	*Catenomyces* (BL)
12	Otu012	P	*Tremella* (B)	12	Otu034	S	*Conidiobolus* (Z)
13	Otu035	S	*Pleurotus* (B)	13	Otu007	S	*Mortierella* (Z)
14	Otu036	S	*Coprinopsis* (B)	14	Otu029	S	*Malassezia* (B)
15	Otu060	S	*Mortierella* (B)	15	Otu052	S	*Boothiomyces*(C)
16	Otu029	S	*Malassezia* (B)	16	Otu022	S	*Mortierella* (Z)
17	Otu044	AMF	*Paraglomus* (G)	17	Otu020	P	*Tremella* (B)
18	Otu021	EM	*Scleroderma* (B)	18	Otu044	AMF	*Paraglomus*(G)
19	Otu010	S	*Mortierella* (B)	19	Otu012	P	*Tremella* (B)
20	Otu039	P	*Catenomyces* BL)	20	Otu071	S	*Clitocybe* (B)

Table 5 continued

	T7				DF		
	OTU #	Niche	Genus (Phylum)		OTU #	Niche	Genus (Phyla)
1	OTU 1	S	*Hygrocybe* (B)	1	Otu011	EM	*Russula* (B)
2	OTU 5	S	*Hygrocybe* (B)	2	Otu008	S	*Monoblepharidales* (C)
3	OTU 9	S	*Inocephalus* (B)	3	Otu016	EM	*Hydnellum* (B);
4	OTU 15	S	*Coprinopsis*(B)	4	Otu018	EM	*Inocybe* (B)
5	OTU 19	S	*Clitopilus*(B)	5	Otu010	S	*Mortierella* (Z)
6	OTU 7	S	*Mortierella*(Z)	6	Otu027	EM	*Russula*(B)
7	OTU 12	P	*Tremella* (B)	7	Otu032	EM	*Russula* (B)
8	OTU 26	EM	*Hydnellum* (B)	8	Otu023	EM	*Xerocomus* (B)
9	OTU 3	S	*Gamsiella*(Z)	9	Otu033	EM	*Inocybe* (B)
10	OTU 37	EM	*Cortinarius*(B)	10	Otu021	EM	*Scleroderma* (B)
11	OTU 20	P	*Tremella*(B)	11	Otu025	S	*Umbelopsis*(Z)
12	OTU 42	S	*Stropharia* (B)	12	Otu041	S	*Mortierella* (Z)
13	OTU 2	S	*Daedaleopsis* (B)	13	Otu043	EM	*Xerocomus* (B)
14	OTU 14	S	*Mortierella* (Z)	14	Otu003	S	*Gamsiella*(B)
15	OTU 6	S	*Mycena* (B)	15	Otu045	EM	*Inocybe* (I)
16	OTU 53	S	*Rhizophlyctis* (C)	16	Otu046	EM	*Hydnellum* (B)
17	OTU 22	S	*Mortierella* (Z)	17	Otu007	S	*Mortierella* (Z)
18	OTU 62	EM	*Craterellus* (B)	18	Otu022	S	*Mortierella* (B)
19	OTU 34	S	*Conidiobolus* (Z)	19	Otu006	S	*Mycena* (B)
20	OTU 66	S	*Clavaria* (B)	20	Otu049	EM	*Sebacina* (B)

Relative abundance of fungal ecological niches in each treatment

The saprotrophic members of the soil fungal community were more abundant than members of any other type of ecological niche in terms of numbers of reads in T1, T2 and T7 (Table 7 and Figure 9) and ectomycorrhizal OTUs were most abundant in DF, with saprotrophic OTUs being second-most abundant.

T7 had the most diverse ecological types of fungi, with 6 different types being present. T1, T2 and DF each had 4 different ecological types of fungi presented in their soil communities

Table 6. Numbers and relative abundances of fungal phyla in each treatment. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatment	Phylum	# OTUS	# Reads	% OTUs	% Reads
T1	Basidiomycota	216	6037	41.86	68.70
	Zygomycota	39	838	7.56	8.33
	Chytridiomycota	114	1140	22.09	12.97
	Glomeromycota	29	240	5.62	2.73
	Blastocladiomycota	10	263	1.94	2.99
	Ascomycota	1	16	0.19	0.18
	Unclassified	107	354	20.74	4.09
T2	Basidiomycota	220	4922	44.0	64.58
	Zygomycota	44	924	8.80	12.12
	Chytridiomycota	94	854	18.8	11.21
	Glomeromycota	34	297	6.80	3.90
	Blastocladiomycota	13	291	2.60	3.82
	Ascomycota	3	39	0.60	0.51
	Unclassified	92	294	18.40	3.86
T7	Basidiomycota	250	9606	48.92	75.79
	Zygomycota	50	1611	9.78	12.71
	Chytridiomycota	73	693	14.29	5.47
	Glomeromycota	31	291	6.07	2.34
	Blastocladiomycota	11	84	2.15	0.66
	Neocallimastigomycota	1	28	0.20	0.22
	Entomophthoromycota	2	12	0.39	0.09
	Unclassified	93	350	18.20	2.76
DF	Basidiomycota	193	7642	51.88	72.61
	Zygomycota	30	1540	8.06	14.63
	Chytridiomycota	60	964	16.13	9.16
	Glomeromycota	17	56	4.57	0.53
	Blastocladiomycota	5	58	1.34	0.55
	Ascomycota	1	3	0.27	0.03
	Unclassified	66	262	17.74	2.49

Table 7. Numbers and relative abundances of fungal ecological niches in each treatment. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatment	Ecological Niche	# OTUS	# Reads	% OTUs	% Reads
T1	Saprotroph	269	6502	52.13	73.15
	Ectomycorrhizal	66	827	12.79	9.30
	Arbuscular-mycorrhizal	29	240	5.62	2.70
	Pathogen	45	965	8.72	10.86
	Unclassified	107	354	20.74	3.98
T2	Saprotroph	262	5657	52.4	74.23
	Ectomycorrhizal	59	494	11.80	6.48
	Arbuscular-mycorrhizal	34	297	6.80	3.90
	Pathogen	53	879	10.60	11.53
	Anaerobic Saprotroph	1	28	0.20	0.22
	Unclassified	92	294	18.40	3.86
T7	Saprotroph	269	6680	52.64	52.70
	Ectomycorrhizal	68	1466	13.31	11.57
	Arbuscular-mycorrhizal	31	291	6.07	2.30
	Pathogen	47	1122	9.20	8.85
	Biotroph	2	2738	0.39	21.60
	Anaerobic Saprotroph	1	28	0.20	0.22
	Unclassified	93	350	18.20	2.76
DF	Saprotroph	186	4385	50	41.66
	Ectomycorrhizal	68	5448	18.28	51.76
	Arbuscular-mycorrhizal	17	56	4.57	0.53
	Pathogen	34	372	9.14	3.53
	Biotroph	1	2	0.27	0.02
	Unclassified	66	262	17.74	2.49

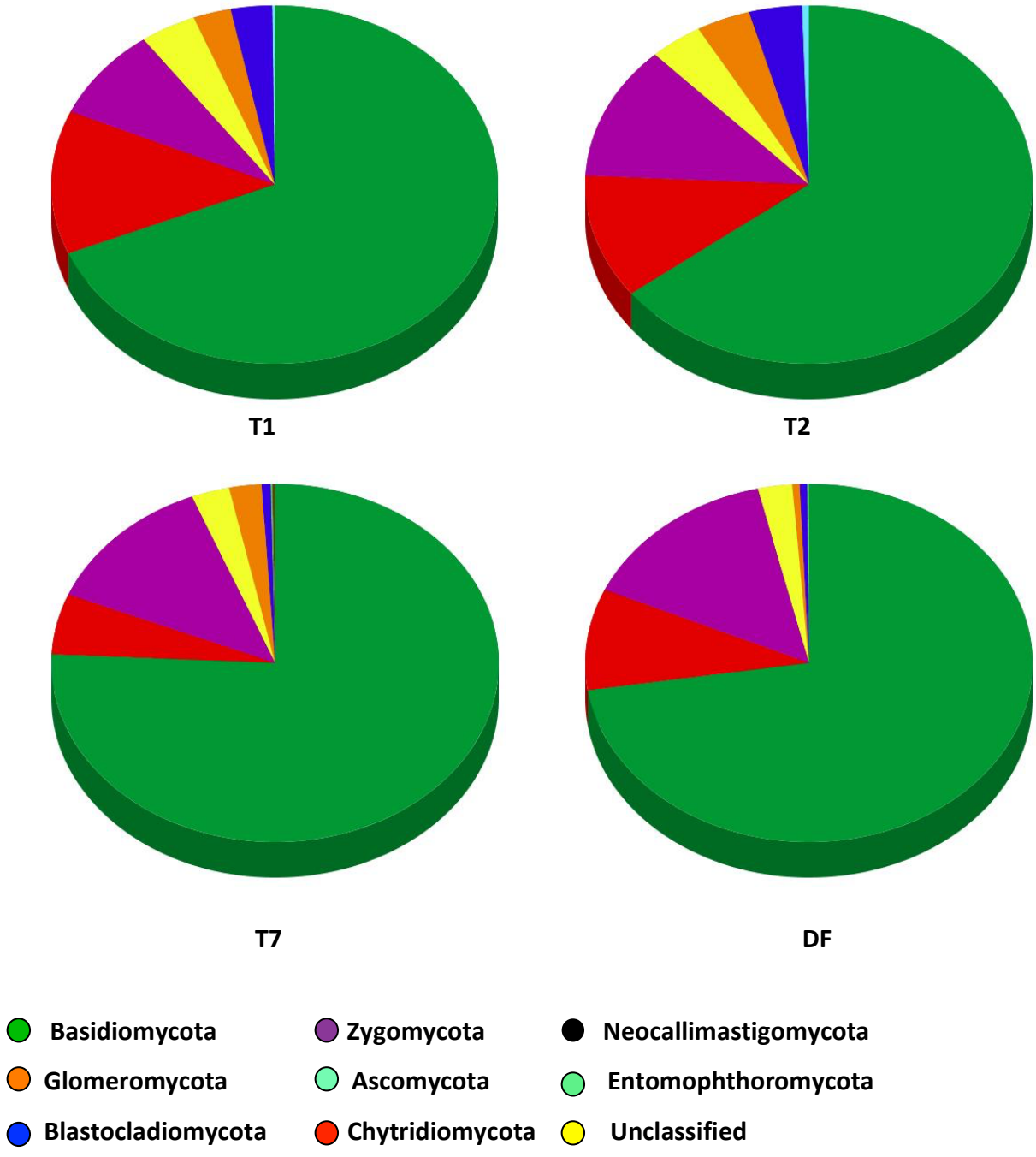

Figure 8. Fungal phyla in each treatment. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

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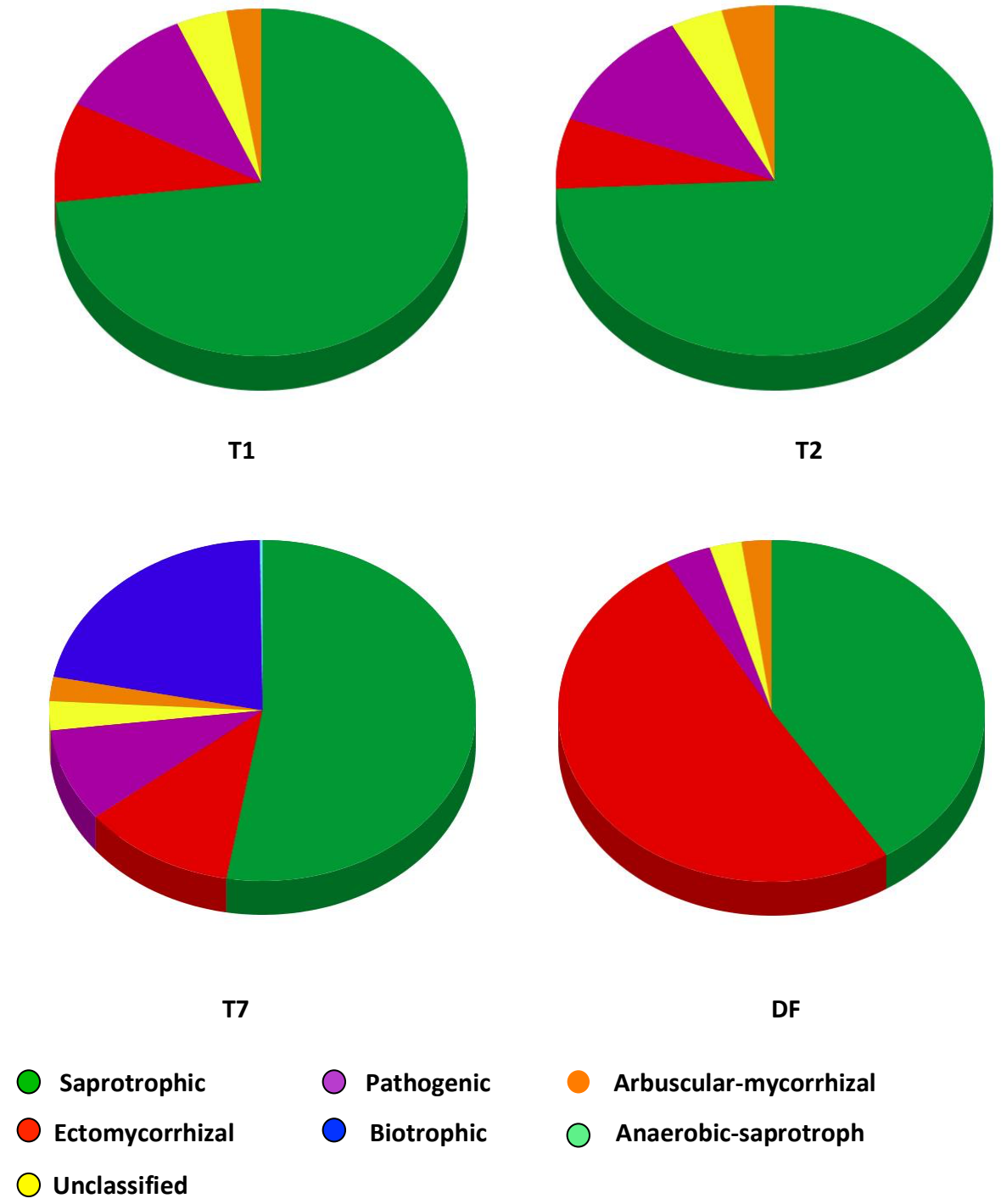

Figure 9. Fungal niches in each treatment. Numbers indicate percentages. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Community structure

Principal components (PCoA) and non-metric multidimensional scaling (NMDS) ordination analyses demonstrate the distinct community structure of each of treatment (Figures 10 and 11). The replicate samples from each treatment clustered together, and independent of the samples from other treatments. NPMANOVA and ANOSIM tests indicate the differences in community structure were statistically significant ($p < 0.05$) (Table 8).

