CHARACTERIZING SOIL MICROBIAL COMMUNITIES OF RECLAIMED ROADS IN NORTH DAKOTA

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

Reclaimed roads on the Little Missouri National Grasslands of southwestern North Dakota have not returned to pre-disturbance conditions. Phospholipid fatty acid analysis was performed on soil samples collected from reclaimed roads and adjacent prairie to assess reclamation effects on the microbial community. Additionally, nutrient cycling capacity was measured by four enzyme assays. Ordination analysis of PLFA data identified a distance gradient indicating microbial communities of reclaimed roads were different from the prairie. Specifically, Gram-negative bacteria and arbuscular mycorrhizal fungi are associated with roads; soil organic matter was associated with prairie sites. Soil enzyme activities associated with prairie sites indicate greater nutrient cycling. The reclaimed road soils have not accumulated sufficient organic matter to sustain both plant and microbial communities characteristic of the surrounding prairie.

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DEDICATION

This work is dedicated to my wife Janessa. I can do anything with her support.

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LITERATURE REVIEW

Introduction

Humans have been modifying the soil environment for agricultural purposes for thousands of years. The industrial revolution elevated the capacity for disturbance with the introduction of fossil fuels. The substantial infrastructure network required to facilitate heavy equipment during resource development makes recovery of fossil fuels an environmentally destructive process. Lands disturbed during resource extraction are required be reclaimed after resource extraction has ceased to mitigate long-term disturbances to ecosystems.

The Little Missouri National Grasslands (LMNG) is a native mixed grass prairie ecosystem located in southwestern North Dakota characterized by a unique, eroded landscape known as the North Dakota badlands. Located above a large deposit of oil and natural gas known as the Bakken shale formation, the LMNG is currently being vigorously developed for resource extraction. Growth in energy development includes the construction of thousands of miles of roads through the native prairie ecosystem. When wells are no longer producing oil they are shut down, dismantled, and the infrastructure is reclaimed.

A road's impact on an ecosystem spans multiple scales affecting an assortment of distinct soil chemical, physical, and biological properties such as pH, electrical conductivity (EC), bulk density, soil structure, and microbial biomass (Spellerberg, 2002; Switalski et al., 2004). Lugo and Gucinski (2009) suggest that roads are dynamic, finite ecosystems separate from natural landscapes; therefore, roads add an element to the landscape matrix. Reed (1996) reports that road effects on an ecosystem can span widths

up to 2.5-3.5 times greater than the actual road. Reincorporating areas disturbed by road construction and maintenance back into the landscape matrix presents challenges as lands around roads have been under different management since disturbance was initiated (Forman et al., 2003).

Guiding succession of disturbed lands on a course that intersects the succession of the surrounding landscape is the job of the land manager. Management of the LMNG is the responsibility of the United States Forest Service (USFS) and the Bureau of Land Management. These agencies have outlined standards for restoration of disturbed federal lands in *Surface Operating Standards and Guidelines for Oil and Gas Exploration and Development,* often referred to as *The Gold Book.* Chapter Six of the *Gold Book* states reclamation objectives are to "…ensure the effect [of development] is not permanent" (USDI and USDA 2007). The final objective of the reclamation process is to guide disturbed lands on a course that will bring about "eventual ecosystem restoration" (USDI and USDA 2007). The statement indicates that agencies, like the scientific community, differentiate the definition of "restoration" from "reclamation".

"Reclamation" has been defined as a "process by which derelict lands are returned to productivity and by which some measure of biotic function and productivity is restored" (Whisenant, 1999). Goals of reclamation projects are often simply to stabilize soil against erosion or further degradation from other mechanisms. The term restoration has been defined as the "process of reestablishing, to the extent possible, the structure, function, and integrity of indigenous ecosystems and sustaining habitats they provide" (Whisenant, 1999). Mitsch and Jorgensen (2004) concur with the previous definition of restoration and reclamation, but add that reclamation can lead sites along a completely

different course than that existing before disturbance. The different definitions indicate that planning for reclamation will not necessarily lead to ecosystem restoration. *The Gold Book* suggests that planning reclamation strategies prior to construction increases the chances of restoration success.

Harris et al. (1996) recognizes three biological components that should be considered in the planning process when attempting to restore an ecosystem: primary producers, consumers, and decomposers. Historically, primary producers and consumers have been the focus of restoration goals, but advances in biogeochemistry have increased the efficiency of studying the decomposer community (Harris et al., 1996). The soil decomposer component is an essential link in nutrient cycles of soil environments (Paul et al., 1979). The importance of decomposers can be summed up with the following statement: "microbial activity is the gate through which all minerals must pass before they can be recycled to plants" (Davidson, 1979). A common effect of road construction in wild lands is reduced energy flow to the decomposer components of the food chain, resulting in a deficiency of plant available macronutrients (Whisenant, 1999). Restoring flow of energy to the decomposer component is an essential mechanism for recharging the macronutrient pool. Soil organic matter is the major source of energy and nutrients for the soil decomposer component and is essential to restoring energy flow. Companies operating within *The Gold Book* directive are advised to "salvage and reuse" topsoil to add organic matter into the degraded system (USDOI and USDA, 2007). Restoring soil organic matter by spreading stored topsoil has proven effective for regenerating the microbial community of the soil (Ingram et al., 2005b).

Reclaimed roads have been studied in the LMNG to determine the effectiveness of reclamation attempts. Simmers and Galatowitsch (2010) measured the plant communities present on the road compared to those of the adjacent native prairie. The authors found that road communities were dominated by species included in the seeding mix planted during reclamation efforts: colonization from the adjacent native range was limited. Lack of native establishment on roadbeds was attributed to three conditions. First, many of the varieties of seeded species are known for their competiveness during establishment after seeding. Second, the harsh climate conditions of the area were considered as a possible barrier to native establishment. Investigations found that late seral species were able to colonize roads indicating that at least some of the native species are able colonize under the unfavorable conditions; therefore, it was concluded that climate was not the main inhibitor to native establishment. Finally, soil compaction was identified as a possible barrier to native colonization. From communication with Forest Service employees, Simmers and Galatowitsch (2010) found that many of the road sites were still compacted in the upper portions of the soil profile. It was suggested that the compaction could inhibit the movement of rhizomes of native grasses into the restored area. The authors suggested investigating "differences in texture, nutrient availability, soil biota, etc. to better understand their relation to plant establishment" (Simmers and Galatowitsch 2010).

The Forest Service subsequently funded a study to investigate the soil properties of the reclaimed roads. Matthees-Dose (2009) investigated multiple soil physical and chemical properties including soil organic matter (SOM), infiltration, particle size distribution including coarse fragments, electrical conductivity (EC), pH, calcium

carbonate equivalent (CCE), and sodium absorption ratio (SAR). In general Matthees-Dose found significantly reduced organic matter, higher proportions of clay, greater soil pH and CCE, reduced water infiltration, and more bare ground on the reclaimed sites compared to the native prairie ecosystems. Coarse fragments were more abundant in reclaimed areas compared to the native prairie. The author attributed this to road surfaces either not being thoroughly removed or not being buried deep enough. Bare ground was also found to increase on reclaimed areas relative to adjacent native areas. These increases could make reclaimed areas inhospitable to native plants. Significantly lower SOM was observed on reclaimed areas compared to that of the native prairie. Lower SOM was attributed to "removal of topsoil, and/or the lack of plant establishment and nutrient cycling after reclamation." The findings of the study indicate that the soil environment was significantly changed because of road construction and subsequent reclamation activities.

Decreased SOM affects the soil environment by reducing nutrients available to the microflora of the soil and by altering the physical environment that serves as habitat to the biological community. Microorganisms utilize energy in SOM for general metabolism and release nutrients as a byproduct. During the degradation process some SOM is incorporated into microbial cells; therefore, soil microbial communities can act as both a source and sink of rapid turnover SOM pools (Tripathi et al., 2007). In many instances a nutrient must pass through multiple microbial populations before it is converted into a plant-available form. Consequently, the composition of microbial communities may have a significant effect on the plant community of a system. Additionally, reduction in habitat quality through loss of SOM may negatively affect the

total biomass of the soil reducing the microbial community's capacity for nutrient transformation. Studying the microbial community of a soil may provide insight as to why a plant community does not return after reclamation attempts.

The microscopic scale of the decomposer component makes direct study difficult. Approximately one percent of soil microorganisms can be cultured using traditional microbial techniques. More recently developed molecular techniques such as phospholipid fatty acid (PLFA) analysis and DNA analysis have made determining the soil microbial community composition more efficient than culture methods. These methods have been utilized in various studies investigating restoration success. Indirect methods for characterizing soil microbial communities (i.e. substrate utilization techniques, enzyme assays, and microbial biomass) are cruder but more cost effective methods of studying soil biological properties than culturing or DNA and fatty acid extractions. Conducting enzyme assays on soil is an effective indirect method of characterizing the function of the decomposer portion of the soil system. Microbial community structure and function of reclaimed roads in the LMNG will be investigated using PLFA analysis and enzyme assays.

Soil Enzymes

Nutrients retained within dead plant material and soil organic matter are often bound to recalcitrant biological materials (Stevenson, 1994). Molecules such as cellulose and lignin are difficult to break down because they are insoluble and contain complex bond structures. Enzymes are biological catalysts that facilitate degradation of recalcitrant molecules. Insolubility and size prevent cellulose and lignin molecules from diffusing to the interior of microbial cells for degradation. The term "extracellular"

describes an enzyme secreted by an organism into its environment (Weetall, 1975). Microbes use extracellular enzymes to mineralize nutrients, such as nitrogen and phosphorus, from large, insoluble organic molecules not easily transported into microbial cells (Burns, 1978). Soil enzymes play an intrinsic role in terrestrial nutrient cycles by degrading recalcitrant molecules and releasing biologically available, mineralized nutrients into the soil solution (Caldwell, 2005). Reactions catalyzed by soil enzymes take place in soil environments and are not associated with specific populations of the soil biological community; therefore, all soil enzymes are extracellular enzymes. Furthermore, it has been found that most enzymes in the soil environment are of microbial origin (Burns, 1978).

Soil enzymes are stabilized by interactions with clay and SOM particles in the soil environment (Weetall, 1975). Stabilization allows enzymes to remain active in soils for extended periods of time since they can withstand extreme environmental conditions (Naseby and Lynch, 2002). Long-term stability of soil enzymes allow organisms to continually obtain nutrients released from degraded SOM molecules (Naseby and Lynch, 2002; Quiquampoix et al., 2002). Long-term stabilization is particularly beneficial when ambient conditions have not favored microbial growth and a boost of nutrients is needed for an increase in biomass once conditions have improved. Although microorganisms produced soil enzymes, it is important to remember that extracellular enzymes are not associated with any one cell but more closely related to the soil itself. Nutrients released by soil enzymes are available to a wide variety of organisms, including plants.

Plants benefit from nutrients released from SOM since soil enzymes release nutrients into the soil environment. In fact, plants “prime” or initiate soil microbial

activity by exuding low molecular weight organic compounds with high nutrient content into the rhizosphere. Large proportions of total plant photosynthate, 5-21%, is exuded into the soil environment in the form of root exudates and root sloughing (Walker et al., 2003). Pulses of root exudates stimulate microbial enzyme secretion leading to mineralization of both fresh inputs and existing soil organic matter. Priming effects allow plants to benefit from byproducts of increased microbial metabolism (Hamilton and Frank, 2001; Hamilton et al., 2008; Paterson, 2003).

In some cases the benefit to plants is so large that feedback mechanisms have evolved. Many studies have found evidence of feedback mechanisms between plants and soil microbial communities (Ehrenfeld et al., 2005; Hamilton et al., 2008; Jordan et al., 2008; Klironomos, 2002). Jordan et al. (2008) defines positive feedback mechanisms as invasive plant species selecting soil microbial communities that increase its chance for success. The authors found increased vigor for a number of invasive plants species grown in containers of soil where invasive species had previously been grown. For example, smooth brome grew significantly better in containers that had previously grown crested wheatgrass. These effects were not seen in containers of sterilized soil. They hypothesized that invasive species were altering microbial communities in the rhizosphere, which favored future establishment of invasive plants thus creating positive feedback mechanisms.

Other studies have found altered community structure and function from invasive species. Li et al. (2006b) studied the effect of an invasive plant on soil chemical and physical properties of a Chinese forest. The authors found that soils under the invasive species had significantly higher enzyme activities than those under native forest

conditions. Using ordination techniques, the authors were able to separate soils supporting native communities from soils containing monocultures of invasive species. Multivariate analysis also allowed the authors to conclude that SOM and available phosphorus were significant factors in determining the enzyme activity of the invaded soils. Other work has characterized the structure and function of soil microbial communities affected by invasive species. Kourtev et al. (2002) used multivariate ordination techniques to separate soils supporting native plants from soils invaded by exotic plants.

While plants have a large impact on soil enzymes and soil microbial communities, the physical and chemical components of the soil can affect soil enzymes as well. Soil microbial biomass was negatively affected by soils with elevated EC values of 7.1 dS m^{-1} (Egamberdieva et al., 2010). Saline soils also had lower total organic carbon values and a smaller proportion of organic carbon derived from microbial carbon. Tripathi et al (2007) studied saline soils during the rainy season, temperate winter, and dry season in India. The authors found saline soils had lower enzyme activities than non-saline soils. The lowest enzyme activities were obtained during the hot, dry summer months, which concentrated salts in the soil surface. The authors stated that reduced enzyme activity could be due to lower microbial biomass from both matric and osmotic stressors.

Soil enzymes respond quickly to changes in land management, a particularly interesting consideration when investigating reclamation success (Pascual et al., 2000). Often, studies compare enzyme activity of restored sites to activity of the target environment. White and Walker (1997) state that reference sites provide essential information about the target environment of the restoration. The goal in these studies is to

match microbial indicies levels on degraded areas to those of target communities. Research has been conducted in southwest Spain where abandoned farmland in arid environments is causing desertification. Scientists are evaluating microbial processes that allow important plant species to recolonize abandoned farmland. Soil enzymes have also been used as an important indicator of restoration success in these fields (Garcia and Hernandez, 1997; Garcia et al., 2005; Izquierdo et al., 2005). Soil enzyme activities have been used to characterize natural successions of spoil piles left after open pit mining (Baldrian et al., 2008). The age of spoil piles dictated the plant community on the site. The authors found enzyme activity at each site changed as plant communities changed. Increased enzyme activity at the sites was associated with increased soil carbon and nutrients such as nitrogen, phosphorus, and potassium.

In the studies discussed above, land degradation was evaluated using soil enzymes. These and other studies conclude that changes in soil organic matter and edaphic properties affect nutrient cycles. Altered plant communities and decreased SOM on reclaimed roads on the Little Missouri National Grasslands suggest soil nutrient cycles have been negatively altered. Investigating enzyme activities of reclaimed roads in the LMNG reveals the general function of the SMC in degraded areas. Enzyme activity does not give any indication of how individual populations within the community have changed. Answering such questions require more detailed molecular techniques.