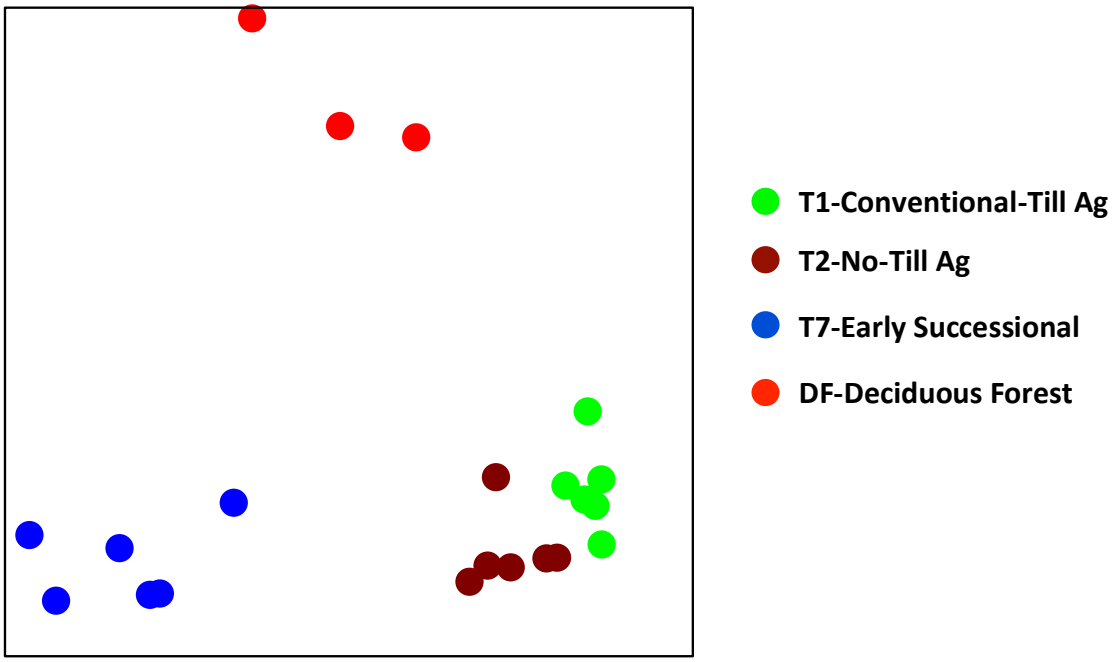

Figure 10. PCoA ordination analysis of species composition by treatment using the Jaccard similarity coefficient. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Table 8. NPMANOVA and ANOSIM p-values for comparisons between treatments using Jaccard similarity coefficient. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatments	NPMANOVA	ANOSIM
T1 vs. T2	0.0147	0.0351
T1 vs. T7	0.0034	0.0025
T1 vs. DF	0.0129	0.0117
T2 vs. T7	0.0002	0.0030
T2 vs. DF	0.0130	0.0128

When examining the edaphic factors that were correlated to the community structure of each treatment, nitrate was found to have the strongest correlation to the agricultural treatments T1 and T2 (Figure 12 and Table 9). POXC, the labile component of total C which is the most

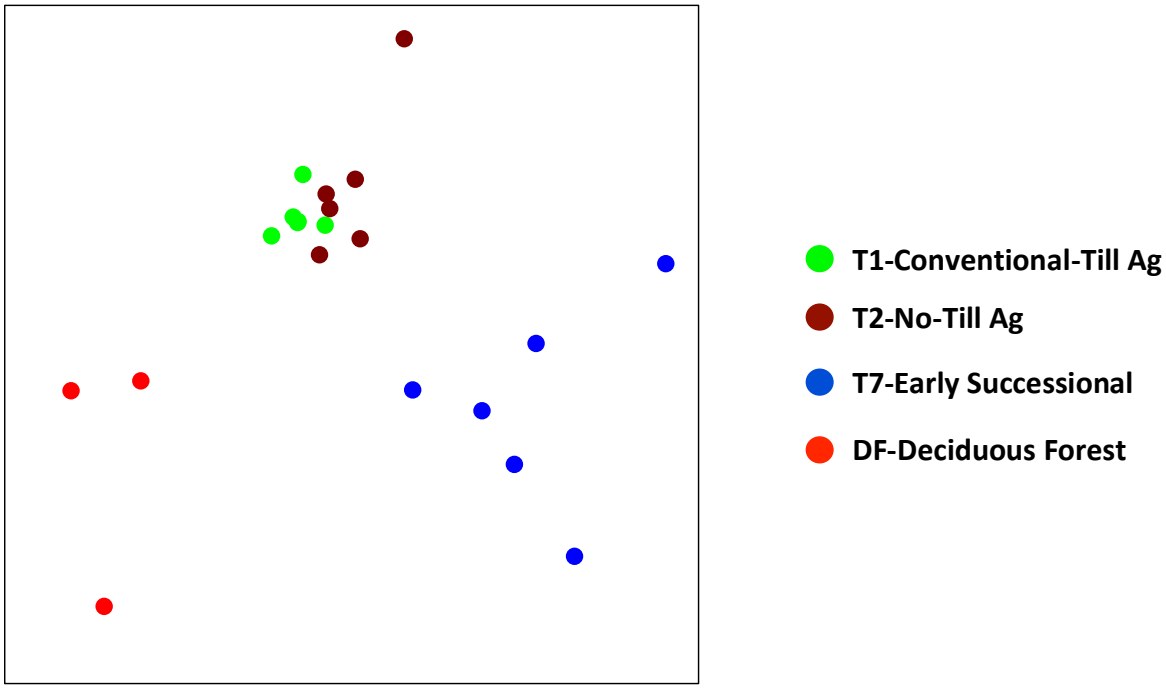

Figure 11. NMDS ordination analysis of species composition by treatment using the Jaccard coefficient.

available C fraction for microbial assimilation, was strongly correlated with separating the structures of the early successional (T7) and forested (DF) sites from T1 and T2, and ammonium was a strongly correlated with separating the fungal community structure of T7.

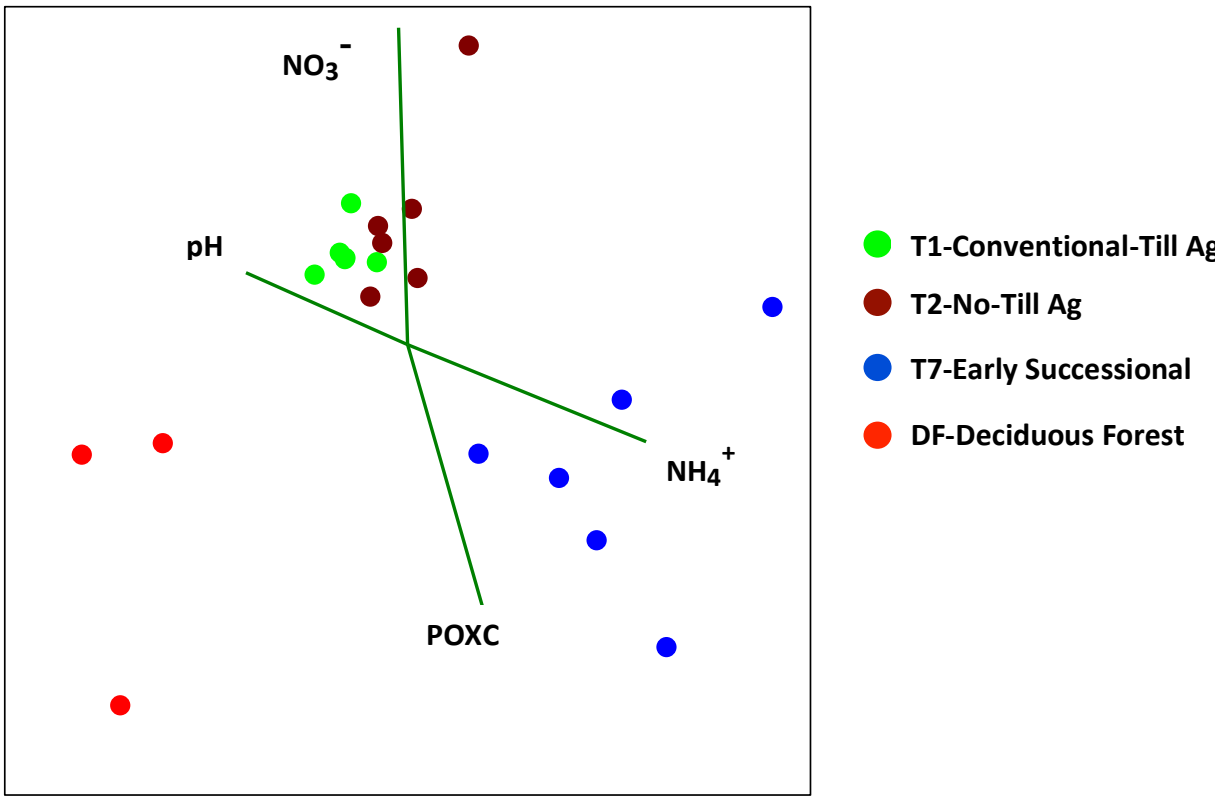

Figure 12. NMDS ordination analysis showing the correlation of nitrate (NO_3^-), ammonium (NH_4^+), pH and permanganate oxidizable carbon (POXC) with the structures of the fungal communities in each treatment.

Table 9. Values for edaphic factors correlated to fungal OTU composition in the replicate treatment plots. POXC analysis was performed on subsamples of the soils used for DNA extraction in the fungal community analysis. Nitrate, ammonium and pH values derived from KBS data tables (http://lter.kbs.msu.edu/datatables/).[1] T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatment	POXC	pH	Nitrate	Ammonium
DF_1	459.99	6.57	0.43	2.57
DF_2	489.16	6.59	0.33	2.61
DF_3	621.12	6.58	1.22	3.41
T1_1	326.9	6.42	1.19	1.42
T1_2	351.81	6.15	1.16	1.32
T1_3	380.8	6.56	1.74	1.65
T1_4	321.97	6.22	1.13	1.37
T1_5	268.95	6.29	0.97	1.3
T1_6	330.75	6.4	1.77	2.04
T2_1	414.71	5.97	1.2	1.66
T2_2	354.71	6.34	1.37	1.49
T2_3	432.09	6.47	1.18	1.62
T2_4	378.65	6.35	1.03	1.53
T2_5	394.39	6.45	0.79	1.76
T2_6	386.1	6.49	0.98	1.52
T7_1	530.08	6.87	0.23	4.49
T7_2	552.36	6.57	2.03	14.61
T7_3	505.26	6.39	0.42	3.08
T7_4	523.12	5.76	0.54	4.06
T7_5	527.5	5.71	0.24	3.72
T7_6	462.73	5.95	0.5	4.76

[1]Values from the KBS data tables were derived from analyses performed on subsamples of the same soils collected during the November 2011, KBS LTER monthly soil sampling of the MCSE that were used for POXC and DNA extraction.

Similarity percentage (SIMPER) analysis

We identified the taxa responsible for the majority of differences between the fungal OTU compositions among the treatments by conducting a SIMPER analysis (Clark, 1993), which compares the differences in the abundances of each taxa among treatments, and identifies which taxa best explain the differences in the compositions among all treatments and between each pair of treatments. We looked at which taxa were responsible for the overall differences among all treatments (Table 10), as well as those taxa that were driving differences between each pair of treatments (Table 11). The main fungal taxa driving differences among all the treatments were *Hygrocybe* OTUs,

Table 10. Top 10 OTUS driving differences between all treatments.

OTU #	Genus (Phylum)[1]	% Contribution
1	Hygrocybe (B)	4.42
2	Daedaleopsis (B)	3.50
5	Hybgrocybe (B)	2.91
3	Gamsiella (Z)	2.69
4	Conocybe (B)	2.43
12	Tremella (B)	2.12
8	Monoblepharidales (C)	1.84
6	Mycena (B)	1.70
9	Inocephalus (B)	1.68
13	Lentinula (B)	1.45

[1] B= Basidiomycota, C = Chytridiomycota, Z = Zygomycota

with 2 different OTUs in the top 3 taxa responsible for the differences in composition between all the treatments. The top taxa found in T7 was *Hygrocybe* OTU 1, which was the main driver of taxonomic differences between T7 and all the other treatments. When comparing T7 and T2*, Hygrocybe* OTUs were the top 2 taxa responsible for differences between the treatments, and a *Conocybe* OTU was the third. The main taxa driving differences between the agronomic treatments, T1 and T2, were *Conocybe*, *Daedaleopsis* and *Trechispora*. *Monoblepharidales* and *Gamsiella* were mainly responsible for differences between T1 and DF, while *Hygrocybe*, *Monoblepharidales* and *Daedaleopsis* drove differences between T2 and DF. The difference in taxonomic composition between T7 and DF was mainly attributable to two *Hygrocybe* OTUs and a *Monoblepharidales.*

Table 11. Top three OTUs driving differences between each pair of treatments.

Treatments OTU #	Genus (Phylum)[1]	% Contribution	% Abundance	% Abundance
T1 vs.T2			T1	T2
OTU 4	Conocybe (B)	5.66	0.08	16.1
OTU 2	Daedaleopsis (B)	3.46	10.16	5.05
OTU 30	Trechispora (B)	2.95	3.24	0.17
T1 vs. T7			T1	T7
OTU 1	Hygrocybe (B)	7.65	0	11.9
OTU 5	Hygrocybe (B)	5.11	0	9.62
OTU 2	Daedaleopsis (B)	5.02	10.16	1.41
T1 vs. DF			T1	DF
OTU 2	Daedaleopsis (B)	5.37	10.16	0.28
OTU 8	Monoblepharidales (C)	4.08	0.42	5.44
OTU 3	Gamsiella (Z)	3.04	6.66	1.82
T2 vs. T7			T2	T7
OTU 1	Hygrocybe (B)	7.57	0.034	11.9
OTU 5	Hygrocybe (B)	5.05	0.04	9.62
OTU 4	Conocybe (B)	4.15	16.1	0.11
T2 vs. DF			T2	DF
OTU 4	Conocybe (B)	3.98	16.1	0
OTU 8	Monoblepharidales (C)	3.92	0.80	5.44
OTU 2	Daedaleopsis (B)	3.10	5.05	0.28
T7 vs. DF			T7	DF
OTU 1	Hygrocybe (B)	6.93	11.9	0
OTU 5	Hygrocybe (B)	4.63	9.62	0
OTU 8	Monoblepharidales (C)	3.78	0.80	5.44

[1] B= Basidiomycota, C = Chytridiomycota, Z = Zygomycota

Discussion

This study investigated the impact of land management and richness of plant litter inputs on the taxonomic richness of fungi in residing in conventional-till and no-till, 3-crop rotational agriculture soils, in a native deciduous forest soil, and in an early-successional field soil of the Midwestern United States. Contrary to our hypotheses, we found that differing land management practices and richness of plant litter inputs did not have a significant impact on overall fungal OTU richness in Midwestern United States soils. The treatments on the contiguous landscape of the LTER MCSE and adjacent forest were shown to house similar numbers of fungal OTUs. However, other studies have noted differences in the fungal carrying capacities of soils under different management. Buée *et al*. (2009) used 454 pyro-sequencing technology to study fungal soil communities of oak, beech and Douglas-Fir plantations. They detected 1000 fungal OTUS in the soil of the oak plantation, while detecting only 590 OTUs in the same volume of soil from the beech plantation. However, the soils in that study were not on a continuous landscape, and differences in the physical and chemical properties of the soils likely influenced the fungal richness of the different sites. Similarly, Penton *et al*. (2013), found 351 to 594 genera of fungi in several Oklahoma grassland soils, and 87 to 351 genera in several Alaska permafrost soils. Again, the sample sites in the Penton *et al*. (2013) study were not on a contiguous landscape, as they were in the present study, and the differing physical and chemical properties of each soil likely played a role in how those communities were structured and how many OTUs were detected in each site.

We did find differences in the ecological niches of the soil fungi residing in the treatments. These differences can partially be attributed to the type of flora found in each treatment. In DF plots, woody plants including oak and hickory are prominent and the predominant fungal guild detected was the ectomycorrhizal fungi, which are symbiotically associated with the roots of woody photobionts (Smith and Read, 1997). Similarly, arbuscular-mycorrhizal fungi, known to be symbiotically associated with crop plants (Jeffries *et al.*, 2003), were found to be among the top twenty ecological groups of fungi in both T1 and T2, but not in DF. T7, which had the highest richness of plant species of the treatments, also had the highest diversity of ecological niches represented by its soil fungal community. This finding is not surprising, as fungi and plants engage in many intimate relationships ranging from harmful to beneficial (Pirozynski, 1981). The increased number of plant species in T7 provides the opportunity for a broader variety of these relationships

The high abundance of biotrophic fungi in T7 is attributed to two of its top three OTUs that classified to the genus *Hygrocybe*. *Hygrocybe* species, also known as waxcaps, are plant-root biotrophic fungi commonly found in forests and nutrient-deficient grassland sites formerly under agricultural management (Halbwachs *et al.,* 2013; Griffith *et al*, 2002). In the United Kingdom, where 65% of the land mass is covered by grasslands due to centuries of agronomic activity, *Hygrocybe* spp. are commonly found in abandoned grasslands (referred to as "waxcap grasslands") but never in agricultural fields originating from reclaimed grasslands. One notable feature of waxy grasslands is that they have not been recently fertilized; it can take 10 to 30 years after the last application of inorganic N before *Hygrocybe*s spp. reappear (Griffith *et al*. 2002). This corresponds to our finding of *Hygrocybe* spp. in T7, abandoned from agriculture 22

years prior to the present study, and the fact that the agricultural treatments T1 and T2 were both depauperate of *Hygrocybe* spp.

The addition of N fertilizer to soils has also been documented to alter the relative abundance of other soil fungi, thus impacting the taxonomic structure of soil fungal communities (Tolijander *et al.,* 2008; Paungfoo-Lonhienne *et al.,* 2015). However, the overall impact of N fertilization on *Hygrocybe* spp., whether N fertilization simply inhibits the fruiting of *Hygrocybe* spp. or whether the mycelium cannot survive eutrophication, has not been previously characterized. Griffith *et al*. (2002), who conducted their study based on visual censuses of *Hygrocybe* carpophores rather molecular interrogations of fungal soil communities, as was done here, stated they did not know whether *Hygrocybe* mycelium in agronomic fields reclaimed from waxy grasslands was killed by eutrophication, or whether eutrophication simply inhibited fruiting body production. Our results indicate that *Hygrocybe* mycelium cannot survive eutrophication, as *Hygrocybe* OTUs were not detected in the soils of either T1 or T2, both of which receive annual inputs of inorganic N, but were detected in both DF and T7, neither of which receive additional N inputs . While we initially hypothesized that tillage would be the main agricultural disturbance to affect the taxonomic membership of fungal soil communities, these results indicate that inorganic fertilization may have a more dramatic impact than tillage on structuring soil fungal communities. In fact, T1 and T2, which differ only in T1 being conventionally tilled and T2 being under no-till management, had the most similar taxonomic composition of all the treatments. The relative impacts of tillage and eutrophication on fungal taxon richness in soils is an area that merits further investigation.

Interestingly, both agricultural treatments T1 and T2 had a higher abundance of ectomycorrhizal fungi than arbuscular-mycorrhizal fungi, despite a complete absence of woody photobionts and a predominance of AMF-dependent crop plants in those treatments. In total, ectomycorrhizal fungi were found to comprise 12.79% and 11.8% of all OTUs, as well as 9.30% and 6.48% of reads in T1 and T2 respectively. The abundance of ectomycorrhizal OTUs and sequences detected in the agronomic treatments was significant, and cannot be reasonably explained by random spore dispersal from the nearby forests. Therefore, it is possible that the putative ectomycorrhizal fungi found in T1 and T2 are living saprotrophically. Although ectomycorrhizal fungi are traditionally thought to live as obligate symbiotic partners with woody plants, recent studies have shown that many ectomycorrhizal fungi possess ligninolytic genes (Bodeker *et al.*, 2009;Talbot *et al.*, 2013), and it is speculated that they may have a more important role in recalcitrant plant litter decomposition that previously thought. Many ectomycorrhizal fungi are culturable *in vitro* as well (personal observation), providing further evidence of their ability to survive saprotrophically. Recent genomic evidence also supports the idea that some ectomycorrhizal fungi may have the capacity to live saprotrophically (Koher *et al*. 2015). Further, it has been demonstrated that ectomycorrhizal fungi have convergently switched from saprotrophs, and vice versa, several times (Bruns *et al*., 1998; Kretzer and Bruns, 1999), and that lineages of ectomycorrhizal fungi retain genes for lignocellulose degradation (Hibbet *et al.*, 2000; Chen *et al.*, 2001). The presence of these and other genes historically associated with saprotrophic fungi may enable putative ectomycorrhizal fungi to facultatively survive saprotrophically or to mine N from lignin and recalcitrant sol C. The role of

ectomycorrhizal fungi living as saprotrophs in soils and other environments is an area that deems further exploration

We found the Basidiomycota to be the most abundant phylum in each treatment, although the individual members and dominant ecological niches of the Basidiomycota found in each treatment were substantially different. This can be attributed in part to the wide diversity of ecosystem services provided by different members of the Basidiomycota, including saprotrophic, arbuscular-mycorrhizal symbionts, pathogens and ectomycorrhizal symbionts, dependent in part on the flora of the ecosystem. Wallenstein *et al*. (2007) examined bacterial and fungal communities in arctic tundra soils and also found Basidiomycota to be the predominant fungal phylum in the soils they examined. Others have found the Ascomycota to be the predominant phylum of fungi in soils (Jumpponen et al, 2009). Similar discrepancies have arisen in metagenomic studies of bacteria. For example, two separate studies of arctic soil communities found *Actinobacteria* to comprise 64% and 5% of all sequences (Desslippe *et al.,* 2012; Wallenstein *et al*., 2007).

While we found a high number of Basidiomycota in the soils of this study, we also found a correspondingly low number of Ascomycota. The most plausible explanation for this is primer bias against the Ascomycota. While it is possible that there may be a low relative abundance of Ascomycota in the soils we interrogated, the extremely low proportion of Ascomycota we found (4 out of 945 fungal OTUs detected) implicates a primer bias against Ascomycota. A tendency for the forward primer used here (LR22) to bias against Ascomycota was noted early on through Sanger sequencing (Rytas Vilgalys, *personal communication),* but this primer set had

never been used in pyrosequencing before. Further study with a different primer set is needed to resolve this discrepancy.

We identified four edaphic factors that correlated with changes in the taxonomic structure of the soil fungi in the treatments we examined. Nitrate was shown to be highly correlated with the shaping of the fungal communities in the agronomic treatments T1 and T2, which is not surprising given that both treatments receive annual amendments of inorganic N. POXC, which represents the labile C component of soils most available for microbial assimilation, was found to be strongly correlated to separating DF and T7 from T1 and T2, while ammonium was strongly correlated to the in shaping fungal community in T7. Previous studies have noted that it is not land use *per se* that shapes microbial communities, but rather it is the changes land management practices makes to edaphic properties such as pH, moisture content, labile C and the availability of N that are the important factors in shaping microbial community structure (Singh *et al*., 2008; Lauber *et al*., 2008). Stochastic forces of dispersion, preemptive colonization and niche exclusion are other major factors in shaping fungal communities, although they are not completely understood.

SIMPER analysis indicated the two *Hygrocybe* OTUs in T7 accounted for the main taxonomic differences between T7 and all the other treatments. The other eight OTUS in the top ten fungi that explained the differences in community composition between all the treatments as a whole, as well as those that explained the differences between each individual pair of treatments, were all saprotrophs. Although this underscores the relative abundance and importance of saprotrophic fungi in soils, this is also somewhat surprising, as DF had a predominance of ectomycorrhizal fungi in its top 20 taxa, yet SIMPER analysis did not identify

any ectomycorrhizal taxa as being in the top three taxa responsible for differences between DF and the other treatments. This is attributable to the fact that SIMPER analysis examines differences in abundances of individual taxa rather than differences in abundances of ecological niches of groups of fungi. While DF had a much larger proportion of ectomycorrhizal fungi in its community composition, there were several ectomycorrhizal OTUs represented and the differences of the individual ectomycorrhizal taxa in DF, when compared to individual ectomycorrhizal taxa in the other treatments, were not as disparate as the differences between the saporotrophic members of DF and saprotrophic members of other treatments.

It was of interest that *Lentinula edodes* , OTU 13, the fungus that produces the shiitake mushroom, was found in all treatments. *L. edodes* was the fourth most abundant taxa in T1, and the seventh most abundant taxa in T2. Using the NCBI-BLAST to compare the fasta sequence from OTU 13 with the GenBank database found it to have a 99% sequence similarity over 100% coverage with other *L. edodes* sequences deposited with GenBank. *L. edodes* does not naturally occur in the Midwestern United States, but is extensively cultivated as a food around the world, including in the Midwestern United States. This underscores the need for mushroom growers to understand possible ecological consequences of introducing foreign species for domestic cultivation.

Conclusions

The conversion of a native, oak-hickory forest terrestrial ecosystem to agricultural use, and early succession back towards the native community in the Midwest United States did not have a strong impact on the overall fungal OTU richness of the underlying soil communities of those ecosystems. However, agronomic land management practices and succession did have a

profound effect on shaping the composition of soil fungal communities, even along a contiguous landscape. Saprotrophic fungi are consistently among the predominant types of soil fungi found in differing terrestrial ecosystems, underscoring their importance in C cycling and soil fertility. Aboveground plant composition can partially explain changes in fungal soil community composition, especially in terms of which fungal ecological niches are selected for in each ecosystem. Other factors, such as the addition of inorganic fertilization, also have an impact in shaping fungal soil communities. While we are presently able to identify individual edaphic and other environmental factors that have either have an impact on, or are correlated to shaping fungal communities in soil, it is probable that many of these factors act synergistically. Accordingly, there is a need to develop tools and methods to investigate the underlying mechanisms that ultimately cause shifts in fungal taxonomic structure in soil ecosystems.

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III. DYNAMICS OF FUNGAL LIGNINOLYTIC GENES IN MIDWESTERN UNITED STATES SOILS

ABSTRACT

The dynamics of how ligninolytic genes are distributed in terrestrial ecosystems is not well understood. Here we employed FungChip, a functional gene microarray containing oligonucleotide probes for fungal genes, to evaluate the distribution and richness of the four main types of fungal ligninolytic genes including lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases in the soil metagenomes of conventional-till and no-till, 3-crop rotational agriculture soils, a native deciduous forest soil, and in an early-successional field soil of the Midwestern United States. In the soil metagenome from conventional-till, 3-crop, rotational agriculture treatment replicates there were a total of 337± 27 ligninolytic genes detected. In the soil metagenome of the no-till, 3-crop, rotational agriculture replicates there were 344 ± 24 ligninolytic genes detected. In the soil metagenome of the early successional field replicates there were 340 ± 18 ligninolytic genes detected, and in the soil metagenome of the deciduous forest replicates there were 341 ± 12 ligninolytic genes detected. We found that quality of plant litter did not have a statistically significant impact on the absolute numbers of ligninolytic genes detected in each treatment. However, we did discover a pattern of ligninolytic gene composition that paralleled the differences of types plant litter being input into each system. The till and no-till, 3-crop agriculture treatments, which have similar inputs of plant litter, showed ligninolytic gene compositions similar to each other, but distinct from those of the early-successional field and deciduous forest, both of which had a unique composition ligninolytic genes in its soil's metagenome. This suggests that different assemblages of ligninolytic genes provide the ecosystem service of lignin decomposition in soils of different

ecosystems, dependent on the composition of plant litter being input. This implies a high degree of functional redundancy and plasticity for ligninolytic processes in soil fungi.

Introduction

The deconstruction of lignin is key process in the recycling of carbon (C) in terrestrial ecosystems, and constitutes the rate-limiting step in the global C cycle (Paul, 2007). Lignin, the only natural polymer with an aromatic backbone, is heterogeneous in structure, and is biologically synthesized in plants from repeating units of the phenolic monomers *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These monomers form the hydroxyphenyl, guiaiacyl and syringyl subunits of lignin respectively, and are incorporated into lignocellulose in different ratios and combinations, depending on the species of plant (Vanholme *et al*., 2010). The resulting structure makes lignin stereochemically complex and recalcitrant to decomposition.

Lignin is almost exclusively metabolized by fungi (Tien and Kirk, 1983; de Boer *et al.,* 2005; Kubicek *et al*. 2010). One group of fungi, the white-rot fungi of the Basidiomycota, which derive their name from the whitish appearance of wood they decay after the lignin component is decomposed, harbor the enzymatic capacity to completely mineralize lignin to CO_2 and H_2O (Cullen and Kersten, 1996). Although fungi cannot rely on lignin decomposition as a sole source of energy or C, lignin degradation provides access to energy-rich polysaccharides such as cellulose and hemicellulose, which it shields from decomposition in lignocellulose (Hammel, 1997).