Soil Microbial Community Analysis

Soil microbial communities are composed of diverse microbial populations interacting with each other and their environment. Changes in soil environments may be reflected in changes in microbial community structure. Directly quantifying populations

of microorganisms has been traditionally difficult (Hill et al., 2000). Direct count methods with microscopes are labor intensive and create precision problems (Hill et al., 2000). Culture techniques have only isolated approximately 1% of soil microorganisms (Paul, 2007). Indirect quantification methods such as chloroform fumigation-extraction are popular when measuring microbial biomass C of the community. This method is not sensitive enough to differentiate populations within the microbial community (Hill et al., 2000). Techniques developed in the last 20 years allow soil microbiologists to characterize and quantify the soil community (Frostegard et al., 1991). Phospholipid fatty acid (PLFA) analysis quantifies concentrations of fatty acids that have been identified as signatures for specific groups of soil organisms (Kandeler, 2007).

Phospholipid fatty acids are the major cell membrane component of most organisms. The cell membrane is a lipid bilayer that forms the exterior of the cell creating a barrier between the aqueous cytoplasm and the environment. PLFA molecules are composed of two fatty acids and one phosphate group ester-linked to a glycerol backbone. Differences in bonding structure of the fatty acid (FA) portion of the molecule allow researchers to differentiate groups of organisms. Molecular structure changes include carbon-chain length, location of unsaturated carbons, and isomeric structure. Relative microbial biomass is another item of information obtainable from PLFA (Frostegard and Baath, 1996). Biomass is measured “when the functional role of an organism is important” (McCune and Grace, 2002) and quantifies the magnitude of the whole community. Biomass values measured using PLFA are expected to represent the metabolically active portion of the soil microbial community as PLFA’s are degraded

quickly in the soil environment after cell death. Changes in microbial biomass have been associated with management changes and disturbance (Kandeler, 2007).

Unlike other methods for determining microbial biomass, PLFA provides a unique opportunity to quantify specific fractions of the microbial community. For example, fungal biomass can be separated from bacterial biomass by totaling different signatures from the extract. Fungal-to-bacterial ratio has been widely used in the literature when comparing microbial communities of two soils under different management (Baath and Anderson, 2003; Frostegard and Baath, 1996). Fungal-to-bacterial ratios are commonly used indicators of how reclamation is progressing. Studies have found that the fungal-to-bacterial ratio in newly reclaimed land is narrower than older reclamation sites resulting from smaller fungal communities and larger bacterial communities (Allison et al., 2005; Dangi et al., 2010; Mummey et al., 2002a). These three studies agreed that, after a certain period of time, widening of the fungal-to-bacterial ratio was caused by a decrease in total bacterial biomass rather than an increase in the amount of fungal biomass.

Phospholipid fatty acid analysis has been widely utilized by soil microbiologists to assess effects of land management on the soil microbial community (Hill et al., 2000). Card et al (2010) utilized PLFA's to determine the microbial community of restored wetlands relative to target wetlands in the prairie pothole region of Canada. Two studies conducted in Wyoming used fatty acid methyl ester (FAME) analysis, a technique similar to PLFA, to investigate the microbial community structure of reclaimed mine soils (Mummey et al., 2002a; Mummey et al., 2002). Card et al. (2010) found that PLFA's of fungal origin were associated with older reclamation sites. Mummey et al. (2002a)

reported wider fungal-to-bacterial ratio of older reclaimed sites and native prairie than the narrower ratio of the younger restorations. Mummey et al. (2002b) observed that fungi in native sites were not highly correlated with soil organic matter nor were they affected spatially. The authors attributed this to larger populations of arbuscular mycorrhizal fungi (AMF). Arbuscular mycorrhizal fungi are able to obtain energy from the symbiotic host plants allowing dispersal throughout the soil system independent of soil organic matter concentrations.

Eighty percent of plant species are able establish a relationship with mycorrhizal fungi with some plant species and all of the AMF species relying on the symbiosis for survival. Plant roots are infected by AMF through contact with hyphae, spores, or infected plant roots remaining in the soil from the previous growing season (Peterson et al., 2004). Symbiotic interactions between plant and fungi help plants access inorganic nutrients such as phosphorus while plants provides fungi with photosynthate carbon for growth and development (Alexander, 1977). Because of their greater ability to branch out and explore the soil and provide inorganic nutrients, mycorrhizal-plant relationships in an ecosystem are usually an adaptive measure by plants to survive in low-nutrient systems (Schultz et al., 2001). Arbuscular mycorrhizal fungi are not host specific, but are obligate biotrophs required to infect plants to access a carbon source (Peterson et al., 2004).

The mutualistic relationship fostered between plants and AMF is an important consideration when restoring disturbed lands. The hyphal network established over long periods of time is an important factor in the infection of plant roots. Areas that have been severely disturbed do not have the vast network of hyphae available for inoculum of newly established plants. Introduction of inocula by incorporating plants already infected

may help with vegetation reestablishment (Peterson et al., 2004). White et al. (1992) found that top soil scraped and hauled directly to a restoration site without storage greatly increased the number of propagules for AMF in the soil. Frost et al. (2001) observed AMF infection density was greater 14 years after reclamation than when the sites were sampled soon after time of reclamation. Increasing the propagules bank in the soil increases probability of colonization by the fungus on a plant root. Unfortunately, quantifying the infection rate of mycorrhizal fungi in the root system is time consuming and not entirely accurate or reliable (Wright, 2005). Olsson et al. (1995) identified a PLFA signature molecule correlated with AMF hyphae counts. This AMF signature molecule has been utilized by a number of different studies to quantify AMF biomass during restorations (Card and Quideau, 2010; Kulmatiski and Beard, 2008; Olsson et al., 1999). This biomarker is different from that used to identify saprophytic fungal populations in the soil community.

Soil fungi are an important component of soil microbial communities. Fungi are often associated with stable systems because these systems tend to accumulate recalcitrant forms of organic material distributed in pockets across the landscape. Fungi have an advantage in stable environments dominated by recalcitrant forms of organic matter because of their ability to degrade complex organic materials with wide C:N ratios (Dix and Webster, 1995). Mineralization of recalcitrant molecules releases biologically available, inorganic nutrients making fungi an important part of nutrient cycles (Dix and Webster, 1995). Ability to move through the soil environment to explore for pockets of organic material and moisture is another advantage fungi exhibit over bacteria in stable environments (Thorn and Lynch, 2007). The ability for fungi to explore is dependant on a

continuous hyphal network to deliver nutrients to the exploring portion of the fungus. Advantage over bacteria is lost when disturbance homogenizes the nutrients in the system and breaks the hyphal network.

A number of studies have identified higher fungal populations in undisturbed reference sites than in disturbed areas using PLFA analysis. Bardgett et al (1999) found significant increases in fungal-bacterial ratios along a management gradient from high input pastures to natural grasslands. Grayston et al (2001) collected a greater proportion of fungal biomarkers in unimproved grasslands over those of improved grasslands. Allison et al (2005) found relative fungal abundance to increase with time in a tallgrass prairie restoration chronosequence. All three studies found higher biomass in stable ecosystems that tended to have heterogenous nutrient distribution.

Bacteria also play an important role in the soil microbial community. They are able to degrade readily-available nutrients from the soil environment (Baath and Anderson, 2003). Bacterial communities are often more active in disturbed soils because of increased resource availability (Allison et al., 2005; Bardgett et al., 1999). Disturbed sites offer easy access to readily mineralizable substrates that would normally be protected by aggregates in more stable environments. As mentioned earlier, PLFA is able to extract biomarkers that have been used to identify different groups of bacteria as well as the whole bacterial biomass (McKinley et al., 2005). Breaking down the whole biomass into individual components allows analysis of proportional changes within the bacterial community. As a general example, the overall bacterial biomass may not have changed between a disturbed site and its reference, but an increase or decrease in the

proportion of Gram-negative signatures indicates a change in the microbial community structure (McKinley et al., 2005).

Bradshaw (1997) stated four biological properties that must return to a restored site: soil organic matter, soil nitrogen capital, other available nutrients, and nutrient cycling. Although fungi are important for soil organic matter accumulation and nutrient cycling in prairie ecosystems, bacteria have a monopoly on the primary nitrogen cycling processes - nitrification, nitrogen fixation, and denitrification - vital to terrestrial ecosystems (Brady, 1974). Specialized bacteria are responsible for the enzymatic transformations important to rebuilding a soil's nitrogen capital. It may take years or decades before these organisms return to a degraded site (Mitsch and Jorgensen, 2004). Allison et al. (2005) found a larger number of general bacterial biomarkers in early road restorations, but through time the population became dominated by specialists like actinomycetes and other Gram-positive bacteria. Higher proportions of actinomycetes may indicate that a portion of the microbial community is shifting to more specialized nitrogen-fixing bacteria. This shift would indicate a less stressed system where an important link of the nitrogen cycle is starting to be restored.

For microbial indices mentioned above to actively reflect what is happening in a disturbed system, comparison to a reference site must be made. In restoration, this often refers to a target site. A target site is representative of the final goal and provides a reference when measuring restoration progress (White and Walker, 1997). Nutrient cycles are temporally variable so utilizing reference sites could help explain discrepancies in the background data. In other situations, background information may

not have been collected before disturbance occurred. Having a target site is essential for meeting any restoration goals for such a sites.

Objectives

Increased energy production in western North Dakota is increasing the number of oil well access roads in the LMNG. Existing and new roads must eventually be reclaimed to pre-disturbance conditions. The reclamation practices used in the past have not restored plant communities or SOM to pre-disturbance conditions based on comparisons of road data with the adjacent native prairie (Matthees-Dose, 2009; Simmers and Galatowitsch, 2010). Plant community and SOM affect the soil microbial community. Identifying how the microbial community differs from the surrounding native prairie and identifying those soil physical/chemical properties driving the differences could indicate which areas of the reclamation process should be reconsidered for future reclamation projects. We may also be able to determine if the plant community is driving differences in microbial community or if soil physical/chemical changes are contributing as well. This research is also one of the first efforts to classify soil microbial communities in the semi-arid mixed grass prairie of southwestern North Dakota. There has been other work done on reclaimed mine lands in Wyoming in similar environments. Other work has been done on restorations of tallgrass prairie ecosystems in the central plains (Allison et al., 2005; Jangid et al., 2010; McKinley et al., 2005). Reclamation effects will be tested using enzyme assays and PLFA analysis since they are sensitive in measuring effects of both plant community and soil physical/chemical changes.

During the vegetative study conducted by Simmers and Galatowitsch (2010), the authors observed that plant communities on the roads were based on the seed mixes used

at the time of restoration. Seed mixes were found to corresponded to policies in place at the time of reclamation. A second study of road soils reported lower soil organic matter on the road compared to the adjacent native prairie. We hypothesized that there would be a disturbance gradient with different microbial communities on the road relative to the adjacent native prairie. Our second hypothesis was that three distinct microbial communities would be present on the road following a pattern similar to differences in plant community identified by Simmers and Galatowitsch (2010). The goals of this research are 1) to determine the effect that time since reclamation has on the soil microbial community, 2) to determine the effect of disturbance gradients on the soil microbial community, and 3) to characterize the soil microbial community of a soil typical of the LMNG.

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CHAPTER 1. INVESTIGATING THE SOIL MICROBIAL COMMUNITY STRUCTURE UNDER RECLAIMED ROADS IN NORTH DAKOTA

Introduction

From 2003 to 2010, North Dakota's oil production nearly quadrupled and production is projected to be second only to Texas by 2013 (Associated Press, 2012; North Dakota Industrial Commission, 2011). Increased oil production has led to expansion of infrastructure, including roads, well sites, and pipelines, reminiscent of earlier oil booms in North Dakota (North Dakota Industrial Commission, 2011). A portion of the region developed for oil extraction is on federal lands managed the United States Forest Service (USFS) and the Bureau of Land Management (BLM). Many wells drilled during previous booms have ceased oil production and have been decommissioned. Government agencies managing these lands require oil and gas companies to reclaim developed areas after oil production ceases.

Government policies regarding restoration and reclamation have changed as ecological understanding of succession has increased. Reclamation techniques are outlined in the *Gold Book* published by the USFS and US Department of the Interior (USDI) (USDI and USDA, 2007). Goals of reclamation include creating a stable environment that will conform to the pre-disturbance, natural environment (USDI and USDA, 2007). In the most recent edition of the *Gold Book*, agencies' recommendations for road reclamation include removal of road-beds, re-contouring disturbed areas, and re-establishing plant cover. Reclamation of many roads in North Dakota occurred during periods when outdated standards for plant materials were approved for reclamation purposes (Simmers and Galatowitsch, 2010).

The goal of habitat reclamation leading to re-establishment of native environments has not been met on reclaimed roads in the LMNG as shown by two previous studies documenting

plant communities and soil physical and chemical properties (Matthees-Dose, 2009; Simmers and Galatowitsch, 2010). Plant communities on reclaimed sites were more likely to reflect species seeded than incremental stages of succession towards a native plant community and may reflect changes in USFS seeding policy (Simmers and Galatowitsch, 2010). Furthermore, a significant distance effect on vegetation distribution was documented from road-center to the adjacent prairie. The distance effect indicated a greater proportion of seeded relative to native species on the roads, suggesting that most native species had not successfully colonized roadbeds. Matthees-Dose (2009) identified several soil properties that differed between roads and the adjacent native prairie. A significant distance effect on soil organic matter was identified along the same transects established by Simmers and Galatowitsch (Matthees-Dose, 2009). Soils on the road had lower soil organic matter relative to surrounding prairie.

Plant species and soil properties, especially soil organic matter, influence the soil microbial community. Soil microbial communities are an important, yet often underappreciated component of nutrient cycles. Microorganisms' unique enzymatic abilities are essential for nutrient mineralization from the complex molecular structures comprising soil organic matter. Measuring the functional status of the soil microbial community can be accomplished by measuring enzyme activity of the soil. Soil enzymes are sensitive measures of soil nutrient cycling since they are respond to subtle environmental changes. Enzyme activity has been used in reclamation studies to asses nutrient cycles on sites undergoing secondary succession (Baldrian et al., 2008). Differences in enzyme activities have been documented between soils supporting either native or invasive plant communities (Kourtev et al., 2002; Li et al., 2006a). Plant species have been found to condition soil microbial communities promoting the success of future generations (Jordan et al., 2008; Klironomos, 2002). Two species reported to be

antagonistic to native species (Jordan et al., 2008) were commonly found on reclaimed roads. Soil physical and chemical properties such as salinity, electrical conductivity (EC), and pH effect soil microbial communities as well (Egamberdieva et al., 2010; Tripathi et al., 2007). More importantly, many studies report positive correlations between soil organic matter and enzyme activity (Garcia and Hernandez, 1997; Garcia et al., 2005; Izquierdo et al., 2005).

Soil microbial community structure is useful for determining community response to reclamation practices. Phospholipid fatty acid (PLFA) analysis is one of many methods used to assess soil microbial community structure. Phospholipids comprise the cell membrane of microorganisms and all other living organisms. Extracted phospholipid fatty acids from soil samples quantify the metabolically active microbial component since PLFA's are quickly degraded in the soil environment (Kandeler, 2007). Phospolipid fatty acid analysis has been used to gauge reclamation effects on the soil microbial community of natural systems including tallgrass prairie, Prairie Pothole Region wetlands, boreal forests, and shrub-steppe (McKinley et al., 2005; Card and Quideau, 2010; Dimitiu et al., 2010; Kulmatiski and Beard, 2008). Reclaimed roads in the LMNG exhibit measurable changes in plant community and soil properties, which suggest changes to the microbial community. Changes in soil physical and chemical properties may have exceeded a threshold preventing native establishment on roads. Identifying soil properties most strongly correlated with gradients in the soil microbial community may result in more ecologically appropriate strategies to encourage native plant colonization of disturbed areas.

Increased oil exploration activity in western North Dakota will inevitably lead to development of new infrastructure on lands managed by the USFS. Eventually these roads, pipelines, and well sites will become obsolete and will have to be reclaimed, like the

infrastructure for wells drilled during the early 1980's. Identifying factors that contributed to previous reclamation shortcomings will help land managers make well-informed decisions on future reclamations. Simmers and Galadowitsch (2010) identified three plant communities that corresponded to changes in seeding policy. Since USFS policy changes represent an evolving set of technical criteria over different reclamation intervals, we hypothesized three distinct microbial communities would exist on roads based on time since reclamation. Secondly, because a transition zone between the road and native prairie was observed during reconnaissance trips, we hypothesized that three distinct microbial communities could be measured from road center to the native prairie. The goals of this research are 1) to determine the effect that time since reclamation has on the soil microbial community, 2) to determine the effect of a disturbance gradient on the soil microbial community, 3) to determine how sampling time affects microbial community, and 4) to characterize the soil microbial community of thin loamy ecological sites which are extensive within the LMNG.

Methods

Study Sites

Study sites were located in the Little Missouri National Grasslands (LMNG) in southwestern North Dakota (46 ° 16' N to 48 ° 08' N and 102 ° 39' W to 104 ° 02' W). The LMNG have a semi-arid environment receiving 355- to 430mm of precipitation annually and with an average annual temperature of 5 to 7 °C. The plant community is classified as a mixed grass prairie (Laurenroth et al., 2008). Dominant species are western wheat grass (*Pascopyrum smithii* (Rydb.) Á. Löve), blue grama (*Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Griffiths), upland sedge (*Carex spp.)*, green needlegrass (*Nassella viridula* (Trin.) Barkworth), and needleandthread (*Hesperostipa comata* (Trin. & Rupr.) Barkworth). The soils of the area are

classified as mollisols, entisols, and inceptisols with frigid temperature regimes and ustic to aridic moisture regimes (Champa, 1998)

All roads sampled for this study were sampled in the previous studies, but only roads classified as thin loamy ecological sites were included. Three roads from the three reclamation intervals identified by Simmers and Galatowitsch (2010) were sampled to test for an effect of time since reclamation (Age). The three categories were defined as roads reclaimed between 1983-1987 (Age 1), 1988-1994 (Age 2), and 1995-2002 (Age 3). Their study observed vegetation composition at 3, 10, and 50 m extending both directions from the center of the roads. We used GPS to locate and sample identical sites on one side of the road, which enabled testing for Distance effects on the soil microbial community. Samples were collected three times throughout the summer to ensure timely laboratory processing, which consequently enabled analysis of Seasonal effects.

A grid with a 3 m radius was centered on each GPS point and ten soil cores were collected from 10 locations within the grid. Cores were collected with a hand probe to 5 cm and placed on ice during transport, and refrigerated in the laboratory. Samples were bulked for enzyme analysis and physical/chemical tests by cutting each core in half and combining the 10 partial cores from each sample unit. Bulked cores were sieved to 2 mm and stored at 8 °C until enzyme assays were performed. The remaining half of each core was bulked in groups of three producing 3 samples per sample unit for PLFA analysis. Samples for PLFA analysis were stored at - 20 °C for up to eight months and lyophilized before analysis.

Plant community composition was documented during the final soil sampling conducted as described by Ganguli et al. (2008). Canopy cover was estimated for the ten sampling quadrats at each sample unit with the following classifications: trace-0.5%; 0.5-1.0%; 1-5%; 6-10%; 11-

25%; 26-50%; 51-75%; 76-95%; 96-100%. Quadrats were approximately 0.5 m x 0.5 m. Plants rooted within a quadrat were counted during determination of cover class. Each species' total cover was averaged across all 10 quadrats for each sample unit. Relative cover was calculated by dividing each species' average cover by the total cover of each sample unit.

Enzyme Assays

Enzyme assays were performed to measure activity of alkaline phosphatase, acid phosphatase, beta-glucosidase, and phenol oxidase. Assays of phosphatase and beta-glucosidase activity were performed within three weeks of sample collection. Acid and alkaline phosphatase was conducted according to the method described by Tabatabai and Bremner (1969). One gram of soil was placed in a 50 ml Erlenmeyer flask and 4 ml of modified universal buffer (MUB), pH 6.5 or 11 (acid and alkaline phosphatase, respectively), 200 µl of toluene, and 1 ml of 0.05M *p*-nitrophenyl phosphate (PNPP) were added. An identical set of samples was prepared to serve as a control and did not receive substrate before incubation. Rubber stoppers were placed in flasks and incubated for 1 h at 37 °C. One milliliter of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH was added to all flasks and PNPP was added to control flasks. Samples were filtered through Whatman no.2 filter paper and the absorbance of the filtrate was measured at 400 nm.

Beta-glucosidase activity was assayed with a method modified from Eivazi and Tabatabai (1988) and Tabatabai (1982). One gram of soil was placed in a 50 ml Erlenmeyer flask and 4 ml of MUB pH 6.0, 250 µl toluene, and 1 ml 0.05 M *p*-nitrophenyl-D-β-glucoside (PNG) were added to the flasks. An identical set of flasks was prepared without PNG to serve as a control. Sample and control flasks were stoppered and incubated at 37°C for 1 h. One milliliter of 0.05 M $CaCl_2$ and 4 ml of tris-(hydroxymethyl)aminomethane (THAM) buffer, pH 12 was added to all flasks and 1 ml PNG was added to control flasks after $CaCl_2$ and THAM buffer additions.

Solutions were filtered through Whatman no. 2 filter paper and filtrate was collected and analyzed for PNP with a spectrophotometer at 400 nm.

Phenol oxidase activity was measured with a modified method from Floch et al. (2007). Ten milliliters of MUB pH 2.0 and 200 µl of 0.1 M 2,2′-azinobis-(-3 ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was added to 0.1 g soil in a 25 ml centrifuge tube. The solution was incubated in a 30 °C water bath for five minutes then centrifuged at 12,500 rpm at 4 °C for 3 min to stop the reaction. Supernatant was decanted into cuvettes and absorbance was measured at 420 nm. There were two controls for each sample. One contained soil and MUB pH 2.0 and no substrate added, while the other contained autoclaved soil, MUB pH 2.0, and substrate. Controls were subtracted from samples after all other calculations were performed.

PLFA Analyses

Phospholipid fatty acid extraction was conducted with a modified Bligh and Dyer (1959) single phase extraction method (Frostegard et al., 1991; Buyer et al., 2002). Briefly, 5 g of lyophilized soil were weighed into a 30 ml culture tube and 20 ml of extraction buffer was added. Tubes were sealed with PTFE lined screw caps, placed on an inversion shaker for 2 h, and lastly, centrifuged at 2300 rpm for 13 min. Supernatant was decanted and saved, while the soil pellet was re-suspended by vortex in 5 ml of chloroform for a second extraction. The soil and chloroform solution was then centrifuged at 2300 rpm for 13 min and the chloroform was added to the original supernatant. Finally, 5 ml of phosphate buffer, pH 7.4, was added to the combined supernatants, vortexed to fully mix phases, and centrifuged at 2300 rpm. The aqueous phase was aspirated from the culture tube and the organic phase was evaporated under a constant stream of nitrogen.

Phospholipids were collected by washing the extracts through silica gel chromatography (Aligent) columns with chloroform, acetone, and methanol, which eluted the phospholipids from the columns. Extracts were dried under a constant stream of nitrogen. A mildly alkaline saponification reaction was performed to produce fatty acid methyl esters (FAMES) recognizable to the detection software. Finally, extracts were purified with amino solid phase extraction chromatography columns and dried under a constant nitrogen stream. The dry purified extracts were suspended in 200 µl of Final Solvent, transferred to glass vials, and sealed with PTFE septa. Vials were stored at –20 °C until shipped on dry ice for gas chromatography (GC) analysis. Lipid recognition software (MIDI, MIDI, Inc.) was used in tandem with GC to identify known lipid signatures.

Statistical Methods

Univariate statistical analyses were conducted using SAS 9.2 for Windows (SAS, Inc., Cary, NC, 2011). Analysis of variance was performed on all soil and biochemical properties measured using a mixed model approach (PROC GLIMMIX). Data were analyzed with a split-plot design with Age (time since reclamation) being the whole plot and Distance (distance from road center) the split plot. Three sampling dates necessitated a repeated measures design to test for Seasonal (sampling date) effects. Mean separations were performed on variables with significant main effects utilizing the Tukey-Kramer HSD procedure. The PLFA data analyzed with univariate statistics are Gram-positive bacteria ((14:0 iso + 15:0 iso + 15:0 anteiso + 16:0 iso + 17:0 iso 17:0 anteiso) / Total PLFA),
Gram-negative Bacteria ((16:1 w9c + 17:0 cyclo + 18:1 w9c) / Total Biomass), fungi (18:2 w6c / Total Biomass), arbuscular mycorrhizal fungi (AMF) (16:1 w5c / Total Biomass), protozoa (20:4 w6c + 20:3 w6c / Total Biomass), Gram-positive to Gram-negative ratio (Gp:Gn) ((14:0

iso + 15:0 iso + 15:0 anteiso + 16:0 iso + 17:0 iso + 17:0 anteiso) / (16:1 w9c + 17:0 cyclo + 18:1 w9c)), fungal to bacterial ratio (F:B) (18:2 w6c / (14:0 iso + 15:0 iso + 15:0 anteiso + 16:0 iso + 17:0 iso + 17:0 anteiso + 16:1 w9c + 17:0 cyclo + 18:1 w9c)), iso to anteiso ratio (I:A) ((15:0 iso + 15:0 anteiso) / (17:0 iso + 17:0 anteiso)), and biomass (Σ all PLFA with of chains ≤ 20 Carbons). Soil enzyme activities (biochemical properties) for alkaline phosphatase, acid phosphatase, beta-glucosidase, and phenol oxidase were analyzed with univariate statistics as well as soil properties measured by the North Dakota State University Soil Testing Lab (organic matter, nitrogen (N), phosphorus (P), potassium (K), electrical conductivity (EC), and pH).

Ordination analyses and Multi-Response Permutation Procedures (MRPP) were performed with PC-ORD software (Version 5, MjM Software Design, Gleneden Beach, OR). Ordination techniques are a family of statistical procedures designed to simplify large, multivariate data sets by identifying variation gradients concealed within the data sets. Detrended correspondence analysis (DCA) was performed on vegetation data. Axes, or variation gradients in the data, are calculated by performing a reciprocal averaging procedure to create the highest correspondence between species space and sample space (McCune and Grace, 2002). The detrending and rescaling processes remove compression and bending common in correspondence analysis and scales the axes in units of beta diversity. Environmental (or response) variables are represented in most ordination procedures as correlation vectors, which are depictions of variables' correlations with the ordination axes. Vectors are scaled, meaning they provide information on both the magnitude and direction of the correlation. For example, if two vectors associated are pointed in the same direction but one vector is longer than the other; the longer vector has a stronger correlation with the axes.

The DCA was performed with relative plant cover data in the main matrix. Rare species were downweighted and rescaled axes were selected by PC-ORD. Axis 1 was broken into 26 segments (default) during the detrending procedure. All biochemical and soil properties measured in this study and listed above were contained in the secondary matrix and correlated with axes from the DCA.

Nonmetric multidimensional scaling (NMS) is another ordination technique utilized for ecological data analysis since it does not assume normality and can manage variable data similar to our PLFA data (McCune and Grace, 2002). Like DCA, NMS calculates axes, or gradients, in the data that help explain major sources of variation, but NMS is in a different ordination family than DCA. The NMS procedure attempts to balance the number of axes with stress in the data. Usually, the greater the number of axes the lower the stress, but adding too many axes without large reductions in stress complicates interpretability.

Phospholipid fatty acid signatures were considered the species data for the NMS analysis. All signatures with carbon chains ≤ 20 carbon atoms long were expressed as mole percent and included in the main matrix. Euclidean distance was used as the distance measure, time of day was used to generate the starting point of the analysis, and 250 runs were performed on the real data. Data defined as environmental variables included all soil and biochemical properties listed above. Correlation vectors of these variables were calculated, overlaid on the axes, and rotated 90 ° to aid interpretation.

Multi-response permutation procedures were used to test site scores of DCA and NMS for effects of Age, Distance, and Season with PC-ORD software (Version 5.0). The analysis can be used to test multivariate data for main effects and pairwise comparisons without the assumptions of the MANOVA procedure available in SAS.

Results

Effects of Age, Distance, and Season

Time since reclamation effects (Age) were not statistically significant for 19 the 20 soil and biochemical properties measured in this study (Table 1). Significant Distance effects (distance from road center) were found for 11 of the 20 properties measured (Table 1). Significant Seasonal effects (month sampled) were found to be a significant effect for 17 of the 20 properties (Table 1).

Means separation on soil and biochemical properties with significant Distance effects revealed a uniform pattern throughout the data. The 3 m sites (road) and 10 m sites were similar to one another and distinct from 50 m sites (reference), for the soil and biochemical properties except pH, Gram-negative bacteria, and fungi (Table 2). Most soil and biochemical properties were significantly greater at reference sites than road sites. However, Gram-negative bacteria and pH decreased from the road to reference sites (Table 2). Fungal signatures were not significantly different between the 10 m points and the reference sites.

Means separation of significant Seasonal effects was variable across soil and biochemical properties. Activity of alkaline phosphatase and beta-glucosidase increased significantly during July, while June and August were statistically equivalent (Table 2). Acid phosphatase declined throughout summer; phenol oxidase activity was greatest in August (Table 2). Fungal signatures peaked in July and remained elevated into August as shown by increased Fungi and fungal to bacterial ratio (F:B) (Table 2); both months' fungal signatures were statistically greater than June.