Fungal lignin modifying enzymes

There are four main types of fungal ligninolytic enzymes: lignin peroxidases (LiPs), manganese-dependent peroxidases (MnPs), versatile peroxidases (VPs), and laccases. LiPs are

glycosylated heme-containing proteins that house a tryptophan residue on the surface of the enzyme, which facilitates the direct oxidation of phenolic and non-phenolic compounds via long-range, multi-step, electron transfer. The products of this oxidation are intermediate radicals, which can then undergo non-enzymatic reactions including side-chain cleavage and demethylation (Dashtban *et al.*, 2010). MnPs are also heme-containing proteins, and possess two to three residues that bind and oxidize Mn^{2+} to Mn^{3+}. MnPs are the most common class II peroxidases produced by fungi, and are believed to be one of the most important fungal enzymes in lignin deconstruction (Hofrichter, 2002). VPs are hybrid enzymes that possess both the tryptophan residue found in LiPs, as well as the Mn-binding sites found in MnPs. They may oxidize both phenolic and non-phenolic compounds, as well as Mn^{2+} (Heinfling *et al*., 1998). LACs are glycosylated oxidoreductases that couple the oxidation of phenolic and other aromatic and non-aromatic substrates with the reduction of molecular oxygen (O_2) to water (H_20). The free-radical products of this oxidation are unstable, and may undergo further enzymatic or non-enzymatic reactions (Kunamneni et al., 2007).

FungChip

FungChip (Tu et al, 2014), a functional gene microarray containing oligonucleotide probes for over 4798 fungal genes, including 567 ligninolytic genes, was used to analyze the metagenome from the soil of each treatment for the presence and abundance of genes encoding the four main types of ligninolytic enzymes. The probes for ligninolytic genes on the FungChip microarray include 27 for LiP genes, 107 for MnP genes, 3 for VP genes and 430 for LAC genes. The probes on the microarray are each 50 bp in length, and are designed to be able

to differentiate between target sequences with 90-92% identity. Similar microarrays such as Phylochip and Geochip have been used to investigate the functional structure of various environmental metagenomes, providing novel insights on how different environmental conditions impact the functional potential of microbial communities in different ecosystems (Brodie *et al.,* 2007; Zhou *et al.,* 2008).

Relevance of this study to current topics in ecology

The impact of ligninolytic fungi in the biogeochemical cycling of C in soil systems is believed to be inversely correlated with the quality of litter inputs into that system, with higher quality litters having lower lignin contents and C:N ratios (Blackwood *et al*., 2007). It has been demonstrated that the addition of inorganic N will increase the carbon content of soils receiving lower quality litter, putatively as a result of the repressive, regulatory effect N has on the ligninolytic enzyme systems of soil fungi, as lignolytic enzymes also degrade humus and other soil C compounds (Deforest *et al.,* 2004; Waldrop *et al*., 2004). By contrast, the addition of inorganic N decreases the carbon content in soils receiving higher quality litter, presumably because it diminishes N limitations on cellulytic microbes (Sinsabaugh *et al*., 2002). Accordingly, inputs of N into an ecosystem, whether by fertilization or atmospheric deposition, can either suppress or stimulate microbial activity, dependent on the biochemistry of litter inputs into the system (Berg and Matzner, 1997; Knorr *et al.,* 2005), with a more repressive impact on ligninolytic fungi in soils receiving lower quality litter.

Because of the importance of lignin degradation to the cycling of soil C and the health and fertility of soils, an understanding of how human-induced disturbance, such as agronomic

land management, may affect the occurrence and distribution of ligninolytic genes in a soil's metagenome is crucial to developing sustainable land management practices, as well as to developing models of soil C (Talbot and Treseder, 2011). Although some studies have investigated ligninolytic enzyme activity in soils, there are few that have queried soil metagenomes for the occurrence and distribution of lignin-degrading genes, or have investigated how land disturbance and quality of litter inputs impact that distribution and the metagenomic potential for lignin degradation.

Here, we investigate how agricultural land management impacts the occurrence and distribution of fungal ligninolytic genes in the soil metagenomes of two agronomic sites, an early-successional field, and a native deciduous forest representing the original ecosystem from which the others originated. We hypothesized that the metagenome from the soil of the native deciduous forest, which receives the lowest quality of litter inputs in comparison to the agricultural and early-successional soils, will have the highest richness of ligninolytic genes. Conversely, we hypothesized that the soil metagenomes from the agricultural soils, which receive the highest quality litter inputs, will have the lowest richness of ligninolytic genes, while the soil of the early-successional community, which receives an intermediate quality of litter inputs, will have an intermediate richness of ligninolytic genes.

Materials and Methods

Field treatments

The Long Term Ecological Research (LTER) site of the Main Cropping Site Experiment (MCSE) at the W.K. Kellogg Biological Station (KBS) offers an opportune location to study the

ecology and relationship between land management strategies and the resident, soil microbiota. There are a broad variety of agronomic treatments laid out on a contiguous landscape, each with its own agro-ecosystem. The physiography of the KBS site is characteristic of a mature, glacial outwash plain and moraine complex. Taxonomically, the soils of the treatments are Alfisols (Gray Forest soils), formed under deciduous and oak-savanna vegetation, with the texture of a fine-to-course loamy, mixed, mesic, Typic Hapludalf (Paul, *et al.,* 2015). The LTER MCSE is comprised of four annual, rotational crop treatments (corn/soybean/wheat), two perennial systems (continuous alfalfa, *Medicago sativa*) and poplar (*Populus* sp.) clone, an early successional treatment abandoned from agriculture in 1989, and a never-tilled, annually mown perennial, grassland control site. There are also nearby successional fields, abandoned from agriculture for 40 to 60 years, and two different forested sites, one coniferous and one deciduous. The treatments examined in this study are described in Table 12.

Table 12. KBS-LTER treatments investigated in this study[1].

	Treatment	Inorganic N added	Tillage	Cover Crop	Plant Species Richness	Lignin Content	# Replicates
T1	Corn/Wheat/ Soybean Rotation	Yes	Yes	No	Low[2]	Low	6
T2	Corn/Wheat/ Soybean Rotation	Yes	No	No	Low[3]	Low	6
T7	Early Successional Field	No	N/A	N/A	High[4]	Moderate	6
DF	Deciduous Forest	No	N/A	N/A	Moderate[5]	High	3

[1] For further information see: http://lter.kbs.msu.edu/research/long-term-experiments/main-cropping-system-experiment/

[2] No more than 1 plant species per replicate other than crop reported.

[3] No more than 2 plant species per replicate other than crop.

[4] Average of 35.3 plant species per replicate plot.

[5] Average of 11.3 plant species per replicate plot. http://lter.kbs.msu.edu/datatables/40.csv, http://lter.kbs.msu.edu/datatables/182.csv.

Soil sampling

Samples from the November 2011, KBS LTER monthly soil sampling of the MCSE were used in this study. We sampled after the growing season when the soil fungal communities were likely to be temporally stable, minimizing potential short-term effects associated with tillage (Calderon *et al*., 2000; Calderon *et al*., 2001). Sampling followed the normal, monthly KBS soil sampling protocol (http://lter.kbs.msu.edu/protocols/11). Briefly, two soil cores were extracted from each of five sampling stations per replicate plot. Soils were sampled to a depth of 25 cm using a 2.5 cm diameter soil corer. In the case of the agricultural treatments (T1 and T2), there were an equal number of cores taken within and between each row at 10 cm

intervals. After sampling, the cores from each plot replicate were composited, sieved through a 4 mm screen to remove pebbles, and frozen at -80^0 C until downstream processing.

DNA extraction

Total community DNA was extracted from 0.5 grams of each sample on the Eppendorf epMotion 5075 robot using the PowerMag® Soil DNA Isolation Kit (optimized for epMotion), (Mo Bio Laboratories, Carlsbad, CA), according to the manufacturer's instructions. Briefly, 0.5 g of each sample were placed into individual wells of a Eppendorf epMotion 5075 bead plate, to which 750 µl of PowerMag^{tm} Bead Solution and 60 µl PowerMag^{tm} Lysis Solution were added. The bead plate was placed on a 96-well plate shaker (Mixer Mill MM 400, Retsch Corporation, Haan, Germany) and shaken for 10 minutes at a vibrational frequency of 20 Hz (1200 min^{-1}), rotated 180^0, and shaken for an additional 10 minutes at 20 Hz. The bead plate was then removed and centrifuged at room temperature for 6 minutes at 4,500 x g. The robot then transferred the supernatant from each well to a new 96-well collection plate. 450 µl PowerMag^{tm} Inhibitor Removal Technology Solution was added to each well and the plate was horizontally vortexed for 5 sec. The plate was then incubated at 4^{0}C for 10 minutes and centrifuged at room temperature for 6 minutes at 4,500 x g. The robot then transferred 850 µl supernatant to a new collection plate. The robot added 850 µl ClearMag^{tm} Binding Solution/ClearMag^{tm} Beads to each well. The plate was then shaken for 10 min and incubated for 15 minutes on a magnet. The beads were washed twice with 500 µl ClearMag^{tm} Wash Solution, and DNA was eluted with 100 µl ClearMag^{tm} Elution Buffer.

FungChip analysis

The DNA extracts were prepared for FungChip microarray hybridization as previously described (Van Nostrand *et al*, 2010). Briefly, 100 ng of each sample was amplified using the TempliPhi Amplification Kit (Amersham Biosciences, Piscataway, NJ) in a buffer containing 200 ng/µl single strand binding protein and 0.04 mM spermidine and incubated for 3 h at 30^0 C. The DNA was then denatured, fluorescently labeled with Cy-3 using random primers (Wu et al., 2006) and purified using the using the QIA quick purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The labeled DNA was lyophilized and resuspended in hybridization buffer (50% formamide, 3 X SSC, 0.3% SDS, 0.02m dithiothreitol, 0.7 µg/ µl herring sperm DNA). Labeled DNA was hybridized to the FungChip microarray on an HS4800 Hybridization Station (TECAN US, Duram NC) at 42^0 C for 10 h. Unhybridized DNA was removed with Wash Buffer I (1 x SSC, 0.1%SDS) for 5 min, Wash Buffer II (0.1 x SSC, 0.1%SDS) for 20 min, Wash Buffer III (0.1 x SSC) for 6 min, and Wash Buffer IV (0.01 x SSC) for 10 sec at room temperature. The FungChip microarray was then dried using purified air.

Following hybridization, the FungChip gene array was scanned using a ScanArray 5000 Microarray Analysis System (PerkinElmer, Wellesley, MA) at 95% laser power and 68% photomultiplier tube gain. The signal intensities of each spot were measured using IMAGENE 6.0 (Biodiscovery Inc., El Segundo, CA). Spots automatically scored as positive were used for further analysis. The signal intensities used for the final analysis were deducted from the background. Cleaning settings were programmed to delete signal intensities below 750 and signal-to-noise ratios below 2.5.

Data analysis

The normalized hybridization data for all genes was sorted in Excel (Microsoft Corporation, Redmond, WA) by lineage and then by gene category (to select for LiP, MnP, LAC and VP genes). Data from non-fungal lineages and non-ligninolytic genes were removed from the dataset after sorting. Each gene was hand-curated by checking its associated annotation using the GenBank accession number.

Statistical Analyses

Alpha diversity and beta diversity analyses were performed using the tools and pipeline from the Institute for Environmental Genomics, University of Oklahoma, Norman, Oklahoma (http://ieg.ou.edu/). All other statistical analyses were performed in PAST (Hammer *et al*., 2001), including non-parametric multivariate analysis of variance (NPMANOVA; Anderson, 2001), which is used to test whether groups have similar distributions; detrended correspondence ordination analysis (Hill and Gauch Jr., 1980), which is used to help visualize similarity in community structure of genes between treatments, and the Mantel test (Mantel, 1967), which tests for correlation between two distance matrices. The Bray-Curtis similarity index was used to calculate similarity in abundance of ligninolytic genes in the sample sets for all ordination analyses (Bray and Curtis, 1957).

Results

Genes detected in each treatment by FungChip

Analysis of DNA extracted from the soils across all replicate plots of all treatments resulted in detection of 440 out of a possible 567 (77.6%) ligninolytic genes represented on the FungChip microarray including 343 out of 430 (79.8%) LAC genes, 23 out of 27 (85.2%) LiP genes, 70 out of 107 (65.4%) MnP genes, and 3 out of 3 (100%) VP genes. The average numbers of genes detected in each replicate plot of each treatment are listed in Figure 13. There was no statistically significant difference in the total number of ligninolytic genes detected among all of the treatments. The average numbers of each type of ligninolytic gene detected in each replicate plot of each treatment are listed in Table 13. There was no statistically significant difference in the total number of each type of ligninolytic gene detected among all the treatments.

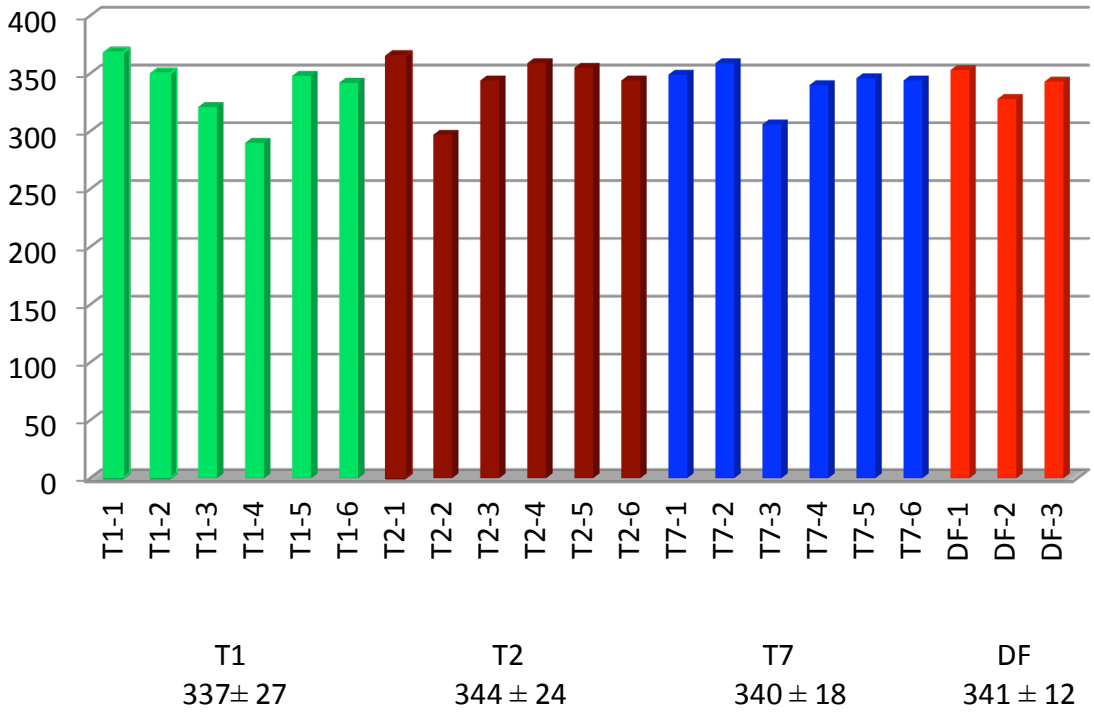

Figure 13. Number of ligninolytic genes detected, out of 567 ligninolytic genes represented on FungChip, in each treatment replicate. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Table 13. Numbers of each type of ligninolytic gene detected in each treatment. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatments	Laccase	LiP[1]	MnP[2]	VP[3]
T1	260±21	18±2	55±6	3±1
T2	266±19	19±3	56±4	3±0
T7	261±17	20±1	57±4	3±0
DF	262±9	20±1	56±3	3±0

1. LiP = Lignin Peroxidase
2. MnP = Manganese Peroxidase
3. VP = Versatile Peroxidase

Alpha diversity and evenness of all ligninolytic genes in the treatments

There was not a statistically significant difference in the alpha diversity and evenness of ligninolytic genes detected in the replicate plots of each treatment, as indicated by the Shannon and inverse Simpson diversity indices (Figure 14). The box-plots demonstrate that of all the treatments, T1 has the most variation between its replicate plots in the structure of its lignolytic genes, and T2 has the highest median. However, the overall differences in ligninolytic gene structure between all treatments are extremely low.

All of the soil metagenomes from each treatment were found to have a high degree of structural evenness in the distribution of their ligninolytic genes. The replicate plots of T7 were found to have slightly more variation in the structural evenness of their ligninolytic genes than the other treatments, and the replicate plots of T2 had the second-most variation. However, the overall differences in the structural evenness of ligninolytic genes between all treatments were found to be extremely low.

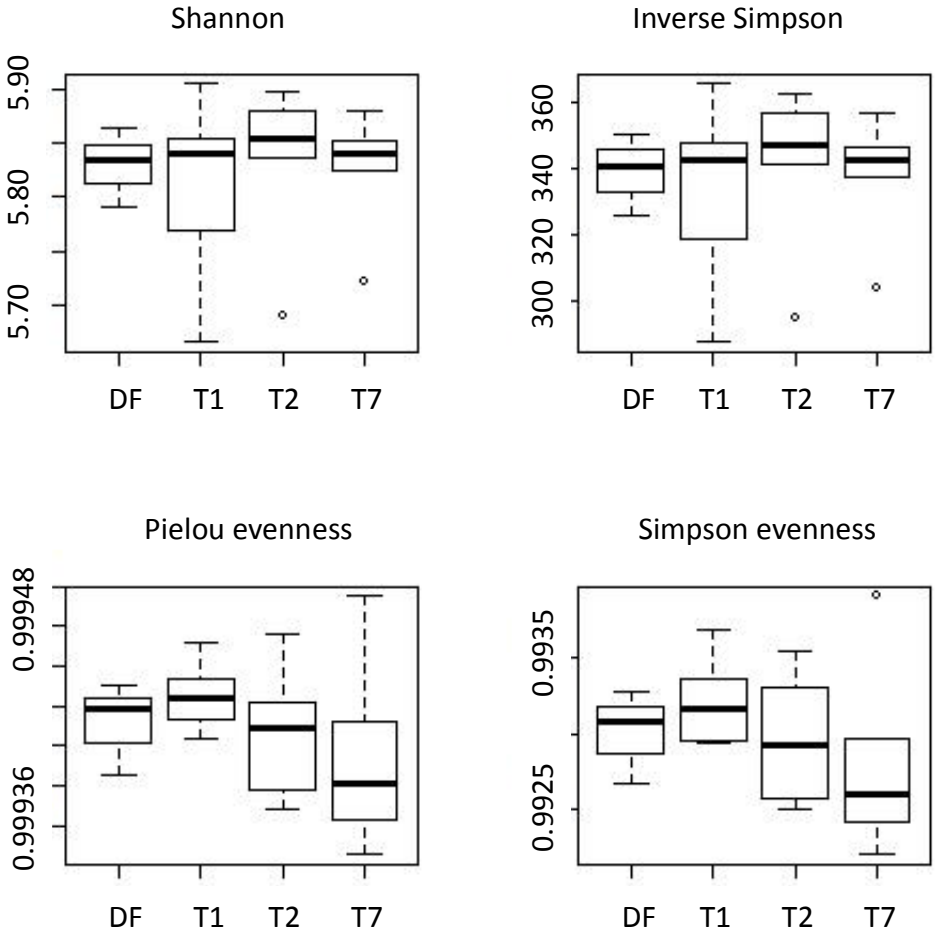

Figure 14. Alpha diversity and evenness in the distribution of ligninolytic genes in all treatments. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Ordination analyses

To help visualize how closely related the gene composition of each replicate plot of each treatment are to each other, we employed a detrended correspondence ordination analysis using the Bray-Curtis dissimilarity index (Figure 15). Analysis of all ligninolytic genes in the treatments by detrended correspondence ordination showed the replicate plots of the two agronomic treatments, T1 and T2, clustered together but separate from those of the replicate

plots from T7 and DF, which clustered separately by treatment. NPMANOVA analysis indicates there was no statistically significant difference between the gene compositions of T1 and T2 ($p = 0.1873$) but the differences in ligninolytic gene composition between T7 and DF were statistically significant ($p = 0.035$) (Table 14). The differences between T1 and T2 together and the other treatments, T7 and DF, were both statistically significant ($p<0.05$). Similar analysis of laccase genes in the treatments shows the same trend as for all ligninolytic genes, but analysis of the class II peroxide genes shows no statistically significant difference between T2 and T7 ($p = 0.145$).

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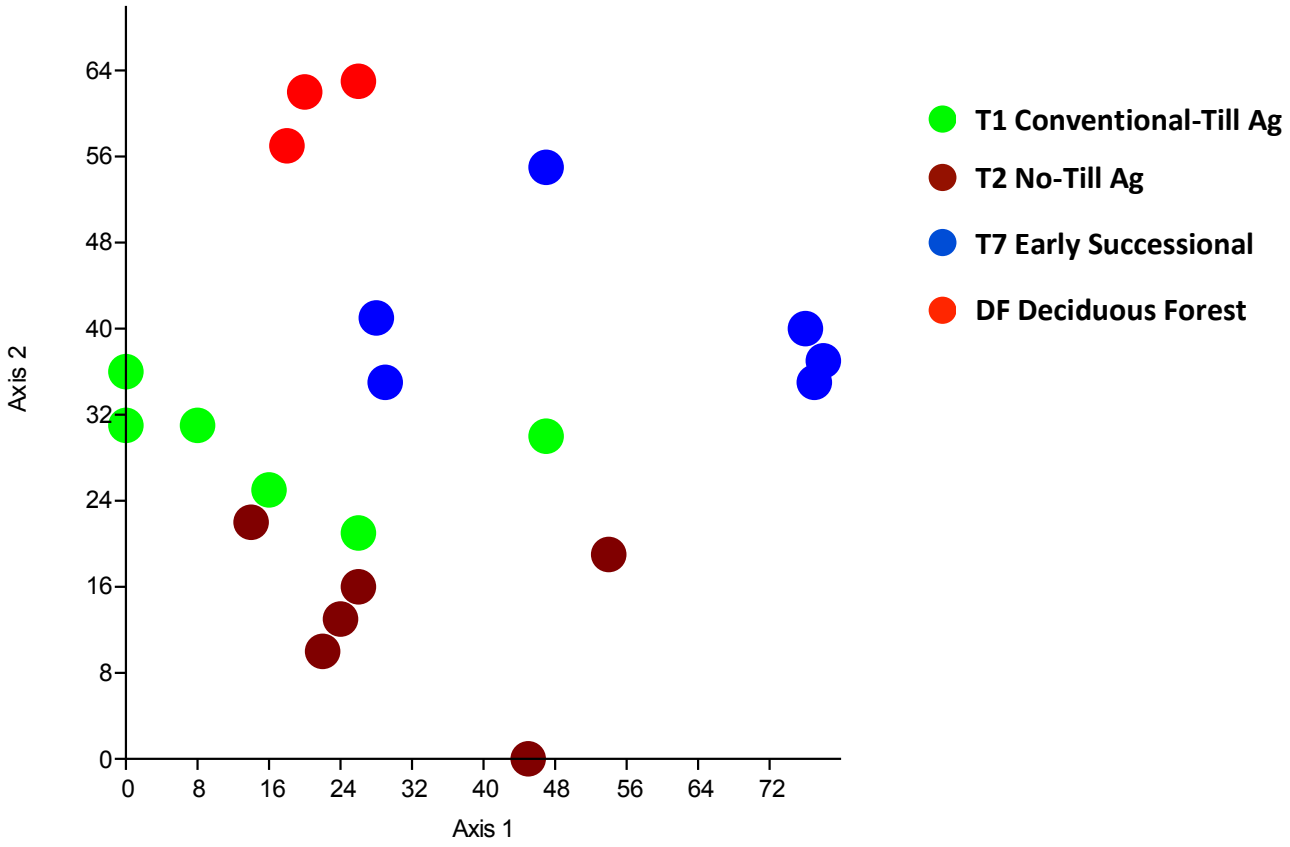
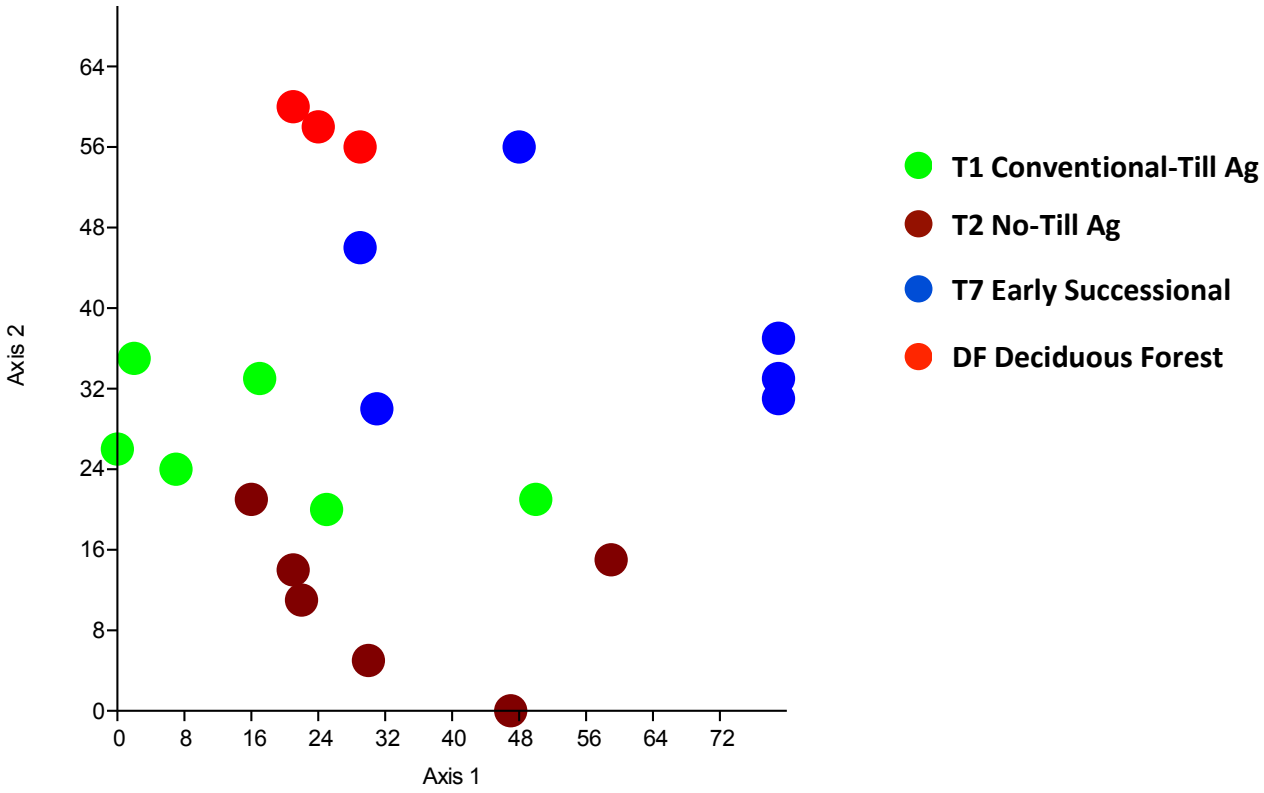


A

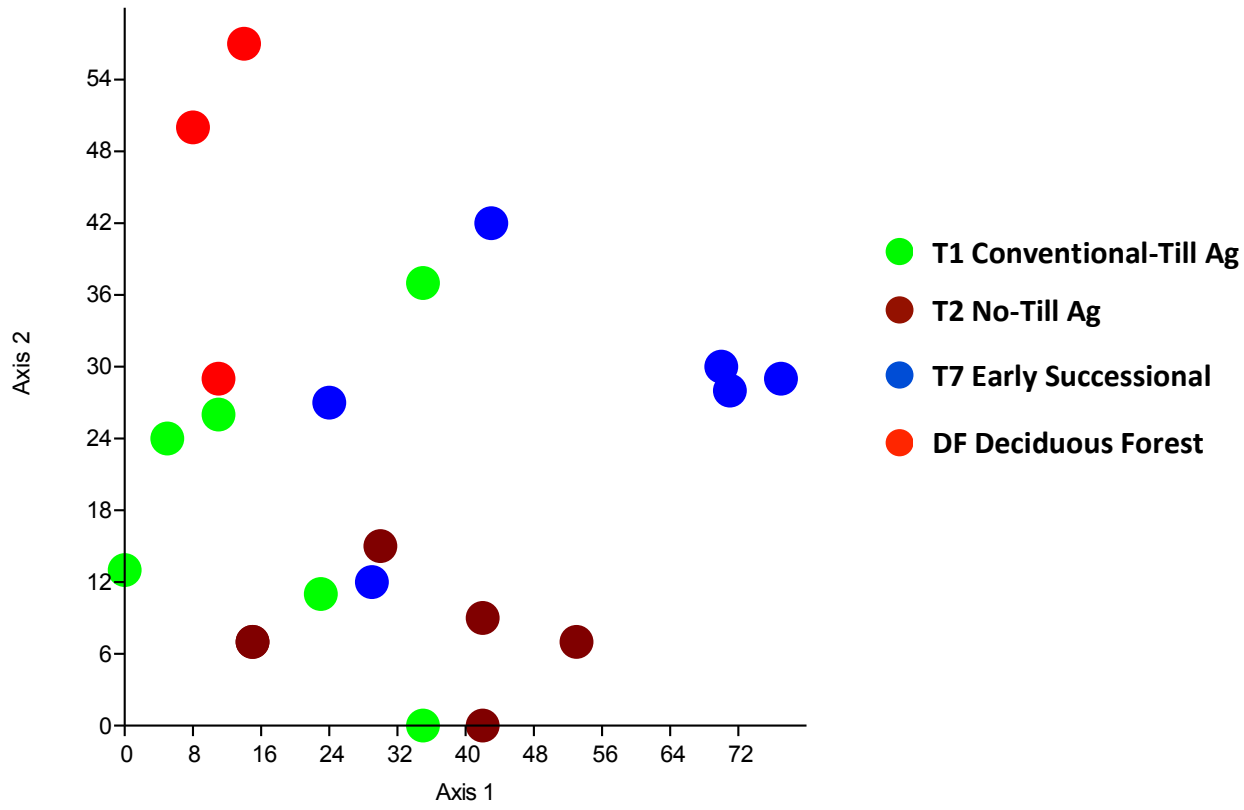
Figure 15. Detrended correspondence ordination analysis of fungal ligninolytic genes employing the Bray-Curtis dissimilarity index. A = All ligninolytic genes, B = Laccase genes, C = Class II peroxidase genes.