Table 1. P-values of ANOVA tests for effects of Age, Distance, and Season and interactions

	Alkaline[a]	Acid[b]	Beta[c]	Phen[d]	Θ_g[e]	Nitrogen[f]	Potassium[g]	Phosphorus[h]	EC[i]	pH[j]
Age[†]	0.4267	0.4409	0.6127	0.7168	0.0492*	0.7697	0.2545	0.8224	0.7109	0.578
Distance[‡]	0.0013*	0.0061*	0.0073*	0.1258	0.0212*	0.6724	0.9378	0.423	0.4198	0.0015*
Season[§]	<.0001*	<.0001*	<.0001*	0.0002*	<.0001*	0.0108*	0.032*	0.2512	0.6293	<.0001*
Age*Distance	0.0021*	0.0374*	0.1854	0.8679	0.0933	0.0585	0.1689	0.1286	0.453	0.0989
Age*Season	0.7435	0.002*	0.9355	0.1699	0.0027*	0.1663	0.0329*	0.1884	0.193	0.0547
Distance*Season	0.2204	0.3547	0.0426*	0.3267	0.4601	0.059	0.6227	0.4294	0.7687	0.1482

	OM[k]	Gramp[l]	Gramn[m]	AMF[n]	Fungi[o]	Protozoa[p]	Biomass[q]	F:B[r]	Gp:Gn[s]	I:A[t]
Age[†]	0.2784	0.4071	0.8565	0.9953	0.56	0.7397	0.2431	0.55	0.4176	0.2538
Distance[‡]	0.0046*	0.325	0.0023*	0.5957	0.0152*	0.0981	0.0036*	0.0224*	0.0342*	0.5892
Season[§]	<.0001*	<.0001*	0.8994	0.0082*	<.0001*	<.0001*	0.0014*	<.0001*	0.0295*	<.0001*
Age*Distance	0.0702	0.3231	0.0117*	0.0159*	0.1533	0.0044*	0.0212*	0.2896	0.0277*	0.005*
Age*Season	0.8457	0.323	0.068	0.1521	0.0184*	0.5673	0.0301*	0.089	0.5105	0.0002*
Distance*Season	0.1859	0.7361	0.9571	0.3927	0.4752	0.1831	0.2429	0.5178	0.7836	0.7306

[a] alkaline phosphatase activity; [b] acid phosphatase activity; [c] beta-glucosidase; [d] phenol oxidase; [e] gravimetric water content; [f] nitrate nitrogen; [g] potassium; [h] bicarbonate extractable phosphorus; [i] electrical conductivity; [j] soil pH; [k] organic matter; [l] Gram-positive bacteria; [m] Gram-negative bacteria; [n] arbuscular mycorrhizal fungi; [o] fungi signatures; [p] protozoa; [q] biomass; [r] fungal to bacterial ratio; [s] Gram-positive to Gram-negative ratio; [t] iso to anteiso ratio

* Designates significance at the 0.05 level

†Age indicates the time since reclamation

‡Distance indicates distance from road center

§Season 1, 2, and 3 represents sampling in June, July, and August, respectively

Table 2. Mean separation of significant effects. Significance was tested using the Tukey-Kramer HSD at the alpha = 0.05. Means of Distances are averaged over all Ages and Seasons. Means for Season are averaged over all Ages and Distances. Different letters indicates significant difference between the different means. Number in parentheses represent SEM. N=27

	Alkaline[a] (µg PNP g^{-1} Dry Soil)			Acid[b] (µg PNP g^{-1} Dry Soil)			Beta[c] (µg PNP g^{-1} Dry Soil)		
	1	2	3	1	2	3	1	2	3
Age†	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Distance‡	469 (37)a	481 (31)a	765 (80)b	225 (26)a	234 (25)a	310 (35)b	132 (13)a	135 (12)a	199 (22)b
Season§	535 (58)a	673 (55)b	508 (62)a	409 (20)a	246 (15)b	113 (19)c	104 (16)a	237 (17)b	125 (2)a

	Phen[d] (µmol $ABTS^{+}$ g^{-1} DM min^{-1})			Gravimetric Water[e] (% by wt)			Nitrogen[f] (NO_3^{-1} lbs $^{-1}$acre)		
	1	2	3	1	2	3	1	2	3
Age†	N/S	N/S	N/S	0.1277a	0.1023ab	0.1446ac	N/S	N/S	N/S
Distance‡	N/S	N/S	N/S	0.111 (.01)a	0.116 (.01)a	0.147 (.02)b	N/S	N/S	N/S
Season§	0.148 (.01)a	0.134 (.01)a	0.20 (.02)b	0.197 (.01)a	0.074 (.01)b	0.103 (.01)c	2.67 (.36)a	1.48 (.18)b	2.30 (.21)a

	Potassium[g] (mg/kg)			pH[h]			OM[i] (%)		
	1	2	3	1	2	3	1	2	3
Age†	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Distance‡	N/S	N/S	N/S	8.1 (.05)a	8.0 (.05)a	7.9 (.07)b	2.87 (.13)a	2.95 (.14)a	4.11 (.24)b
Season§	247(14)a	234(12)ab	229(10)b	8.0 (.05)a	8.2 (.05)a	7.8 (.07)b	3.51(.24)a	3.49(.16)a	2.92(.20)b

[a] alkaline phosphatase activity; [b] acid phosphatase activity; [c] beta-glucosidase; [d] phenol oxidase; [e] gravimetric water content; [f] nitrate nitrogen; [g] potassium; [h] Soil pH; [i] Organic Matter.

†Age 1, 2, and 3 represents reclamation conducted in years 1984-1987, 1988-1994, and 1995-2002, respectively

‡Distance 1, 2, and 3 represents 3m, 10m, and 50m from road center, respectively

§Season 1, 2, and 3 represents sampling in June, July, and August, respectively

N/S represents no significant main effect

Table 2. (Continued)

	Gram-Negative Bacteria[j] (mol %)			AMF[k] (mol %)			Fungi[l] (mol %)		
	1	2	3	1	2	3	1	2	3
Age[†]	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Distance[‡]	16.6 (.13)a	16.4 (.23)a	15.5 (.23)b	N/S	N/S	N/S	5.3 (.27)a	6.0 (.28)ab	6.5 (.43)b
Season[§]	N/S	N/S	N/S	5.26 (.13)a	5.43 (.11)ab	5.61 (.11)b	4.60 (.20)a	6.80 (.33)b	6.48 (.11)b
	Protozoa[m] (mol %)			Biomass[n] (nmols g^{-1} DM)			F:B[o]		
	1	2	3	1	2	3	1	2	3
Age[†]	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Distance[‡]	N/S	N/S	N/S	59.2 (3.5)a	58.8 (2.7)a	80.6 (6.1)b	0.160 (.01)a	0.186 (.01)ab	0.204 (.01)b
Season[§]	1.8 (.08)a	2.1 (.09)b	1.8 (.08)a	68.6 (5.0)a	59.4 (4.8)ab	70.9 (4.24)ac	0.143 (.01)a	0.213 (.01)b	0.196 (.01)b
	Gram-Positive Bacterial[p] (mol %)			Gp:Gn[q]			I:A[r]		
	1	2	3	1	2	3	1	2	3
Age[†]	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S	N/S
Distance[‡]	N/S	N/S	N/S	0.989 (.02)a	0.985 (.02)a	1.094 (.03)b	N/S	N/S	N/S
Season[§]	16.1(.22)a	16.1(.34)a	17 (.21)b	1.001(.02)a	1.014(.04)ab	1.054(.02)b	1.26(.01)a	1.305(.02)b	1.382(.02)c

[j] Gram-negative Bacteria; [k] Arbuscular Mycorrhizal Fungi; [l] Fungi Signatures; [m] Protozoa; [n] Biomass; [o] Fungal to Bacterial Ratio; [p] Gram-Positive Bacteria; [q] Gram-positive to Gram-negative ratio; [r] Iso to Anteiso Ratio.

[†]Age 1, 2, and 3 represents reclamation started between the years 1984-1987, 1988-1994, and 1995-2002, respectively.

[‡]Distance 1, 2, and 3 represents 3m, 10m, and 50m from road center, respectively.

[§]Season 1, 2, and 3 represents sampling in June, July, and August, respectively.

N/S represents no significant main effect

Arbuscular mycorrhizal fungi signatures (AMF) significantly increased between June and August (Table 2). Protozoa signatures peaked in July but subsequently declined from July to August (Table 2). Biomass was significantly lower in July relative to June and August (Table 2). Gravimetric soil water content was greatest in June, lowest in July, and significantly increased in August (Table 2). Gram-positive-to-Gram-negative bacterial ratio (Gp:Gn) showed a significant increase from June to August; July was intermediate. The iso-to-anteiso ratio (I:A) steadily increased throughout the summer (Table 2).

Eight biochemical properties displayed significant Age*Distance interactions (Table 1). Values of alkaline and acid phosphatase activity, biomass, Gp:Gn, and I:A were significantly greater for Age 1 reference sites than those of Ages 2 and 3 (data not shown). Gram-negative bacteria and I:A had significant Age effects within 10 m sites and on road sites, respectively (data not shown).

To summarize, the cause of the significant Age*Distance interactions noted in this study are primarily due to the elevated biochemical properties of Age 1 reference sites. Six of the 8 biochemical properties with significant Age*Distance interactions had significant Distance effects within Age 1 (Table 3). Ages 2 and 3 demonstrate the same, but non-significant, trend for alkaline phosphatase, acid phosphatase, and biomass seen in Age 1, i.e., larger values for reference sites than road and 10 m sites (Table 3). Gram-negative and AMF signatures were the only biochemical properties that were elevated on the roads relative compared to reference sites (Table 3). Alkaline and acid phosphatase, biomass, Gp:Gn, and I:A were significantly greater between the road and reference sites within Age 1 (Table 3). Protozoa signatures in Age 2 demonstrated greater values for the reference sites than the 3 m and 10 m locations; this was the only property to have a significant Distance effect for an Age other than Age 1 (Table 3).

Table 3. Mean separation of variables found to have significant Age*Distance interactions. Significance was determined using Tukey-Kramer's HSD at the 0.05 level. Different letters following values indicate a significant difference between Distances within Ages. Significant differences are not indicated between the ages. Means for each distance within ages have been averaged over all months. Numbers in parentheses represent SEM. N=9

	Age 1[†]			Age 2[†]			Age 3[†]		
	3 m[‡]	10 m[‡]	50 m[‡]	3 m[†]	10 m[†]	50 m[†]	3 m[†]	10 m[†]	50 m[†]
Alkaline Phos[a§]	471 (79)a	401 (63)a	1163 (126)b	406 (61)a	549 (36)a	526 (126)a	530 (50)a	492 (53)a	607 (65)a
Acid Phos[b§]	236 (41)a	202 (43)a	403 (58)b	187 (40)a	222 (44)a	235 (56)a	251 (54)a	278 (46)a	291 (61)a
Gram Negative[c¶]	16.2 (.22)a	17.3 (.34)a	14.9 (.46)b	16.7 (.26)a	16.1 (.12)a	16.0 (.22)a	16.9 (.21)a	15.9 (.53)a	15.6 (.39)a
AMF[d¶]	5.65 (.15)a	5.51 (.11)ab	5.07 (.22)b	5.20 (.26)a	5.58 (.21)a	5.57 (.32)a	5.45 (.14)a	5.41 (.22)a	5.47 (.16)a
Protozoa[e¶]	1.86 (.17)a	1.92 (.10)a	1.73 (.12)a	1.67 (.16)a	1.97 (.15)a	2.47 (.21)b	1.87 (.08)a	2.07 (.20)a	1.92 (.09)a
Biomass[f#]	74.1 (4.4)a	49.7 (4.4)a	104.3 (8.7)b	52.1 (4.2)a	67.5 (1.7)a	73.5 (13)a	52.3 (5.5)a	59.1 (5.3)a	64.1 (6.3)a
Gram P: Gram N[g]	1.06 (.05)ab	0.91 (.03)a	1.20 (.08)b	0.96 (.04)a	1.02 (.03)a	1.02 (.03)a	0.94 (.03)a	1.03 (.05)a	1.06 (.05)a
I:A[h]	1.27 (.03)a	1.34 (.03)ab	1.36 (.01)b	1.39 (.03)a	1.34 (.03)a	1.33 (.02)a	1.27 (.03)a	1.26 (.03)a	1.30 (.04)a

[a]Alkaline Phos = Alkaline Phosphatase activity; [b]Acid Phos = Acid Phosphatase activity; [c]Gram negative = Sum of Gram negative PLFA signatures; [d]AMF= Arbuscular mycorrhizal fungi PLFA signature; [e]Protozoa = Sum of Protozoa PLFA signatures; [f]Biomass = Sum of all PLFA signatures; [g]Gram P:Gram N = Ratio of gram positive to gram negative PLFA signatures; [h]I:A = Ratio of iso to anteiso PLFA signatures.

[†]Ages 1, 2, and 3 represent reclamations started 1984-1987, 1988-1994, and 1995-2002, respectively.

[‡]3m, 10m, and 50m represent distance from road center

[§] μg PNP g^{-1} Dry Soil

[¶] mol %

[#] nmol g^{-1} Dry Soil

Acid Phosphatase activity, potassium, fungi, and I:A showed a significant Age*Season interaction (Table 1). Biomass was significantly lower between Ages 1 and 3 within the July sampling trip. The I:A ratio showed a significant decrease for both Ages 1 and 3 and Ages 2 and 3 during July (Table 4). Potassium was significantly higher in Age 1 compared to Age 3 during the June sampling period.

Table 4. Mean separation of variables found to have significant Age*Season interactions. Significance was determined using Tukey-Kramer's HSD at the 0.05 level. Different letters following values indicate significant difference between Ages within Seasons. Significant differences are not indicated between the ages. Means for Ages within months represent averages of 3 m, 10 m, and 50 m sites. Number in parentheses represent SEM. N=9

	June†			July†			August†		
	Age 1‡	Age 2‡	Age 3‡	Age 1‡	Age 2‡	Age 3‡	Age 1‡	Age 2‡	Age 3‡
Acid[a§]	433 (45)a	354 (29)a	440 (24)a	251 (28)a	221 (22)a	267 (30)a	156 (43)a	70 (21)a	113 (26)a
Potassium[b#]	293 (28)a	227 (21) ab	220 (14)b	227 (13)a	238 (22)a	238 (28)a	254 (24)a	203 (14)a	230 (11)a
Biomass[c&]	80 (9.6)a	65 (9.7)a	60 (5.6)a	71 (10)a	61 (7.7)ab	44 (4.0)b	76 (9.7)a	66 (7.5)a	70 (4.3)a
I:A[d]	1.26 (.03)a	1.27 (.03)a	1.26 (.02)a	1.34 (.02)a	1.37 (.02)a	1.20 (.03)b	1.37 (.03)a	1.41 (.02)a	1.37 (.03)a

[a]Acid = Acid Phosphatase activity; [b]Potassium = soil potassium; [c]Biomass = Sum of al PLFA signatures; [d]I:A = ratio of iso to anteiso PLFA signatures.
†June, July, and August represent the month that samples were collected
‡Ages 1, 2, and 3 represent reclamations conducted from 1984-1987, 1988-1994, and 1995-2002, respectively
§ μg PNP g^{-1} Dry Soil
¶ mol %
# PPM Soil Potassium
& nmol g^{-1} Dry Soil

Ordination Statistics

Microbial Community Structure

The NMS analysis of PLFA signatures extracted from soils of reclaimed roads on the LMNG suggested a 2-dimensional solution after 89 iterations with a final stress of 8.78 and final instability of less than 10^{-4}. The Monte Carlo permutation analysis of the NMS procedure showed that the 2-dimensional solution was significant (P=0.0040). Site scores were subjected to a multi-response permutation procedure (MRPP) to test for effects of Age, Distance, and Season. Significant Distance effect was identified during the MRPP analysis, but no significant Age effect was identified in NMS results (Table 5).

Significant Season effects were identified in the data, but the low agreement statistic (A-value) suggesting low ecological significance (Table 5). Pairwise comparisons of each Distance indicated the same pattern shown by univariate statistics for PLFA data. The road sites and 10 m sites were not significantly different as indicated by the low A-values but both were significantly distinct from reference sites (Table 5).

Table 5. Results from MRPP of microbial community NMS scores and Plant community DCA scores. Main effects were determined to be significant at the 0.05 level. Pairwise comparisons were considered significant at the 0.017 level after a Bonferonni adjustment (p = 0.05/3).