Figure 15 cont'd



B

Figure 15 cont'd



C

Table 14. P-values for the significance of difference between treatments for ligninolytic genes using Non-Parametric Multivariate Analysis of Variance (NPMANOVA) employing the Bray-Curtis dissimilarity index. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatments	NPMANOVA All Genes	NPMANOVA Laccase Genes	NPMANOVA Class II Peroxidase Genes
T1 vs. T2	0.187	0.187	0.258
T1 vs. T7	0.019	0.017	0.030
T1 vs. DF	0.024	0.023	0.048
T2 vs. T7	0.033	0.034	0.145
T2 vs. DF	0.011	0.012	0.026
T7 vs. DF	0.035	0.037	0.024

Similarity percentage (SIMPER) analysis

We identified the individual genes that contributed the most to differences in the community structure of ligninolytic genes between the treatments by conducting a SIMPER analysis (Clarke, 1993). SIMPER analysis compares the differences of the mean abundance of each gene across treatments, and identifies which genes best explain the differences in the gene compositions among treatments by identifying which genes have the largest differences in mean abundance. We used SIMPER to analyze the differences in gene structure among all treatments (Table 16), and between each set of treatments (Table 16).

The main individual gene types driving differences among treatments were the LACs and MNPs. Both LAC and MnP genes were among the top genes responsible for differences between T1 and T2 and between T1 and T7, and combinations of LAC genes were the top genes responsible for the differences between all other pairs of treatments.

Table 15. Top ten ligninolytic genes driving differences between all treatments.

Gene[1]	GenBank Accession Number	Order (Phylum)[2]	% Contribution to Differences
LAC	116205994	Sordariales (A)	0.70
LAC	32399641	Polyporales (B)	0.65
MnP	169289	Polyporales (B)	0.63
LAC	38479528	Auriculariales (B)	0.62
LAC	169846188	Agaricales (B)	0.61
LAC	578092	Corticiales (B)	0.61
LAC	367043112	Sordariales(A)	0.60
LAC	259480215	Eurotiales(A)	0.60
LAC	123228419	Uncultured[3]	0.59
MnP	169643677	Corticiales (B)	0.59

[1]LAC = Laccase, MnP = Manganese Peroxidase.

[2] A = Ascomycota, B = Basidiomycota.

[3]Uncultured fungus.

Correlation between differences in community structure of ligninolytic genes and taxa

To determine whether there was a correlation between the differences in the species composition between treatments (Chapter 2) and community structure of ligninolytic genes between the treatments, a Mantel test was performed. The Pearson product-moment correlation coefficient, or R value was 0.0951 (p= 0.2388), indicating that there is not a statistically significant correlation between the differences in the species composition and differences in the structure of ligninolytic genes between the treatments.

Table 16. Top three ligninolytic genes driving differences between specific pairs of treatments. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest. T1 = corn/wheat/soybean conventional tillage, T2 = corn/wheat/soybean no till, T7 = successional field, DF = deciduous forest.

Treatments Gene[1]	Genbank Accession Number	Order (Phylum)[2]	% Contrib	% Abund.	% Abund.
T1 vs. T2				T1	T2
LAC	116205994	Sordariales (A)	0.981	0.899	0
LAC	32399641	Polyporales (B)	0.802	0.730	0
MnP	169289	Polyporales (B)	0.746	0.375	0.936
T1 vs. T7				T1	T7
LAC	123228419	Uncultured[3]	0.879	0	0.965
LAC	169846188	Agaricales (B)	0.812	0	0.892
MnP	169643677	Corticiales (B)	0.807	0	0.881
T1 vs. DF				T1	DF
LAC	41367334	Agaricales (B)	1.142	1.03	0
LAC	218564148	Uncultured	1.030	0	0.933
LAC	169846188	Agaricales (B)	0.128	0	0.931
T2 vs. T7				T2	T7
LAC	169846188	Agaricales (B)	0.878	0	0.892
LAC	116205994	Sordariales (A)	0.813	0	0.821
LAC	218564148	Uncultured	0.746	0	0.757
T2 vs. DF				T2	DF
LAC	32399641	Polyporales (B)	1.096	0	1.05
LAC	41367334	Agaricales (B)	1.091	1.04	0
LAC	37791151	Agaricales (B)	1.047	0.999	0
T7 vs. DF				T7	DF
LAC	578092	Corticiales (B)	1.056	1.050	0
LAC	41367334	Agaricales (B)	1.045	1.040	0
LAC	37791151	Agaricales (B)	1.030	1.030	0

[1]LAC = Laccase, MnP = Manganese Peroxidase. [2] A = Ascomycota, B = Basidiomycota.

[3]Uncultured fungus.

Discussion

This study focused on the impact of land management and quality of litter inputs on the distribution and richness of ligninolytic genes in the soil metagenomes of conventional-till and no-till, 3-crop rotational agriculture soils, a native deciduous forest soil, and in an early-successional field soil of the Midwestern United States. Our results indicate that agricultural land management and litter quality did not make a statistically significant difference in the absolute number of fungal ligninolytic genes detected in soil metagenomes of each treatment. However, ordination and MPMANOVA analysis clearly demonstrate that although there was not a statistically significant difference between the ligninolytic gene composition of the fungal soil metagenomes from T1 and T2, ruling out an impact of tillage, there was a statistically significant difference among the ligninolytic gene composition of the agricultural treatments and T7 and DF, both of which had unique ligninolytic gene compositions. This finding parallels differences in the types of litter inputs into each treatment. The litter inputs into T1 and T2, which are mainly crop residues, were similar to each other, but differ from those of both T7 and DF, each of which each have their own distinct litter inputs. This suggests that the litter biochemistry of an ecosystem, including the quality of the ecosystem's litter, is a strong selective force for the specific ligninolytic gene composition of the soil metagenome, and that different assemblages of ligninolytic genes provide the ecosystem services of lignin decomposition in soils of different ecosystems. Thus, our results demonstrate functional redundancy and plasticity of ligninolytic processes in soil fungi.

The low correlation between the species composition and ligninolytic gene structure of the treatments, as shown by the Mantel test, indicates that the structural profiles of ligninolytic

genes in the soil metagenomes of the treatments are not dependent on the specific fungal species compositions of the treatments. This finding suggests that different groups of fungi are providing the ecosystem service of lignin deconstruction in the soils of each treatment, and that the potential for ligninolytic activity, rather than being confined to a few key organisms, may be more common among fungi than previously realized. Our findings support widespread functional redundancy and plasticity in ligninolytic potentials of fungal soil communities, indicating that rather than environmental selection for individual fungal species that can carry out specific ligninolytic functions, there may instead be selection for genes shared among species of evolutionarily-related taxonomic lineages. Thus, each ecosystem selects for lineages that contain genes best suited for metabolizing the type of litter being deposited into its ecosystem.

Our finding of functional redundancy for plant-decomposing processes in soil metagenomes is consistent with the results of a recent, long-term soil incubation study of Midwestern United States, where after a 707 day incubation soils retained the ability to decompose corn and wheat litter at a rate similar to that of freshly collected soils, despite a 50% depletion in microbial biomass (Birge *et al.* 2015). Whereas cellulases and other hydrolytic enzymes involved in enzymatically depolymerizing fresh plant residues are implicated in the study, the non-specific nature of ligninolytic enzymes provide a mechanism by which functional redundancy may be widespread for ligninolytic enzymes. In contrast to hydrolytic enzymes, which target specific chemical linkages that are often repeated in large-chain polysaccharides, ligninolytic enzymes non-specifically attack a wide diversity of chemical bonds. Consequently, there is a high degree of overlap among the substrates different types of ligninolytic enzymes

can effectively degrade, resulting in functional redundancy of the types of ligninolytic enzymes that are encoded by different fungi for lignin deconstruction. Functional redundancy for ligninolytic process is advantageous for ecosystems as ligninolytic processes are crucial to the flow of energy and nutrients through an ecosystem.

Different isozymes of LAC and MnP were identified by SIMPER analysis as being the only types of ligninolytic genes in the top 10 genes driving the differences among all the treatments, with differing LAC isozymes being the top 3 genes driving the differences between most pairs of treatments. This underscores the importance of laccase in lignin depolymerization in soil ecosystems, as well as its cosmopolitan distribution, despite having a lower redox potential than either MnP or LIP (Leonowicz *et al*., 2001). An Ascomycota laccase gene was the top gene responsible for differences among all treatments, indicating that members of the Ascomycota may play a more important part in lignin deconstruction in soils than presently believed. This also implicates a primer bias against the Ascomycota in the taxonomic assessment of the fungal soil communities in the treatments (Chapter 2). An investigation into the role Ascomycota fungi may play in lignin deconstruction in soils is an interesting topic for future investigation.

Finally, the remarkably distinct set of ligninolytic genes found in DF in comparison to the other treatments is most likely attributed to the difference in predominant ecological niche occupied by the of fungi of DF compared to the other treatments. Although some ectomycorrhizal fungi were detected in T1, T2 and T7, saprotrophic fungi constituted the most abundant fungal ecological niche in those treatments. In contrast, the fungi in DF were predominantly ectomycorrhizal. These differences in ecological niche also support the proposition that selection may be occurring for different evolutionary lineages of ligninolytic

genes. Although lignin modifying enzymes were previously documented only in saprotrophic fungi, notably the white-rot fungi of the Basidiomycota (Tien and Kirk, 1983), it has since been shown that ectomycorrhizal fungi have convergently switched from saprotrophs (and vice versa) several times (Bruns et al., 1998; Kretzer and Bruns, 1999), and that many lineages of ectomycorrhizal fungi have retained genes for lignin degradation (Hibbett et al, 2000; Chen et al, 2001). In fact, recent studies have shown that most ectomycorrhizal fungi possess ligninolytic genes (Bodeker *et al.*, 2009;Talbot *et al.*, 2013), with one study finding that 90% of ectomycorrhizal fungi tested were able to metabolize a phenolic polymer (Talbot and Treseder, 2010). Since lineages of ectomycorrhizal fungi have convergently evolved with those of saprotrophic lineages (Hibbett et al, 2000), the specific ligninolytic genes they encode have also convergently evolved with saprotrophic ligninolytic genes. Therefore, differences in the specific isozymes encoded by each guild would be expected.

Conclusions

The quality of plant litter inputs and agricultural land management practices such as tillage do not have a significant impact on the overall richness of fungal ligninolytic genes detected in Midwestern United States soils. However, the litter biochemistry of these ecosystems, as well as the flora associated with the predominant ecological niches of fungi in each ecosystem, are strong selective forces for the types of ligninolytic genes contained in each soil's metagenome. Each ecosystem selects for lineages of fungi encoding ligninolytic genes that are optimal for metabolizing specific litter inputs into that system, and different assemblages of ligninolytic genes provide the ecosystem services of lignin decomposition in soils of different ecosystems. Accordingly, there exists a great deal of functional redundancy and plasticity in the

potential for lignin decomposition in soil fungi. These results call for further research to identify the roles of different ecological guilds of fungi in ligninolytic processes in ecosystems, as well as further investigation into the role of the Ascomycota in lignin decomposition.

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IV. MICROBIAL BIOMASS OF MIDWESTERN UNITED STATES AGRICULTURAL AND FORESTED SOILS

ABSTRACT

Microbial biomass is an indicator of the overall health, quality, ecological stability and potential for primary productivity of soil. Microbes carry out the majority of biogeochemical processes and nutrient cycling in soils, and also constitute a substantial reserve of energy and nutrients. We examined how several agronomic land management practices impact the microbial biomass of soils in the Midwestern United States. The treatments we examined included four annual, rotational crop treatments (corn/soybean/wheat), two perennial systems (continuous alfalfa (*Medicago sativa*) and poplar clone (*Populus sp.*), an early-successional treatment, and a never-tilled, annually mown, perennial grassland control site. In addition, we also examined soils from an adjacent mid-successional field, deciduous forest, and coniferous forest. The microbial biomass of each soil was measured by chloroform-fumigation-incubation analysis (CFIA), as well as by measuring total DNA content. In the four corn/wheat/soybean rotational treatments, tillage was found to have the largest negative impact on microbial biomass, with the conventionally-tilled treatment without a cover crop having microbial biomass values of 8.2 ± 0.5 μg DNA g^{-1} dry soil and 165.6 ± 38.4 g^{-1} C CFIA, compared to 10.9 ± 0.4 μg DNA g^{-1} dry soil and 217.5± 47.2 g^{-1} C CFIA in the similar no-till treatment. However, the negative effects of tillage were demonstrated to be overcome by the inclusion of a cover crop in the other two conventionally-tilled rotational crop treatments, one that receives reduced inputs of nitrogen and the other that is managed organically, with respective microbial biomass

values of 10.8± 0.6 µg DNA dry soil and 11.9 ± 0.5 µg DNA g^{-1} dry soil, and 225.9 ± 27.0 g^{-1} C CFIA and 236.3 ± 28.4 g^{-1} C CFIA. Both the continuous poplar and continuous alfalfa treatments had higher microbial biomass values than the 3-crop agriculture treatments when measured by CFIA, but approximately equal values when measured by extracted DNA. The soil of the early-successional treatment had a higher microbial biomass than any of the 3-crop or continuous agricultural treatments, and the yearly-mown grassland site had an even higher microbial biomass. The soils of the forested sites and mid-successional field, as measured by chloroform fumigation-incubation, did not vary significantly from those of the agricultural and mid-successional treatments, but when measured by DNA were found to have twice to three times the values of the agricultural and successional treatments. In summary, we found that the style of agricultural land management has a significant impact on the soil microbial biomass. However, a decrease in soil microbial biomass did not necessarily equate with lower crop yields due to the offsetting benefits of inorganic nitrogen enrichment. Whether application of nitrogen fertilizer to offset losses of microbial biomass benefits is sustainable in the long term has yet to be determined, and should continue to be explored.