	NMS Site Scores			DCA Site Scores		
	T†	A‡	p§	T	A	p
Distance	-8.514	0.1092	<0.0001	-2.975	0.1047	0.0099
3 m vs.10m	-3.384	0.045	0.013	1.033	-0.037	0.8910
3 m vs. 50m	-11.795	0.1614	<0.0001	-4.377	0.1652	0.0021
10m vs. 50m	-3.101	0.0389	0.0171	-2.942	0.0980	0.0113
Age	-0.458	0.0059	0.2530	-0.560	0.0197	0.2518
1 vs. 2	NA	NA	NA	NA	NA	NA
1 vs. 3	NA	NA	NA	NA	NA	NA
2 vs. 3	NA	NA	NA	NA	NA	NA
Season	-1.985	0.0255	0.0483			
June vs. July	-2.812	0.037	0.0230			
June vs. August	-1.570	0.0216	0.0755			
July vs. August	0.133	-0.002	0.4102			

* Indicates significance at the 0.05 level
†T is the test statistic
‡A is the agreement statistic
§p is the p-value
¶3m, 10m, and 50m represent distance from road center, respectively
#Ages 1, 2, and 3 represent reclamations started 1984-1987, 1988-1994, and 1995-2002, respectively
††June, July, and August represent the month samples were collected
NA Indicates no pairwise comparisons were made

The axes of the final NMS solution were rotated 90 degrees to facilitate interpretation of the ordination (Fig. 1). After rotation, the ordination accounts for 96.5% of the variation in the original data. Axis 1 accounts for 88.1% and Axis 2 for 8.1% of the variation. Axis 1 represents

the Distance gradient identified by MRPP (Table 5). Reference sites clustered to the right side of Axis 1, while road sites clustered to the left of Axis 1 (Fig. 1). Correlation of the PLFA data indicated that two signatures were highly correlated with Axis 1. These two signatures were iso 17:1 G and iso 17:1 at 9, with r^2-values of 0.850 and 0.931, respectively. The signature 17:1 at 9 had a negative correlation coefficient while iso 17:1 G had a positive correlation coefficient with Axis 1 (data not shown). The high correlation of these two signatures indicates that they change more than any other PLFA signature from roads to reference sites. Two other signatures, 17:0 anteiso and 18:1 ω9c, representing Gram-positive and Gram-negative bacteria, respectively, have correlations (r^2-values) with Axis 1 of 0.302 and 0.201, respectively.

Correlations of soil (i.e. organic matter and pH) and biochemical (i.e. alkaline phosphatase and fungi) properties with Axis 1 showed that Gram-negative bacteria had the greatest r^2-value (0.337). Other biochemical properties correlated with Axis 1 were alkaline phosphatase (r^2=0.314), Gp:Gn (r^2=0.202), acid phosphatase (r^2=0.156), AMF (r^2=0.165), and biomass (r^2=0.178). Soil properties significantly correlated with Axis 1 were organic matter (r^2=0.188) and pH (r^2=0.129). The correlation vectors for gram-negative bacteria and AMF were pointing toward the 3 m road points while the Gp:Gn was indicating a correlation with the reference sites (Fig. 2). All correlation vectors of soil and biochemical properties except pH, AMF, and Gram-negative bacteria point to reference sites, indicating that most soil and biochemical properties measured were higher in the native prairie than on disturbed sites (Fig. 1).

When site scores were classified by Season, there is an apparent, but slight Seasonal gradient along Axis 2 (Fig. 2). Fungi and protozoa were correlated with Axis 2 with r^2-values of 0.422 and 0.194, respectively. The MRPP analysis showed a statistically significant effect of Season in NMS scores, but the A-value was low indicating vague ecologic significance,

additionally paired Seasonal comparisons failed to show significance (Table 5). Therefore, interpretation of Axis 2 as a weak Seasonal gradient in the ordination is not warranted.

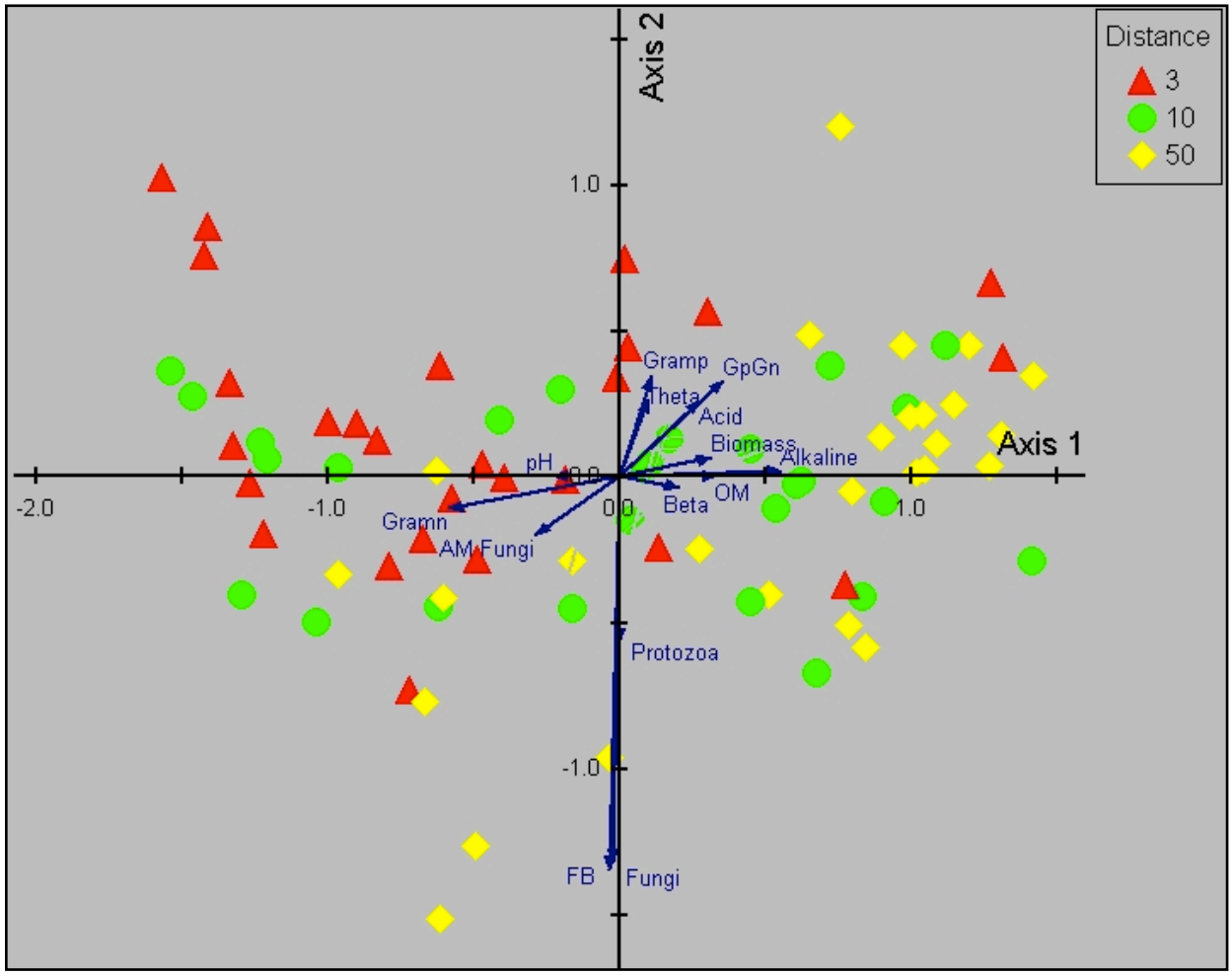

Figure 1. NMS ordination of PLFA community scores. Points represent sites plotted along the axes, which represent gradients detected within the PLFA data. Environmental vectors show the scale and direction of the correlation with each axis. Axes were rotated 90 degrees to aid interoperability. Gramp = Gram positive bacteria PLFA signatures; Gramn = Gram negative bacteria PLFA signatures; FB = fungal to bacterial ratio; Protozoa = protozoa PLFA signatures; Fungi = fungal PLFA signatures; GpGn = Gram positive to Gram negative ratio; Biomass = sum of bacterial and fungal PLFA Signatures; AM Fungi = arbuscular mycorrhizal fungi PLFA signatures; pH = Soil pH; Theta = water content; Beta = beta-glucosidase activity; Alkaline= alkaline phosphatase activity; Acid = acid phosphatase activity; OM = organic matter.

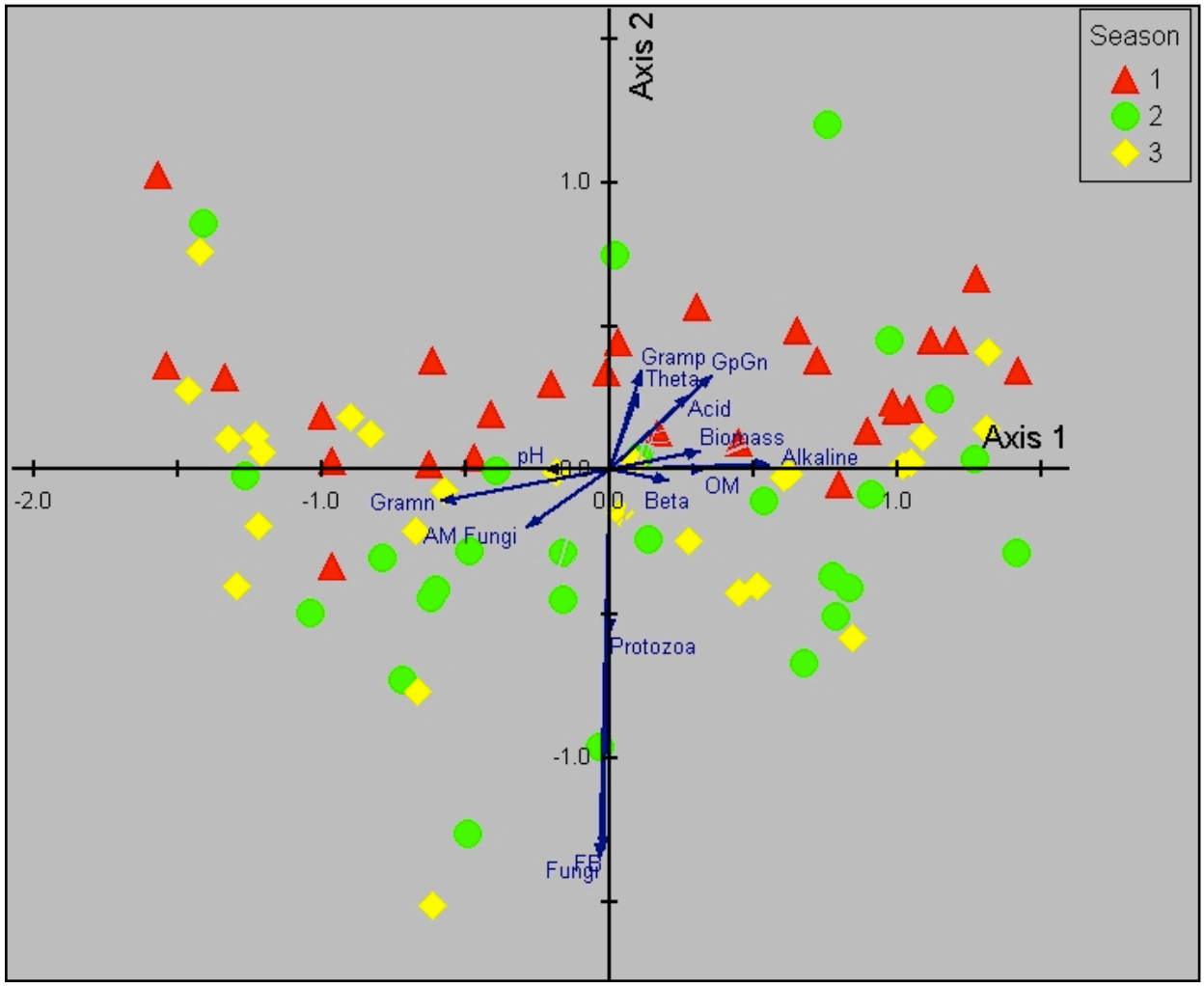

Figure 2. NMS ordination of PLFA community scores. Points represent site scores plotted along axes representing gradients in the microbial community data. Site scores are classified by Season: Season 1, 2, and 3 represent June, July, and August, respectively. Axes were rotated 90 degrees. Gramp = Gram positive bacteria PLFA signatures; Gramn = Gram negative bacteria PLFA signatures; FB = fungal to bacterial ratio; Protozoa = protozoa PLFA signatures; Fungi = fungal PLFA signatures; GpGn = Gram positive to Gram negative ratio; Biomass = sum of bacterial and fungal PLFA Signatures; AM Fungi = arbuscular mycorrhizal fungi PLFA signatures; pH = Soil pH; Theta = water content; Beta = beta-glucosidase activity; Alkaline= alkaline phosphatase activity; Acid = acid phosphatase activity; OM = organic matter.

Vegetation Community

Detrended correspondence analysis (DCA) of the vegetation data resulted in a 3-dimensional solution and accounted for 31.5% of the variance in the original data. The two axes that explained the majority of the variance were Axis 1 (22.8%) and Axis 2 (4.9%). The MRPP analysis of the DCA site scores indicates a significant Distance effect in the plant data; no Age effect was identified (Table 5)

Axis 1 represents the Distance gradient identified by MRPP analysis (Table 5). Species' scores plotted in ordination space portray relatively distinct plant communities associated with road and reference sites (Fig. 3). Species scores that plot proximate to road sites have higher relative abundances on roads. Species' correlations with DCA axes provide a quantitative means to segregate the vegetation communities of the sites. Species plotting with the road sites were *M. officinalis* (r^2-value 0.461), *A. frigida* (r^2-value 0.134), and *A. cristatum* (r^2-value 0.332). Species associated with the native prairie include *B. gracilis* (r^2-value 0.258), *Carex spp.* (r^2-value 0.354), and *Opuntia spp.* (r^2-value 0.132).

Correlation vectors indicate soil and biochemical properties were greatest in the native plant community of the reference sites (Fig. 3). Organic matter (r^2-value 0.115), Gram-negative bacteria (r^2-value 0.119), AMF (r^2-value 0.156), Beta-glucosidase activity (r^2-value 0.108), and Gp:Gn (r^2-value 0.098) were associated with the native plant community (Fig. 3). Gram-negative bacteria and AMF, conversely, were correlated with the plant community of roads with r^2-values of 0.119 and 0.156, respectively.