Introduction

Microorganisms are critical components of healthy soils, and are responsible for the majority of biogeochemical processes and transformations therein. Microbes manufacture the precursors required for humification, and are the driving forces behind the decomposition of organic matter inputs, thereby recycling elements and nutrients necessary for sustainable primary production (Paul, 2007; Baaru *et al.*, 2007). Soil microbial biomass is part of the labile component of the organic fraction, and serves as an important reservoir of soil nutrients (Jenkinson and Ladd, 1981; Garcia and Rice, 1994). Microbes in soil can account for 1-3% of the soil's total carbon (C), as well as 3-5% of its total nitrogen (N). Values for microbial C in soils range from 100 to < 1000 µg C g^{-1} (Paul *et al*., 1999). On a global scale, soil microbial C is estimated to be 16.7 pentagrams in the 0 to 30 cm profile, and 23.2 pentagrams in the 0-100 cm profile (Xu *et al*., 2013).

The microbial biomass of a soil reflects the amount of energy being stored in that segment of an ecosystem's biota, (Atlas and Bartha, 1998; Jenkinson and Ladd, 1981), and is an indicator of soil quality, health, ecological stability and potential for primary productivity (Sparling *et al*, 1997; Ajwa *et al*., 1999). In agro-ecosystems, where great amounts of crop biomass are being exported, the efficient recycling of organic inputs is critical to sustainability (Baaru *et al*., 2007). Accordingly, understanding how agronomic land management affects the microbial community is essential to planning and maintaining strategies for long-term, sustainable agriculture.

The size, activity and structure of microbial communities are dependent on the climate, topographic, edaphic and vegetative characteristics of a soil (Zorzona *et al.,* 2009). The

dynamic reactivity of soil microbes to changes in soil has led some to report that microbial biomass can be used as an indicator of changes in soil due to management practices long before other measures such as C or N can be used as indicators (Christiansen, 1996). In fact, one study investigating the effect of burning crop residues, as compared to letting them decompose over time, found no significant differences in total soil C between the burned and unburned plots, whereas microbial biomass C decreased from 150 to 100 Kg C/ha in the burned plots (Hoyle *et al*., 2006). Thus, the microbial biomass was found to be a more sensitive indicator of change than total soil C.

Several studies have noted the impacts of some land management practices on the soil microbial community. Differing agricultural processes can alter the chemical, physical and biological components of soil, thereby reducing the quality of the soil and microhabitats in which microbes function (Joergensen and Emmerling, 2006). For example, tillage has been noted to initially increase soil organic matter and dissolved organic matter availability, with a corresponding increase in microbial biomass due to the increased contact between the microbes and litter inputs (Gregorich *et al*., 2000). However, after several consecutive seasons of tillage, increased microbial contact and organic matter availability can lead to a depletion of both dissolved organic matter and the light fraction of soil organic matter, with a corresponding decrease in microbial biomass (Malhi *et al*., 2006). Such decreases can result in changes to microbial-mediated, biogeochemical processes (Booth *et al*., 2005).

There are several methods for measuring microbial biomass including enzyme-base assays that measure the metabolic activity of soil microbes such as phosphatase, esterase, protease, dehydrogenase, b-glucosidase, urease and deaminase activities (e.g. Ajwa *et al.,*

1999). However, these measurements are specific for metabolic functions that may be differentially expressed in response to temporal or environmental conditions or both. To address total microbial biomass, rather than evaluate those members of the microbial community that might be metabolically active in one or more specific process(es) at a particular point in time, we employed two independent assays of total microbial biomass; the chloroform incubation fumigation analysis (CFIA) method (Jenkinson and Powlson, 1976), and a measurement of total extracted soil DNA. A regression was then employed to test how well the two methods resolved with each other.

We explored how differing agricultural management practices in Midwestern United States agricultural systems, including four 3-crop rotational (corn/wheat/soybean) treatments under differing management styles, continuous alfalfa and continuous poplar, would impact the microbial biomass of their soils. We also explored an early-successional field, a mid-successional field treatment, a yearly-mown grassland and two native forested systems, one deciduous and one coniferous. We hypothesize that in the 3-crop rotational treatments, tillage will negatively impact the microbial biomass due its the negative impact on soil structure. We further hypothesize that microbial biomass will be higher in the organically managed than in the inorganically N enriched 3-crop treatments, and that the presence of a cover crop will also increase microbial biomass by providing a continuity of photobionts for the rhizosphere and arbuscular-mycorrhizal (AMF) microbes. In addition, we hypothesize there will be a lower microbial biomass in all of the 3-rotation crop treatments than in continuous alfalfa, continuous poplar, mown grassland, successional field and forested sites, all of which have larger aboveground biomass and larger inputs of litter and root exudates.

Materials and methods

Field treatments and soil sampling.

The W. K Kellogg Biological Station's (KBS's) Main Cropping Site Experiment (MCSE) is comprised of four annual, rotational crop treatments (corn/soybean/wheat), two perennial systems [continuous alfalfa (*Medicago sativa*) and poplar clone (*Populus* sp.)], a native, early successional treatment, and a never-tilled, annually mown perennial, grassland control treatment. All treatments. In addition, there are adjacent successional fields, abandoned from agriculture 30-50 years, and deciduous and coniferous forested sites. All of the treatments, fields and forests are on a contiguous landscape. The treatments examined in this study are listed in Table 17.

Soils were sampled from each of the replicate plots of the agronomic treatments, successional field and forested sites as part of the November, 1999 KBS monthly sampling, as previously described (Boone *et al.,* 1999). Briefly, two replicate soil samples were removed from each of five pre-existing sampling stations located within each plot with a 2.5 cm diameter soil corer at a depth of 0-25 cm. The samples from each plot were composited, picked free of roots and other litter, and then sieved though a 4 mm screen. Samples were stored at field moisture and a temperature of 4^{o} C for 48 hours before processing.

Table 17. Treatments of the main site cropping experiment, successional and forested sites at the W. K. Kellogg Biological Station.[1]

	Treatment	Inorganic N added	Tillage	Cover Crop	Number of Replicates
T1	Corn/Wheat/Soybean Rotation	Yes	Yes	No	6
T2	Corn/Wheat/Soybean Rotation	Yes	No	No	6
T3	Corn/Wheat/Soybean Rotation	Reduced	Yes	Yes	6
T4	Corn/Wheat/Soybean Rotation	No	Yes	Yes	6
T5	Perrenial *Poplus spp.* (10 year Rotation)	No	No	No	6
T6	Continuous Alfalfa	No	No	No	6
T7	Early Successional Field	No	No	No	6
T8	Grassland (mown once a year)	No	No	No	6
DF	Deciduous Forest	NA	No	No	3
SF	Mid Successional Field	No	No	No	3
CF	Coniferous Forest	No	No	No	3

[1] For further information see: http://lter.kbs.msu.edu/research/long-term-experiments/main-cropping-system-experiment/ and http://lter.kbs.msu.edu/research/long-term-experiments/successional-and-forest-sites/.

Measurement of microbial biomass

CFIA method

The CFIA method for measuring microbial biomass compares the amount of C released as CO_2 from a soil sample that has been fumigated with chloroform to that of a similar sample that has not been fumigated. This assay relies on the assumption that chloroform fumigation will kill most soil microbes, thereby releasing their cellular components, and that those microorganisms that survive the fumigation will subsequently mineralize (to CO_2) a proportion the cell components that were released during a period of incubation following fumigation (Paul *et al*., 1999). The CFIA protocol followed here was previously described (Jenkinson and Powlson, 1976). Briefly, three 25 g subsamples of each soil sample were placed inside individual 50 ml glass beakers. Two of the replicates were fumigated. One unfumigated replicate served as a control. Fumigation consisted of placing samples in a vacuum desiccator lined with damp paper towels. A 50 ml beaker containing ethanol-free chloroform and three boiling chips was placed in the center of the desiccator. The desiccator was then sealed, placed in a fume hood, hooked up to a vacuum manifold, and evacuated until the chloroform came to a boil. The chloroform was boiled for approximately 30 seconds, after which the vacuum was broken. The evacuation and boiling process was repeated four times. At the end of the fourth chloroform boil, the desiccator valve was closed and the samples were incubated in the chloroform vapor-saturated desiccator for 24 hours. Following the incubation, the desiccator was vacuum flushed with air eight times to remove the vapor. Samples were then removed from the desiccator, placed in quart-sized, wide mouth Mason canning jars, and sealed with lids

that were fitted with rubber septa to allow for gas sampling. At the same time, unfumigated control soil samples were also placed in similar canning jars with similarly fitted lids. All samples were then incubated in the sealed jars for 10 days at room temperature. After 10 days, 1 ml of gas from the head space of each jar was collected. Evolved CO_2 was measured using an infra-red gas analyzer.

Biomass C was calculated using the formula:

$Bc = Fc/Kc$

Where Bc=Biomass C, Fc= CO_2-C evolved from fumigated soil during the 10 day incubation minus CO_2-C evolved from the control during the 10 day incubation, and Kc=0.41.

DNA extraction method

DNA was extracted from each of three half-gram subsamples of the soil from each plot using the FastDNA SPIN Kit for Soil (Bio101, Vista, CA) according to the manufacturer's instructions. Briefly, three 0.5 gram aliquots of debris-free soil were each placed inside individual MULTIMIX 2 Tissue Matrix Tubes used for bead-beating lysis. .5 M Sodium phosphate buffer (978 µl) and lysis buffer (122 µl) were added to each tube. The tubes were then placed on a bead-beating tube adapter attached to a vortex, and vortexed at maximum power for 20 minutes. The tubes were subsequently centrifuged at 1,400 x g for 5 min to precipitate the solids. The supernatant of each tube was then transferred to a clean tube, to which 250 µl protein precipitating reagent was added. The tubes were then hand-mixed for 10 min, followed by centrifugation for 10 min at 1,400 x g to pellet the precipitate.

The supernatant of each tube was transferred to a sterile 15 ml conical tube, and 1 ml of DNA Binding Matrix Suspension was added to each tube. The tubes were then inverted by hand for 2 min, and placed at room temperature in a rack to allow the settling of the silica matrix. 500 µl of the supernatant was removed and discarded, and the silica matrix was then resuspended in the remaining supernatant. The suspension from each tube was then transferred to a spin filter column and centrifuged for 1 min at 1,400 x g, the flow-through was discarded, and the spin filter was washed with 500 µl salt-ethanol wash solution, and air-dried for 5 min. The DNA bound to the spin filter was then eluted using 50 µl DNase/pyrogen-free water.

The DNA concentration of each sample was spectophotometrically measured at 260nm (A_{260}), where an absorbance value of 1 equals a concentration of 0.05 µg/µl. The three values for the subsamples from each plot were then averaged, and the amount of DNA per gram dry soil was calculated. To adjust for moisture, the moisture content of each sample was measured by weighing three 1-gram subsamples into individual tin weighing boats, measuring the soil wet weights, drying the samples in their tins overnight in a 200^0 F oven, and then reweighing the samples. The samples were again placed in the 200^0 F oven in one-hour intervals until the weights remained unchanged. Percent moisture content of the soil was calculated using the formula:

%MC= SWW-SDW/SWW x 100

Where MC = moisture content, SWW = soil wet weight, and SDW = soil dry weight.

The values for µg total DNA per g dry weight soil were calculated using the formula:

µg DNA/g dry soil = µg DNA/g moist soil x 1/MC.

Results

The tilled, 3-crop rotation agriculture treatment (T1) had the lowest microbial biomass of all treatments, with a value of 165.6± 38.4 C g^{-1} soil from chloroform fumigation-incubation analysis (CFIA), and 8.2 ± 49 µg DNA g^{-1} soil (Table 18 and Figure 18). All of the other 3-crop, rotational treatments (T2-T4) had very similar microbial biomass values when measured by either CFIA or extracted DNA, and were approximately 1.5 times higher in microbial biomass than T1. There was no statistically significant difference between the microbial biomass values for T2-T4. The soils of both T5 and T6 had a greater microbial biomass than any of the rotational agronomic treatments, the soil of T7 had a greater microbial biomass than any of the agronomic or perennial biomass treatments, and the soil of T8, which had never been under agronomic management, had the greatest microbial biomass of any of the main site cropping experiment (MSCE) treatments. The microbial biomass of the successional field and forested sites, when measured by chloroform fumigation, had values that approximately spanned those of T2-T7. When using a total DNA measure of microbial biomass, however, the values of the successional field and forested sites were much higher than those of any of the agronomic treatments, with DF having the lowest and CF having the highest values for microbial biomass. CF had a higher microbial biomass than any other treatment when measured by DNA content, and was only second to T8 when measured by CFIA.

Table 18. Microbial biomass of treatments using DNA extraction and chloroform fumigation incubation analysis(CFIA) methods. See Table 18 for description of treatments.