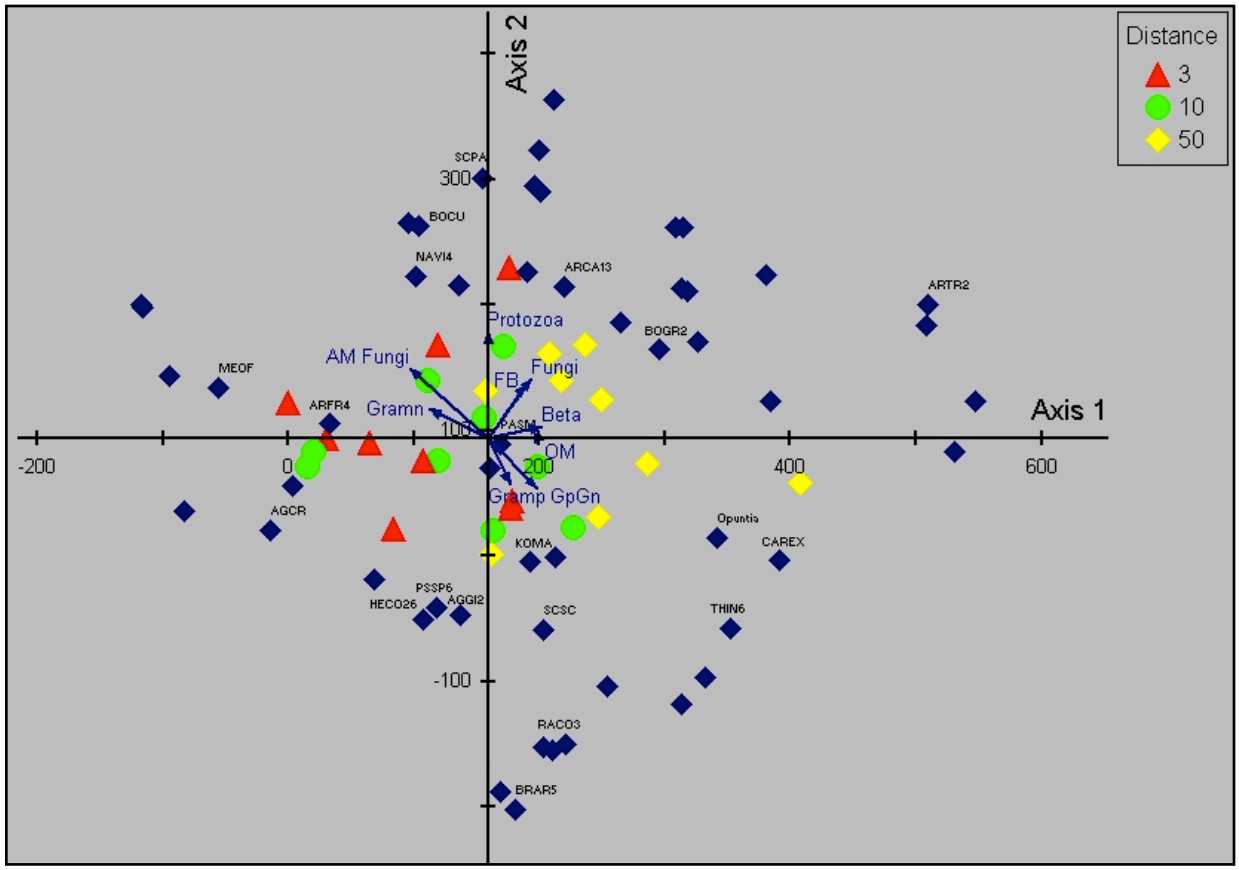

Figure 3. DCA ordination of plant community data. Red, green, and yellow points represent site scores. Blue diamonds represent plant species scores. Sites are classified by Distance with 3 m, 10 m, and 50 m representing distance from road center. Vectors represent scale and direction of the correlation of data with the axes. Gramp = Gram positive bacteria PLFA signatures; Gramn = Gram negative bacteria PLFA signatures; FB = fungal to bacterial ratio; Protozoa = protozoa PLFA signatures; Fungi = fungal PLFA signatures; GpGn = Gram positive to Gram negative ratio; Biomass = sum of bacterial and fungal PLFA Signatures; AM Fungi = arbuscular mycorrhizal fungi PLFA signatures; pH = Soil pH; Theta = water content; Beta = beta-glucosidase activity; Alkaline= alkaline phosphatase activity; Acid = acid phosphatase activity; OM = organic matter. MEOF = *M. officinalis* ; ARFR = *A. frigida* ; AGCR4 = *A. cristatum*; BOGR2 = *B. gracilis*; CAREX = *Carex spp;* Opuntia = *Opuntia spp*; PSSP6 = *Pseudoroengneria spicata;* ARCA13 = *Artemesia cana;* ARTR2 = *Artemesia tridentia;* THIN6 = *Thinopryrum intermedium*; RACO3 = *Ratibida columnfera;* BRAR5 = *Bromus arvensis*; HECO26 = *Hemitomes congestum*; AGGI2 = *Agrostis gigantean*; BOCU = *Bouteloua curtipendula*; NAVI3 = *N. viridula*; KOMA = *Koeleria macrantha*; SCPA = *Schedonnardus paniculatus*; PASM = *P. smithii*; SCSC = *Schizachyrium scoparium*.

Discussion

Effects of Age, Distance, and Season

No biologically significant Age effects (time since reclamation) on soil and biochemical properties were identified by univariate statistical analyses (Table 1). Multi-response permutation procedure (MRPP) conducted on non-metric multidimensional scaling (NMS) site scores also did not indicate a significant Age effect on the microbial community (Table 5). However, Age effects were anticipated based upon plant community changes documented by Simmers and Galatowitsch (2010). Furthermore, our results contrast other studies that identified strong time since reclamation response of the soil microbial community on reclaimed tallgrass prairies (McKinley et al., 2005), Prairie Pothole Region wetlands (Card and Quideau, 2010), and reclaimed mine sites in semi-arid environments (Mummey et al., 2002). The lack of Age effects in this study may be a function of our relatively small data set; only 17 % of the roads studied by Simmers and Galatowitsch (2010) were sampled. Extracting the subset of roads sampled in this study from the Simmers and Galatowitsch (2010) dataset and analyzing them for Age effects would indicate if our small data set was robust enough to control the random variability on the LMNG.

Among the soil and chemical properties measured, significant Distance effects (distance from road center) were identified for water content, organic matter, and soil pH. Water content on road sites was approximately 25 % less than reference sites (Table 2). Matthees-Dose (2009) found average organic matter levels of 4.7 % in reference sites compared to 3.1 % on roads; this study's results of organic matter were quite similar with 4.1 % reference sites, 3.1 % roads (Table 2). Significantly increased values of soil pH on road sites in this study resemble those of a previous study (Table2) (Matthees-Dose, 2009). Organic matter is the principle source of energy

and nutrients for the soil microbial community and therefore drives the size, structure, and function of the soil biological community. Elevated nutrient pools of organic matter-rich environments stimulate enzyme activity and enhance mineralization, often resulting in greater microbial biomass (Ingram et al., 2008).

Most biochemical properties measured in this study had significant Distance effects (Table 1). Gram-negative PLFA signatures were greater on the roads relative to reference sites, while Gp:Gn was significantly greater in the reference sites (Table 2); suggesting that increased Gp:Gn on the reference sites is caused by the significant decrease of Gram-negative bacteria. Therefore, reclamation practices have created an environment more hospitable to Gram-negative bacteria (Table 2). Our findings for Gram-negative bacteria signatures concur with a study of reclaimed boreal forest microbial communities (Dimitriu et al., 2010), while other authors have reported increased Gram-negative signatures in non-disturbed soils (Peacock et al 2001; Card and Quideau, 2010). Although there is no apparent consensus regarding the cause or significance of changes in Gp:Gn along disturbance gradients (McKinley et al., 2005), the consistent observation of changes in microbial community is additional evidence that reclamation processes do affect soil biological processes (Ingram et al., 2005). Identifying target microbial communities and establishing appropriate goals for restoration is advocated during restoration research (McKinley et al., 2005; White and Walker, 1997).

Fungal PLFA concentrations were greater in reference sites compared to roads (Table 2). Fungi form vast hyphal networks throughout soils adapting them to conditions of osmotic and nutrient stress (Dix and Webster, 1995). Disturbance of the hyphal network removes much of the advantage fungi have compared to bacteria for growth in nutrient-poor environments. It is surprising that fungal communities have not reestablished on the LMNG roads given the long

period since reclamation was initiated (10-29 years). Studies on prairie reclamations have identified a sudden increase in fungal biomass soon after disturbance has ceased (e.g. Allison et al., 2005). Since samples were collected long after reclamation was initiated, this flush was not detected in our study.

Significantly lower enzyme activities were identified at road sites. Alkaline phosphatase, acid phosphatase, and beta-glucosidase decreased 38%, 24%, and 34%, respectively (Table 2). Decreased enzyme activities on LMNG roads relative to reference sites demonstrate reduced biological activity and, consequently, nutrient cycling potential of reclaimed road soils. Reduced enzyme activity of road soils is likely due to reduced organic matter leading to limited substrate availability. Decreased nutrient cycling, as measured by enzyme activity, was directly correlated with organic matter on European mine spoils (Baldrian et al., 2008). Changes in enzyme activity have also been documented along plant community gradients. For instance, Li et al. (2006) analyzed enzyme activity with ordination techniques to identify an invasive-to-indigenous plant community gradient of forest soils in China. Reclaimed sites on the LMNG have shown both decreased organic matter content (Matthees-Dose, 2009) and marked plant community changes from the adjacent prairie (Simmers and Galatowitsch, 2010). Therefore, differences in nutrient cycling between roads and reference sites, as measured by enzyme activity in this study, were anticipated and observed.

The significant Age*Distance interaction identified for eight soil and biochemical properties (Table 1) is largely explained by significantly greater biochemical properties at Age 1 reference sites relative to those for Ages 2 and 3 (Table 3). Gram-negative bacteria and protozoa showed significant Age effects within 3 and 10 m sites (data not shown). More than half of the biochemical properties with interactions exhibit significant Age effects within reference sites

(data not shown). Six of the eight properties significantly increased between road and reference sites within Age 1 (Table 3). The protozoa signature was the only measured biological property to display significant Distance effects for Ages 2 and 3 (Table 3).

Inherent variability of the native prairie may account for the lack of Distance effects within Ages 2 and 3. Semi-arid prairies, like the LMNG, are heterogeneous environments with nutrient "islands" dispersed throughout the landscape (Laurenroth et al., 2008). Data collected from such environments are difficult to evaluate statistically because some sample units should be expected to lie within "islands" representing random variability. Controlling random variability in ecological studies is best accomplished with larger samples. Sampling more roads in this study would have resulted in a better representation of the native prairie. However, few roads met both ecological site and time since reclamation sampling criteria, which resulted in fewer roads within each Age. A repeated measures sampling design was implemented to collect a sufficient number of samples from limited roads, manage inherent variability of the landscape, and allow evaluation of Seasonal effects. Continuous sampling of the same sites as mandated by repeated measures analyses did not allow for full representation of variability that would have been possible with a larger sample.

Significant Seasonal effects were found for all soil and biochemical properties measured except Phosphorus, EC, and Gram-negative bacteria, although identifying consistent patterns in the data was difficult (Tables 1 and 2). Even ordination analysis, designed to detect gradients within complex datasets, did not produce a significant Seasonal gradient in PLFA data (Table 5).

Ordination

While Distance effects were statistically non-significant for roads of Ages 2 and 3, biochemical properties did show similar patterns to Age 1 roads and a significant Distance

gradient was produced by NMS (Fig. 1 and Table 5). Reference sites were generally associated and distinct from road sites along Axis 1, indicating different microbial communities between the two sites (Fig. 1). The 10 m sites were dispersed throughout the biplot, indicating they are associated with both the roads and reference sites (Fig. 1). During fieldwork, obvious boundaries between roads and the adjacent prairie were documented, but in some cases the sampling grid at 10 m sites straddled this boundary, contributing to increasing variability.

Increased variability of 10 m sites is quantified by the "chance-corrected within-group agreement statistic (A-value)" (McCune and Grace, 2002). Pairwise comparisons of 10 m sites had A-values closer to zero, indicating low within group homogeneity. For example, the 3 m vs. 10 m comparison is significant ($P<0.05$), but the low A-value of (0.045, Table 5) indicates a small effect size due to low within group homogeneity. Conversely, the significant p-value for the 3 m vs. 50 m test has an A-value of 0.161 (Table 5), indicating high with-in group homogeneity (McCune and Grace, 2002). Therefore, it is the 10 m sites that contribute to the low "chance-corrected within group" statistic for MRPP analysis. Separating the microbial communities between the road and reference sites would be a valid interpretation of our data, but stating that significantly different microbial communities exist between 10 m sites and road or reference sites would be difficult to defend.

Individual signatures associated with Gram-positive and Gram-negative bacteria were significantly correlated with Axis 1. The Gram-positive signature (17:0 anteiso) and the Gram-negative signature (18:1 w9c) had r^2-values with Axis 1 of 0.284 and 0.199, respectively. While individual Gram-positive and Gram-negative signatures were significantly correlated with Axis 1, the PLFA signature iso 17:1 at G had an r^2-value of 0.950 with Axis 1. The signature iso17:1 at G has been used to indicate actinomycetes, another group of soil bacteria (Chatterjee et al.,

2008). The correlation coefficient for this signature is positive (data not shown) indicating that it is strongly associated with reference sites. Correlation of individual bacterial signatures with Axis 1 indicates that bacteria are greatly affected by the Distance gradient.

The PLFA data correlated with Axis 1 reveals how specific microbial groups are dispersed along the Distance gradient. The Gp:Gn is associated with reference sites (r^2-value 0.202), while Gram-negative bacteria are strongly associated with roads (r^2-value 0.337; Fig. 1). These explanatory vectors indicate that Gram-negative bacteria have greater relative abundance on roads than reference sites, contrasting a study that found greater Gram-negative bacteria signatures in non-disturbed settings compared to sites compacted by vehicle use (Peacock et al., 2001). The increase in Gp:Gn in reference sites is probably due to decreased relative abundance of Gram-negative bacteria rather than an increase of Gram-positive bacteria. The AMF signature was clearly associated with road sites (r^2-value 0.165; Fig. 1). Conversely, an absence of AMF was associated with restored Wyoming mine sites (Mummey et al. 2002), which raises a question of why such different AMF responses were documented on similar semi-arid prairies. Other authors report varied results of reclamation on the microbial community i.e., such as conflicting responses for the Gp:Gn (Ingram et al., 2005; McKinley et al., 2005). Identifying specific changes in microbial community structure is beyond the scope of this study since PLFA analysis produces a rather coarse representation of community structure. The results of this analysis establish that differences in the microbial community along the Distance gradient from road-center to native prairie demonstrates that reclamation practices have not attained pre-disturbance conditions on the roads.

Soil properties were correlated with Axis 1 to relate changes in the soil microbial community along the Distance gradient. Organic matter and pH were correlated with Axis 1,

with r^2-values of 0.188 and 0.129, respectively (Fig. 1). The correlation vector representing organic matter points to reference sites, while the pH vector is associated with the roads (Fig. 1). Matthees-Dose (2009) identified organic matter as the primary soil property consistently associated with disturbance caused by road construction through the prairie. Correlation of organic matter with the Distance gradient corroborates findings of univariate analysis results indicating a significant Distance effect on this property. Organic matter and pH are known to influence the soil microbial community (Frostegard et al., 1993; Fierer and Jackson, 2006). It is not surprising that most microbial community signatures measured in this study vary in accordance with organic matter content (Fig. 1). Although correlations do not indicate causal relationships, the influence of organic matter on the soil microbial community is well documented and clearly demonstrated in this study.

Besides organic matter and pH, reference sites exhibited greater enzyme activity as indicated by correlation vectors for alkaline and acid phosphatase and beta-glucosidase, with r^2-values of 0.314, 0.156, and 0.117, respectively. (Fig. 1) Soil enzyme activity has been directly correlated with soil organic matter in several studies (Baldrian et al., 2008; Garcia and Hernandez, 1997). Soil organic matter accounted for the greatest amount of variation in soil enzyme data for one study of invasive species in a Chinese forest soil (Li et al., 2006). Significantly greater enzyme activity in reference sites indicates that reclaimed roads have not acquired the same degree of biological activity and nutrient cycling potential as the surrounding native prairie.

Changes in the plant community cause fluctuations in the soil microbial community (Li et al., 2006; Jordan et al., 2008). Detrended correspondence analysis (DCA) on relative cover of the road vegetation and native prairie indicated a Distance gradient, which was confirmed by MRPP

analysis (Fig. 3, Table 5). Axis 1 shows that reference sites clustered separately from road sites, indicating that the plant community on the roads is different from that of the prairie (Fig. 3). Plant species correlated with Axis 1 reveal that *A. cristatum* (r^2-value 0.332)*, M. officianalis* (r^2-value 0.461), and *A. fridgida* (r^2-value 0.134) are associated with the road environment while *B. gracilis* (r^2-value 0.258)*, Carex spp*. (r^2-value 0.354), and *Opuntia spp*. (r^2-value 0.132) are more closely associated with the native prairie environment (Fig. 3). The plant communities identified in this study generally concur with those identified by Simmers and Galatowitsch (2010).

The vegetation and microbial community appear to follow similar Distance gradients based on correlations of PLFA signatures with the plant community gradient (Figs. 1 and 2). Organic matter is correlated with both the PLFA community gradient and the vegetation gradient. Both vegetation community and soil organic matter have been shown to influence the soil microbial community (Li et al., 2006). It is possible that decreased soil organic matter content on the reclaimed roads is the barrier preventing colonization of the roads by the indigenous plant and soil microbial communities due to decreased "species performance."

The "successional management framework" states that succession occurs because of three processes: "site availability, species availability, and species performance" (Krueger-Mangold et al., 2006). Site availability should not be hindering succession on the LMNG because of the large proportion of bare ground documented on the reclaimed roads (Matthees-Dose, 2009; Simmers and Galatowitsch, 2010). Species availability was considered by Simmers and Galatowitsch (2010), but was dismissed because of a "narrow" disturbance winding through the diverse prairie. Road sites had decreased organic matter content and suppressed biological activity that are not increasing with time indicating that reclaimed sites are not accumulating nutrients needed to increase species performance.

In conclusion, differences in the microbial community were identified between the reclaimed roads and the adjacent prairie. Specifically, the road sites tended to have a greater relative abundance of Gram-negative bacteria and AMF relative to the surrounding prairie, while reference sites had greater total biomass, enzyme activity, and Gp:Gn. The plant community of the roads and was significantly different from the community of the prairie and generally concurred with vegetation data collected in a previous study. Correlations of Gram-negative bacteria and AMF signatures with the DCA axes indicate they are closely associated with road sites, which indicates the plant community and soil microbial community vary along a similar Distance gradient. Organic matter was correlated with the Distance gradient of both the soil microbial community and the plant community. The apparent relationship of organic matter with both ecological components indicate that future reclamation projects should insure inputs of organic matter during the initial phases of reclamation to stimulate microbial activity, thus increasing species performance. No ecologically significant Age or Season effects were identified during this study. Future research could take advantage of the disturbed sites to determine the most economic method of accumulating the soil organic matter characteristic of these semi-arid systems.

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CONCLUSIONS AND MANAGEMENT IMPLICATIONS

The lack of an Age effect in soil properties, enzyme activity, PLFA concentration, and even vegetation data indicates that roads reclaimed at different time periods are not in different stages of succession. Simmers and Galatowitsch (2010) found that plant communities were determined by time since reclamation, but much of the effect was attributed to seeding mixes used during restoration not subsequent establishment of native species on the roads. Establishing a vigorous autotrophic community in degraded sites is thought to be an important step toward soil organic matter accumulation, but the vegetative community of LMNG roads has not induced significant changes to organic matter content in 30 years.

Heterotrophic activity is just as important on primary and secondary succession sites as autotrophic activity since it directly controls nutrient cycling (Hodkinson et al., 2002). Heterotrophic activity requires energy from soil organic matter. Not surprisingly, organic matter was the soil property most associated with the Distance gradient, which explained changes in soil microbial community and vegetative communities. Restoring organic matter to levels observed pre-disturbance would be the first step in creating an environment conducive to restoration. To expect that soil organic matter could attain levels equivalent to the reference sites on LMNG roads with seeding alone would take decades to centuries. Replacing topsoil, and possibly supplementing seedbeds with organic amendments, would provide a seedbed similar to the native prairie for restoration of the LMNG's native plant community.

Establishing the plant community is a vital step in the reclamation process. The *Gold Book* defines the goal of reclamation as creating an ecosystem that will eventually return to the "fully functioning ecosystem" present before disturbance (USDI and USDA, 2007). Reclamation efforts on the LMNG attempted to establish a stable climax plant community prior to

reestablishment of soil properties, which resulted in the condition of the road environment observed in this study. Some native species have colonized the roads i.e., *A. frigida*, but the majority of species have not been able to overcome the barrier imposed by reclamation (Simmers and Galatowitsch, 2010).

Reclamation attempts to establish a climax plant community immediately after major ecological disturbance ignores earlier and required stages of plant community succession. Attempts to skip stages of succession need to overcome energy thresholds, i.e., soil organic matter content, that prevent plant communities from moving forward in succession (Harris, 2009). Seeding a mix of perennial, cool season grasses on roadbeds in the LMNG attempted to by-pass multiple stages of succession without overcoming the energetic and nutritional threshold imposed by a lack of organic matter in the soil. Failure to reestablish organic matter may have caused road reclamations to stall on the LMNG. Relying on the plant community established during reclamation attempts to rebuild organic matter would take decades to centuries, especially since no accumulation of organic matter with time since reclamation was detected in this study.

Experiments designed to identify economically effective methods of reestablishing plant communities on former roads is essential to success of future reclamations. Increased oil exploration and drilling activity in western North Dakota will provide new research opportunities and adequate numbers of study sites as new areas of the LMNG are disturbed. Construction of new roads and drilling pads will provide stocks of topsoil with a microbial population and, possibly, a seed bank indigenous to the region. Developing a "restoration prescription" for the LMNG may provide oil companies a more efficient method of bond release and the US Forest Service restoration that meets the goals defined in the *Gold Book*.

This study is the third to investigate the environment of reclaimed roads in the LMNG. Although this study investigated a limited number of roads, the dataset for the nine roads is robust and included vegetation data, soil physical/chemical properties, and soil microbial community data. Formulating relevant research questions addressing specific soil properties that limit establishment of native plant communities on reclaimed roads is critical to synthesizing the findings of these three studies. A combined analysis of the data collected during the three studies would assist in hypothesis development for field studies on the LMNG. Ideally, the resulting document would identify significant soil, plant, and biochemical properties affecting reclamation success and would subsequently recommend reclamation practices based on the findings of these three studies and others. The reclamation recommendations would be applied to recently disturbed areas in the LMNG and monitored to determine their efficacy and to make changes as needed.

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APPENDIX A. DETAILED DESCRIPTIONS OF FIELD, LABORATORY, AND STATISTICAL METHODS

Site Description

Sites were located in the Little Missouri National Grasslands (LMNG) in Billings County, North Dakota. The LMNG are located in Major Land Resource Area (MLRA) 58C- northern rolling high plains, northeastern part (USDA, 2006). The LMNG environment is a semi-arid mixed grass region of the Northern Great Plains dominated by western wheat grass (*Pascopyrum smithii* (Rydb.) Á. Löve), blue grama (*Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Griffiths), upland sedge (*Carex spp.)*, green needlegrass (*Nassella viridula* (Trin.) Barkworth), and needleandthread (*Hesperostipa comata* (Trin. & Rupr.) Barkworth) (Laurenroth et al., 2008). Average annual rainfall for the region is 355- to 430mm annually, most of which comes in sporadic, heavy storms during early summer. Average temperature of the area ranges from 5 to 7°C. Soils of the region are generally classified as mollisols, inceptisols, and entisols with frigid temperature regimes, ustic to aridic moisture regime, and mixed mineralogy (Champa, 1998).

Since semi-arid environments are characterized by variable soils (Kelly et al., 2008), controlling environmental variability was an important goal of the experimental design. Ecological sites are a classification system utilized by the Natural Resource Conservation Service (NRCS) that incorporate both soil and plant community properties (USDA, 2006). Sampling within ecological sites allowed partial control over the plant community that soil map units (SMU's) could not provide. Samples representative of the LMNG's plant community and soils were desired. Areas classified as thin loamy ecological sites cover approximately 43% of the LMNG in Billings County (Soil Survey Staff). Transects chosen for sampling in this study

were therefore restricted to those originally classified as thin loamy ecological sites. Roads were further blocked by initial reclamation date. Simmers and Galatowistch (2010) documented three different plant communities based on seeding mixes used during initial reclamation periods, i.e., seeding mixes were generally related to the age of the restoration. A subset of the Simmers and Galatowistch (2010) roads and Matthees-Dose (2009) roads containing thin loamy ecological site were identified using GIS and confirmed in the field. Only roads included in during both Simmers and Galatowitsch (2010) and Matthees-Dose (2009) studies were sampled to allow for inclusion of plant and soil data in later analyses. A total of nine transects on nine different roads, including three roads from each time period, were sampled. Each transect had three points located 3-, 10-, and 50m from road center representing on-road environment, transition environment, and native prairie environment, respectively. Three sampling trips were made throughout the summer at one-month intervals in an attempt to capture seasonal changes in microbial communities. The experiment was designed to test the effects of sampling time (season), distance from road center (distance), and time since reclamation (age).

Samples were collected from a 3m radius around previous sampling points. A grid system was designed to standardize the sampling procedure. The total area covered by the grid was $28m^2$. Each square within the grid covered an area of $2300cm^2$. Squares of the grid were numbered 1 through 70. A number between 1 and 10 was randomly selected as a starting point. Samples were taken every seventh square, resulting in 10 cores per point. This method guaranteed uniform sampling of the area around the point. The grid was staked in two places for realignment during subsequent sampling trips. Samples were collected with a soil hand probe 3.5cm in diameter to a depth of 5cm from the same location in every square.

Samples were stored on ice during transport and refrigerated at the lab. Samples were processed by halving each core and mixing the 10 half cores to create one bulk sample for each point. After homogenization, samples were sieved to pass 2mm and stored in the refrigerator. Analysis of bulk samples included enzyme activity and gravimetric water content. A 35g subsample of bulked samples was hand ground, dried, and sent to the NDSU Soil-Testing Lab for nitrogen, potassium, phosphorus, soil organic matter, pH, and EC. The remaining half cores were placed in 50ml centrifuge tubes, frozen at -20°C for up to 8 months, bulked, and lyophilized to be used for PLFA extraction.

Bulking cores for PLFA analysis was done by randomly assigning the 10 cores for each point a number between 1 and 10. These numbers were rearranged in ascending order and grouped in sets of three (1-3, 4-6, and 7-9). The core with the highest randomly assigned value was left out. The three cores where bulked creating three replicates per sampling point. Samples were lyophilized by freezing with liquid nitrogen and placing under vacuum for 48 hours. Cores were homogenized in bags and sieved to pass 2mm. Bulked and lyophilized samples were stored at -20°C until PLFA extraction.

Plant Community

The plant community was recorded during the final sampling August 2010 as described by Ganguli et al. (2008). The system used nine classes (trace-0.5%, 0.5-1.0%, 1-5, 6-10%, 11-25%, 26-50%, 51-75%, 76-95%, 96-100%). Cover class was recorded for all ten sampling locations within the sampling grid. Plants found rooted within a square where counted during determination of cover class. Plants not identified in the field were collected, labeled, and brought back to the lab for identification. Scores from sites were entered into spreadsheet. Class values were replaced with the median percent cover for all 10 squares at each site. The percent

cover for each square was averaged for each site. Total cover was calculated for each site by summing the average cover across all plant species. Relativized data was calculated by dividing each species' percent cover by the total cover for each site. Relativized data were used for detrended correspondence analysis.

Enzyme Assays

Acid and Alkaline Phosphatase

Acid and alkaline phosphatase was evaluated according to the method described by Tabatabai and Bremner (1969). One gram of bulked soil was weighed into a 50ml Erlenmeyer flask and 4ml of modified universal buffer (MUB) at pH 6.5 or 11 (acid and alkaline phosphatase, respectively) was added to maintain optimum pH. Two hundred microliters of toluene was added to all flasks to inhibit microbial activity and 1ml 0.05M *p*-nitrophenyl phosphate (PNPP) was added to sample flasks. Flasks were stoppered and incubated for 1 hour at 37 °C. Another set of samples was prepared in a similar fashion to act as controls but did not receive substrate before incubation.

Flasks were removed from the incubator after 1 hour. One milliliter of 0.5M $CaCl_2$ was added to sample and control flasks to flocculate clays and organic matter. Four milliliters of 0.5M NaOH was added to sample and control flasks to halt enzymatic reactions. Finally, substrate was added to the control flasks to balance absorbance of non-reacted substrate in the sample flask. All samples were filtered quantitatively with Whatman No. 2v filter paper. Filtrate was collected in another 50ml Erlenmeyer flask. Absorbance of sample and control filtrates was measured on a spectrophotometer at 400nm wavelength. If absorbance of a filtrate was greater than the 50µg limit of the standard curve, the solution was diluted 10:1 with de-ionized water.

Absorbance of the new solution was measured. Absorbance of the control was subtracted from each sample's absorbance to account for background absorbance.

Enzyme activity was determined from a standard curve prepared for each set of samples. Standard curves were created by adding 1ml *p*-nitrophenol (PNP) standard solution to 50ml of de-ionized water and diluting to 100ml with de-ionized water in a volumetric flask. The final concentration was 10µg PNP/ml. The diluted solution was added to five Erlenmeyer flasks in 1ml increments creating flasks containing 0-, 1-, 2-, 3-, 4-, and 5ml of standard solution representing 0-, 10-, 20-, 30-, 40-, and 50µg PNP, respectively. De-ionized water was added to each flask to make the final volume of all flasks 5ml. One milliliter of 0.5M calcium chloride and 4ml of 0.5M NaOH were added to the six flasks. Solutions were filtered through Whatman no.2v filter paper and absorbance of the filtrate was measured with a spectrophotometer. Absorbance of each solution was plotted against the known weight of PNP for each flask. A regression line was fit to the plot giving the following equation:

$$A = mx + b$$

Where A is absorbance, x is weight of PNP in µg, m is slope, and b is intercept. The equation was rearranged to isolate concentration. Absorbance from the experiment was entered into the equation to determine mass of product derived from the reaction. Mass from the equation was divided by the mass of soil in the assay to give enzyme activity in units of µg PNP*g^{-1} dry soil. Dry soil weight was calculated by the equation

$$S_D = \frac{S_m}{1 + \theta_g}$$

where S_D is the dry weight, S_m is the field moist weight, and θ_g is the gravimetric water content. Disposable cuvettes were used in the spectrophotometer when measuring absorbance.

Solutions for enzyme assays were prepared as follows. All glassware was acid washed before the assay procedures. Modified universal buffer stock solution is made with 12.1g tris-(hydroxymethyl)aminomethane (THAM), 11.6g maleic acid, 14.0g citric acid, and 6.3g boric acid in 488ml 1M NaOH and diluted to 1l with de-ionized water in a volumetric flask. Making MUB with proper pH required 200ml of MUB stock solution to be titrated with 0.5M HCl or NaOH (acid or alkaline phosphatase, respectively) to the proper pH and diluted to 1l with de-ionized water. *Para*-nitrophenyl phosphate solution was prepared by adding 1.095g *para*-nitrophenyl phosphate to 50ml of MUB of the proper pH and diluting with MUB of the same pH to 100ml in a volumetric flask. Calcium chloride solution was made by adding 5.55g $CaCl_2$ to 50ml of de-ionized water and diluting to 100ml in a volumetric flask. Sodium hydroxide solution was prepared by dissolving 20g pelletized NaOH in 500ml of de-ionized water and diluting the solution to 1l with de-ionized water in a volumetric flask. One molar sodium hydroxide solution was made by adding 40g of pelletized NaOH to 500ml of de-ionized water, dissolving the solids, and diluting the solution to 1l with de-ionized water in a volumetric flask. Half-molar hydrochloric acid was made by adding 41.3ml 12.1M HCl to 500ml de-ionized water and diluting to 1l in volumetric flasks. Standard para-nitrophenyl phosphate solution was made by dissolving 0.5000g para-nitrophenyl phosphate in 500ml de-ionized water.