Treatment	µg DNA g^{-1} dry soil	C g^{-1} soil from CFIA
T1	8.2 ± 0.5	165.57 ± 38.4
T2	10.9 ± 0.4	217.51± 47.2
T3	10.8± 0.6	225.93 ± 27.0
T4	11.9 ± 0.5	236.26 ± 28.4
T5	11.7 ± 0.9	272.02 ± 24.4
T6	11.5 ± 0.3	259.30 ± 53.2
T7	17.0 ± 1.4	287.27 ± 18.1
T8	20.3 ± 1.5	417.35 ± 35.3
DF	25.9 ± 1.8	198.90 ± 51.0
SF	36.6± 1.0	228.50 ± 58.6
CF	40.0± 1.0	288.05± 21.7

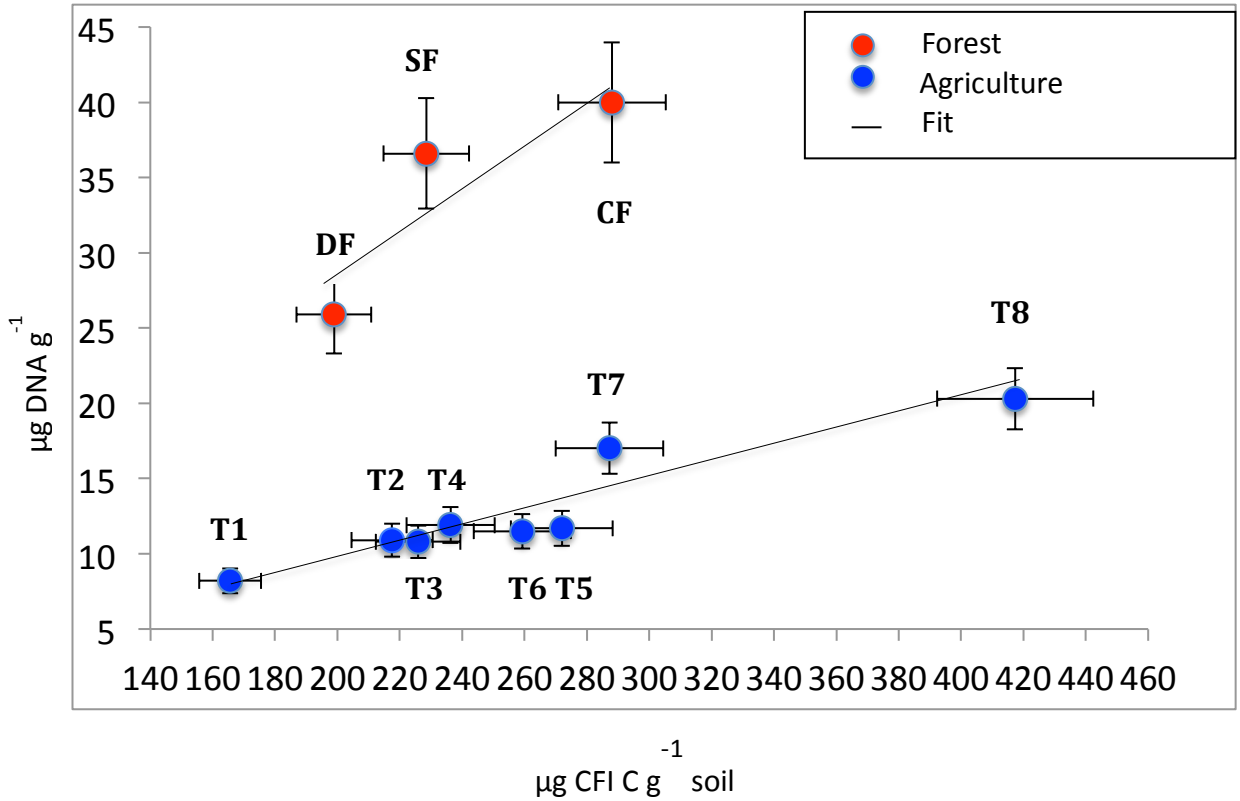

Figure 16. Regression between Chloroform Fumigation Method and DNA extraction methods of evaluating microbial biomass under different land management strategies. See Table 18 for description of treatments.

The correlation between the microbial biomass C as measured by CFIA compared to that of DNA was very strong, as indicated in the regression analysis (Figure 18). This indicates the two methods employed were very consistent with each other in measuring microbial biomass from the treatments. However, there was a significant difference between the slope of the fit between the agronomic treatments and the successional field and forest sites.

Discussion

The tilled 3-crop agricultural treatment (T1) had a much lower microbial biomass than the similar no-till treatment (T2), demonstrating the significantly negative impact tillage can have on a soil's microbial biomass. It has been noted that tillage can result in the depletion and erosion of soils, thereby reducing available nutrient levels and soil organic material (Beare *et al.,* 1994). The disruptive mechanisms of tillage aerate and homogenize soils, and break apart soil aggregates, thereby exposing otherwise protected SOM to oxidation and microbial degradation (Malhi *et al*., 2006). This results not only in a dramatic decrease in soil C, and the potential to sequester C, but also decreases the water holding capacity, total N, cation exchange capacity and aggregate stability of a soil (Caldwell *et al*., 1999; Gardi *et al.,* 2002). The impact of tillage on the AMF community is likely a contributing factor to lowering the microbial biomass detected T1. Tillage has been demonstrated to dramatically impact the size of the AMF community in agricultural soils, with one study finding a decrease of 97% and 497% of AMF active mycelium and colonized root length respectively in a conventionally tilled compared to no-till agronomic site, with an accompanying 75% increase of AMF propagules in the no-till site (Cornejo *et al.*, 2009). Furthermore, as a result of the decreased quantity and size of soil aggregates under tillage, there is a diminished availability of micro-niches to provide sites for

microbial diversity (Joergensen and Emmerling, 2006). All of these factors diminish the mass and activity of the microbial community.

The addition of a cover crop to the tilled 3-crop rotational treatments T3 and T4 was shown to dramatically increase the microbial biomass over the non-cover-crop, tilled treatment (T1). In T3, the addition of a cover crop increased the microbial biomass to one and a half times that of T1, despite a decrease in the amount of organic N being input into the system. Although the addition of inorganic N to an agricultural soil has been demonstrated to increase microbial biomass, albeit to a lesser degree than organic N (Kaur *et al.,* 2005; Masto *et al.,* 2006; Chu *et al*. 2007), the addition of a cover crop in T3 appeared to off-set the impact of decreasing the amount of inorganic N inputs. The organically-managed cover-cropped, tilled treatment T4, realized an even larger increase in microbial biomass. The increases in microbial biomass in the cover-crop treatments may be partially attributable to the incorporation of "green manure" when the cover crops are turned over in the spring, as well as by providing a continuity of photobionts for the AMF and other soil microbes living in the rhizosphere community. Without a cover crop, photobionts are seasonal and intermittent, as are the microbes associated with plant rhizospheres. With an unvarying presence of photobionts, the cover crop treatments provide a seasonal bridge of symbiotic partners to the AMF and rhizosphere communities, ensuring a constant supply of photosynthate. All of the above factors likely contributed to a larger microbial biomass in the soils of the cover-crop treatments.

Interestingly, crop yield data from the four rotational crops did not mirror the differences in microbial biomass found among the different treatments (Table 19). Although lower in microbial biomass, the tilled T1 treatment had a higher crop yield than the no-till T2

treatment that was receiving similar inputs of inorganic N. The reduced-inorganic-N-input, tilled treatment (T3) had the highest crop yield out all the treatments, surpassing the yields of both of the treatments receiving higher inputs of N, as well as the organically managed, treatment (T4), which harbored the highest microbial biomass. These data indicate that although microbial biomass plays an important role in soil health and the potential for crop productivity, crop yields are not necessarily directly correlated with microbial biomass. In addition, agricultural management strategies such as increased inorganic N enrichment may compensate and overcome the losses of microbial ecosystem services that accompany a lower soil microbial biomass. At the time this study was conducted, the treatments of the main LTER cropping site had been under their specific, agronomic land management regimens for 20 years from 1989 to 1999. Whether increased yields at the expense of the microbial community can be sustainably maintained over a longer period of time is yet to be seen and should continue to be monitored.

Table 19. Crop yields from the agronomic, 3-crop rotational plots (*Zea mays* in rotation). [1]

Treatment	Yield Kg/hectare/year
T1	10995.5 ± 757.6
T2	11520.5 ± 736.7
T3	11850.3 ± 627.3
T4	6746.7 ± 545.5

[1] For further information see: http://lter.kbs.msu.edu/datatables/51. See Table 18 for description of treatments.

Both perennial crops alfalfa (T5) and poplar (T6) had higher microbial biomasses than any of the agronomic rotations. This may be attributed to the higher input of organic litter and root exudates from the larger plants in those treatments (Zornoza *et al.*, 2009). Furthermore, the year-round continuity of non-changing photobionts in the perennial treatments also provide a stable niche for both ectomycorrhizal and endomycorrhizal fungi, as well as bacterial rhizosphere microbiota. In the alfalfa, nitrogen-fixing, root symbionts enrich the soil for N, thereby decreasing potential N limitations on the microbial community thereby contributing to an increase in microbial biomass. The slight increase of microbial biomass in the poplar over the alfalfa treatment is most likely attributable to the increased mass of the photobionts, which contribute larger inputs of litter (Zornoza *et al.*, 2009), and harbor a larger biomass of belowground roots, resulting in an increased surface area with greater inputs of exudates for mycorrhizal and other rhizosphere community members.

The successional treatment (T7) demonstrated an increase in microbial biomass over all the agronomic treatments. Successional fields, subsequent to abandonment from agriculture, generally have a higher amount of vegetative cover and litter inputs as they are colonized by surrounding, indigenous vegetation, and demonstrate a correspondingly higher microbial biomass. This increase is often correlated with an increased organic matter content and aggregate stability (Kosmas *et al*., 2000). , However, this was not the case with T7, as evidenced by data on mean % total C from KBS (Table 20). While the microbial biomass increased in the successional treatment compared to all the agronomic treatments, there was not a corresponding increase in total mean percent C, the value of which was instead similar to that of the agricultural treatments. The plots of the successional treatment are burned annually,

which is the most likely reason for the lower value of its soil carbon content. Prescribed burns forge recalcitrant products of pyrolysis some of which, such as ash, are lost into the atmosphere, thereby reducing the levels of labile C and N (Sinsabaugh, 2010). In a recent study by Dooley and Treseder (2012), a meta-analysis of 42 publications on microbial responses to fire reported that the microbial biomass of terrestrial ecosystems decrease, on average, by 32% after a fire. Accordingly, the annual prescribed burning of the successional treatment may be precluding its soil from harboring an even greater microbial biomass.

Table 20. Mean % C in KBS treatments, successional field and forested sites.[1] For explanations of treatments see table 18.

Treatment	Mean % C g/hg soil
T1	0.86 ± 0.05
T2	0.93 ± .04
T3	0.90 ± .05
T4	0.93 ± .07
T5	0.97 ± .07
T6	0.88 ± .05
T7	0.91 ± .06
T8	1.49 ± 0.05
DF	1.52 ± 0.05
SF	0.97 ± 0.03
CF	1.46 ± 0.05

[1]For further information see: http://lter.kbs.msu.edu/datatables/68.

The vastly larger microbial biomass of the yearly-mown, grassland treatment (T8) is typical of grasslands when compared to agronomically managed land, and is due to the increased litter inputs, carbon content and soil structure typical of grasslands (Dilly and Munch,

1995). Accordingly, T8 had much higher biomass than any of the other MSCE treatments, along with a correspondingly higher total mean % C, demonstrating a positive correlation between soil C and microbial biomass C in that treatment.

Regression between CFI and DNA assays

The regression between the fumigation and molecular data was strong. The difference between the slopes between of the agronomic and forested sites indicated that there is a dramatic increase in DNA content per unit of carbon microbial biomass in the forested sites as compared to the agronomic treatments. Previous studies, using methods other than CFIA and total extracted DNA, have found forested soils to exhibit significantly higher values of microbial biomass than either grasslands or agriculturally managed systems (Griffiths *et al*., 1997 (direct microscopy); Dilly and Munch, 1995(fumigation-extraction). The findings of Zoronza *et al*. (2009) were similar. They used phospholipid fatty acid (PLFA) analysis, a technique that is more indicative of the viable microbial community, since PFLAs are quickly turned over after cell death. Therefore, the microbial biomass values of the forest soils as measured by the DNA extraction here are more consistent with previous findings that assessed microbial biomass using other techniques than values derived from the CFIA.

There are several possible explanations why the slopes for the forest and agricultural treatments were so different. First, there has also been some contention that the K value (coefficient of mineralization) of 0.41 used here may not be consistent for soils harboring different ratios of fungi to bacteria (Anderson and Domsch 1978). A different coefficient of mineralization may, therefore, be needed when computing microbial biomass of forest versus

agricultural soils because of differences in the relative proportion of fungi and bacteria, with fungal biomass being demonstrated to be dominant over bacterial biomass in forested communities (Horwath and Paul, 1994; Zornoza *et al*., 2009; Griffiths *et al*., 1997). Another explanation could be that the forested soils harbor a higher concentration of soil biota harboring DNA recalcitrant to fumigation such as nematodes, micro-arthropods and other multi-cellular, eukaryotic organisms. Finally, there could also have been a higher concentration of humic acids co-extracted with the DNA from the forested soils, which would result in artificially high DNA concentration values (Zhou et al., 1996).

Conclusions

The use of conventional tillage in agricultural management has dramatic, negative effects on the underlying soil's microbial biomass. In addition to loss of structure, nutrients, water holding capacity and C, tillage ultimately decreases the size and density of the microbial biomass of a soil. Utilizing a no-till management style significantly improves the size of microbial biomass, as does the inclusion of a cover crop. Thus, the benefits of no-till farming management and incorporation of a cover crop to the underlying microbial community are evident and should provide guidance for future agricultural management strategies. This does not, however, necessarily correlate to higher agricultural yields, as demonstrated by the difference in yields between the till and no-till crop rotations here receiving conventional amounts of N. More investigations into what practices and strategies are viable for a sustainable plan of agricultural management are needed. The data from the successional treatment indicates that over time, 20 years in this study, the soil microbial biomass may recover from agricultural management and in fact become larger than the original, native

ecosystem from which the agricultural and successional ecosystems developed. This holds promise for preserving our microbiota in the future.

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CONCLUSIONS AND FUTURE DIRECTIONS

We have determined that agricultural land management significantly impacts the microbial biomass of Midwestern United States soils, with tillage being the component that has the largest negative impact. However, the addition of a cover crop can compensate for losses in microbial biomass attributable to tillage, and early- to mid-succession from agriculture can increase soil's microbial biomass to a level higher than realized under agricultural land management or in the original native deciduous forest ecosystem. While agricultural land management and succession did not significantly impact the richness of fungal taxa or richness of fungal ligninolytic genes in soil, it did impact the taxonomic membership and distribution of ligninolytic genes of soil fungal communities, indicating niche differentiating on microbial structure and functioning with some degree of functional redundancy and plasticity.

Although we uncovered some of the impacts that agricultural land management and succession have on soil microbial communities, there is a need for further investigations into the mechanisms that shape microbial and fungal communities and control their functions in terrestrial ecosystems. We need a deeper understanding of how guilds of fungi interact, both within and among themselves, and what environmental factors influence those interactions and the metabolic processes they mediate. Microbes provide ecosystem services that are crucial to even the most basic ecosystem functions of terrestrial ecosystems such as primary production and maintaining soil fertility. A deeper understanding on what we can do to preserve and manage the Earth's microbial assets is key to the long-term sustainability of microbial processes and functioning in agricultural and other terrestrial ecosystems.