Beta-glucosidase

Beta-glucosidase activity was measured using the method described by Tabatabai (1982) and Eivazi and Tabatabai (1988). One gram of soil was measured into a 50ml Erlenmeyer flask and 250µl toluene, 4ml of MUB pH 6.0, and 1ml 0.05M of *p*-nitrophenyl-D-β-glucoside (PNG) were added. Flasks were stoppered and incubated at 37°C for 1 hour. Another set of flasks was prepared in a similar fashion to act as controls. Controls did not receive substrate before

incubation. After incubation, 1ml of 0.05M $CaCl_2$ was added to all flasks to flocculate clays and organic matter. Then, 4ml of tris-(hydroxymethyl) aminomethane (THAM) buffer pH 12 was added to all flasks to stop enzymatic reactions. One milliliter of PNG was added to the control flasks after $CaCl_2$ and THAM buffer was added to balance absorbance or non-reacted substrate in the samples. The solution was filtered through Whatman no. 2v folded filter paper. Filtrates were collected in clean 50ml Erlenmeyer flasks. Absorbance of filtrates was measured with spectrophotometer at 400nm.

Standard curves for beta-glucosidase were constructed similar to the method described for acid and alkaline phosphatase, but THAM buffer pH 12 was added instead of NaOH as in the procedure. If absorbance of the solution was greater than the absorbance of 50μg, the sample was diluted 1:10 with THAM buffer pH approximately 10. Solutions for beta-glucosidase were prepared as follows. All glassware was acid washed before the assay procedure. Modified universal buffer pH 6.0 was made by titrating 200ml of MUB stock solution with 0.5M HCl to pH 6 and diluting to 1l with de-ionized water in a volumetric flask. Substrate solution was prepared by dissolving 1.51g *p*-nitrophenyl β-D-glucoside in 50ml MUB pH 6.0 and dilute with MUB pH 6.0 to 100ml in volumetric flask. THAM buffer pH 12 was made by adding 12.1g tris(hydroxymethyl)aminomethane to 500ml de-ionized water and tritrated to pH 12.0 using 0.5M NaOH. De-ionized water was used to dilute the solution to 1l in a volumetric flask. THAM buffer pH approximately 10 was made by adding 12.1g tris(hydroxymethyl)aminomethane to 500ml de-ionized water and diluted to 1l with de-ionized water in a volumetric flask. All other solutions were prepared as described in the *Acid and Alkaline Phosphatase Procedure.*

Phenol Oxidase

Phenol oxidase activity was measured using a method modified from Floch et al (2007). The assay for phenol oxidase was conducted by weighing 0.1g of soil into a 25ml centrifuge tube. Ten milliliters of MUB pH 2.0 was added to the tube along with 200µl 0.1M of 2,2′-azinobis-(-3ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS). The mixture was incubated in a 30°C water bath for 5 minutes. Samples were centrifuged at 12,000rpm at 4°C for 3min to stop the reaction and separate soil from supernatant. Absorbance of the supernatant was analyzed on the spectophotometer at 420nm. Each sample had two controls: soil with no ABTS added, and sterilized soil with ABTS added. Sterilized soil was autoclaved for one hour prior to the assay. One blank without soil was included each time a set of samples was tested.

Phenol oxidase activity was calculated using Beer's Law

$$A = bc\varepsilon_{420}$$

where A is absorption, b is the pathlength, c is the concentration of $ABTS^{+}$ in solution, and ε_{420} is the molar absorptivity of the $ABTS^{+}$ at wavelength 420nm. The published value for ε_{420} is 18,460 M^{-1} cm^{-1} (Floch et al., 2007). Simple manipulation of Beer's Law allowed isolation of concentration

$$c = \frac{A}{b\varepsilon_{420}}$$

Concentration was determined by using absorbance of filtrates, the known path-length and molar absorptivity. Controls of each soil were subtracted from the sample after all calculations. Concentration from the calculation was divided by the weight of the dry soil and by the incubation time resulting in units of µmol $ABTS^{+}$ * g^{-1} dry soil * min^{-1}. Solutions for phenol oxidase solutions were made as follows. 2,2′-azinobis-(3 ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt solution was made by dissolving 0.2743g ABTS in 5ml de-ionized water.

Modified universal buffer stock solution was made using the same method described in *Acid and Alkaline Phosphatase Procedure*. Modified universal buffer pH 2.0 was made by titrating 200ml MUB stock solution with 0.5M HCl and diluting to 1l with de-ionized water in a volumetric flask.

PLFA Analysis

Phospholipid fatty acid (PLFA) extraction was accomplished by the single phase extraction method by Bligh and Dyer (1959) and modified by (Frostegard et al., 2001; Buyer et al., 2002). Lipids were extracted from the soil with a single-phase extraction solution of 1:2:0.8 chloroform:methanol:phosphate buffer. All glassware and metal used during the procedure was heated to 470 °C in a muffle furnace for a minimum of four hours. Plastic utensils were autoclaved or rinsed with acetone, methanol, and chloroform prior to contact with extracts.

Five grams of lyophilized soil were weighed into 30ml culture tubes and 20 ml of extraction buffer was added to the soil. Tubes were sealed with a PTFE lined screw cap and checked for leaks by inverting multiple times. Sealed tubes were spun end-over-end for 2 hours. Tubes were centrifuged at 2200 rpm for 13 min and the supernatant was decanted into a 30 ml culture tube. Five milliliters of chloroform was added to the soil pellet and vortexed to suspend the pellet in chloroform. Tubes were centrifuged again at 2200 rpm for 13 min and the supernatant was decanted into the culture tubes containing the extraction buffer. Five milliliters of 0.05 M phosphate buffer were added to the extraction buffer, tubes were sealed with clean Teflon lined screw caps, and vortex to mix phases completely. The tubes were centrifuged a third time at 2200 rpm for 13 min to separate the organic and aqueous phases. The aqueous phase was aspirated from the organic phase with a clean Pasteur pipette attached to a vacuum pump. The

organic phase was evaporated under a constant stream of Ultra Pure nitrogen (N_2) in a water bath at 38 °C. Tubes were filled with N_2, capped, and stored at -20 °C.

Lipids were purified using 6ml silica solid phase extraction (SPE) columns (product no. 97016-194, VWR) and a vacuum manifold (Agilent Technologies). Silica columns were inserted into the vacuum manifold and washed by draining one volume (about 5 ml) of methanol from the columns, which were dried under vacuum. One volume of chloroform was allowed to drain from the columns, which remained saturated with chloroform. Dried extract was dissolved with approximately 1 ml of chloroform and transferred to SPE columns using a rubber bulb and Pasteur pipette. Sides of each tube were rinsed multiple times to increase transfer efficiency. The transfer process was repeated with another 1 ml of chloroform to ensure complete transfer of lipids to columns. Five milliliters of chloroform was allowed to flow through the SPE columns to remove neutral lipids from soil extracts. Five milliliters of acetone was allowed to flow through the columns and discarded to remove glycolipids. Twelve-milliliter culture tubes were placed beneath the SPE columns to capture phospholipids in the next stage. Five milliliters of methanol containing phospholipids was collected as it flowed from the SPE tubes. Another volume of methanol was collected from the columns and a vacuum was applied to dry the SPE columns. Methanol was evaporated under a constant stream of N_2 at 42°C. Tubes were filled with N_2, sealed with Teflon screw caps, and stored at -20°C.

Lipid methylation was conducted using a mildly alkaline saponification reaction. Dried extracts were suspended in 1ml of 1:1 methanol:toluene solution. One milliliter 0.2M methanolic KOH was added to culture tubes and incubated at 37°C for 15 minutes. Half a milliliter of 1M acetic acid, 2ml of chloroform, and 2ml of sterile de-ionized water were added to tubes containing soil extract, sealed with Teflon lined caps, and vortexed for 15 seconds to thoroughly

mix the phases. Phases were allowed to separate by gravity. The organic (bottom) layer was extracted from the culture tubes using clean Pasteur pipettes and rubber bulbs. Transfer was completed by priming the pipette while moving through the aqueous phase and drawing out the organic phase with the primed bulb. The organic phase was transferred to 8ml culture tubes. Two milliliters of chloroform was added to the aqueous phase. Tubes were capped, vortexed for 15 s, and allowed to separate. The organic phase was drawn from the culture tubes with the pipette using the same "priming" technique and added to the 8 ml tube containing the extract in the organic phase. The organic phase was evaporated under a constant stream of N_2 in a 38 °C water bath.

Samples were purified using amino SPE columns (product no. 97016-142, VWR, Inc.) and the vacuum manifold. Amino columns were placed in the vacuum manifold and a small amount of sodium sulfate was added to the top of the solid column as a desiccant. One milliliter of methanol was allowed to flow through the columns. Columns were dried under vacuum. Five hundred microliters of chloroform was allowed to flow through columns and allowed to saturate the column. Clean, labeled 8 ml culture tubes were placed under columns. Extracts were suspended in 0.5 ml of chloroform and transferred to the columns using a clean Pasteur pipette and bulb for each sample. Culture tubes were rinsed with 0.5ml of chloroform to increase transfer efficiency. The rinse was transferred to SPE columns with the pipettes and drained into clean 8 ml culture tubes. The rinse and transfer steps were repeated to increase yield. Two 1 ml volumes of chloroform were allowed to flow through columns. SPE columns were dried under vacuum. Extracts were allowed to dry under a constant stream of N_2 in a water bath at 38 °C.

Extracts were dissolved in 200 µl of final solvent (1:1 hexane:methyl tert-butyl ether with a 73.3 nmol fatty acid(FA)/ml) and vortexed for 15 s. The final solution was transferred with

Pasteur pipettes to labeled screw cap vials with a 250 µl glass tube insert. Vials were sealed with a screw cap and PTFE septa. Samples were stored at - 20 °C until shipment for analysis. Samples were packed on dry ice and sent overnight.

Samples were analyzed at the University of Wyoming at the Department of Renewable Resources using gas chromatography (GC) with MIDI software and PLFA signature library. Concentration of fatty acids was calculated by the following formula:

$$\mu gFAg_{DW}^{-1} = \frac{PA_i * 73.3nmolml^{-1} * 0.2ml}{PA_S * DW_S}$$

where PA_i is the peak area of the individual fatty acid response. PA_S is the peak area of the standard, 73.3nmol*ml^{-1} is the concentration of the methyl-ester standard, and 0.2ml is the amount of standard solution added to the extracts. DW_S is the dry weight of extracted soil. The final units, nmols FA g_{DW}^{-1} are nanomoles fatty acids per gram of soil dry weight.

Reagents for PLFA extraction were as follows. All solvents used were HPLC grade except chloroform, which was analytical grade. Phosphate buffer pH 7.4 (0.05 M) was made by dissolving 8.7 g K_2HPO_4 in 500 ml de-ionized water and diluting to 1 l in a volumetric flask. The pH of the phosphate buffer was adjusted by adding drops of concentrated phosphoric acid with an eyedropper. Approximately 20 ml of chloroform was added to the solution to deter microbial growth. The solution was transferred to a muffled amber glass container and stored in refrigerator. A 1 : 1 methanol:toluene (vol : vol) solution was prepaired by mixing 50 ml methanol and 50 ml toluene. Contents were placed in an amber bottle and stored in a vented hood. Methanolic KOH (0.2 M) was made by adding 6 ml of 1 M KOH in methanol (catalog number SP220-1, Fisher Scientific, Inc.) to 24 ml of methanol. This solution prepared fresh for each extraction to ensure proper concentration of KOH was maintained. Acetic acid (1 M) was made by adding 14.4 ml concentrated acetic acid to 225.6 ml de-ionized water. Acetic acid was

poured into a muffled amber jar and stored under the fume hood. The PLFA extraction solvent was made before each extraction. Chloroform : methanol : phosphate buffer were mixed in a ratio of 1:2:0.8. Fife hundred fifty milliliters of extraction solvent were made for each extraction. One hundred forty-four milliliters of chloroform were mixed with 289 ml methanol and 115 ml of 0.05 M phosphate buffer (pH 7.4). All liquids were added to an amber pipetting jar which was rinsed with acetone, methanol, and chloroform. Swirling the jar until mixing lines were no longer visible indicated the solution was properly mixed. The auto-pipetter (primed with water) was placed on top of the dispensing jar and pumped until the solution ran clear indicating no water in the system (usually twice). MIDI reagent 3 is made at a 1 : 1 (vol : vol) ratio of hexane and *tert*-butyl ether: 100 ml of each were used resulting in 200 ml MIDI reagent 3.

The final solvent and internal standard was made in a two-step process. First, the stock solution was made by dissolving 100 mg 20:0 ethyl ester powder (product number N-20-E, Nu-chek-prep, Inc.) in 25ml MIDI reagent 3 in a 100 ml volumetric flask. Exactly 100 mg of 20:0 ethyl ester powder was ordered from the company packed in a glass ampule. The neck of the glass ampule was filed and then broken. The contents were transferred directly to the flask and the ampule was rinsed multiple times with MIDI reagent 3 to ensure total transfer to the volumetric flask. The standard was diluted to 100 ml using MIDI reagent 3 in the volumetric flask making a final concentration of 1 mg / ml. Stock solution was stored in a muffled amber jar with PTFE lined lid at – 20 °C. The final solvent was made by adding 2.5 ml of stock solution to 50 ml of MIDI reagent 3 and diluting to 100 ml with MIDI reagent 3. Store final solvent in a muffled amber jar at – 20 °C for up to a year.

Statistics

Two-way ANOVA tests were performed on environmental variables as well as microbial indicators calculated from PLFA data. Microbial indicators were calculated from PLFA signature molecules known to be associated with a specific group of organisms. Signatures included those associated with Gram-positive bacteria (14:0iso, 15:0iso, 15:0anteiso, 16:0iso, 17:0iso, and 17:0anteiso), Gram-negative bacteria (16:1w9c, 17:0cyclo, and 18:1w9c), fungi (18:2w6c), arbuscular mycorrhizal fungi (AMF) (16:1w5c), and protozoa (20:4w6c and 20:3w6c). Phospholipid fatty acid data provided other measures of the microbial community such as Gram positive to Gram negative (Gp:Gn) ratio (14:0iso + 15:0iso + 15:0anteiso + 16:0iso + 17:0iso + 17:0anteiso / 16:1w9c + 17:0cyclo + 18:1w9c), fungal to bacterial (F:B) ratio (14:0iso + 15:0iso + 15:0anteiso + 16:0iso + 17:0iso + 17:0anteiso + 16:1w9c + 17:0cyclo + 18:1w9c / 18:2w6c) and iso to anteiso (I:A) ratio (15:0iso + 15:0anteiso / 17:0iso + 17:0anteiso). Total PLFA biomass was the sum of all PLFA's with less than or equal to 20 carbon chain. Soil properties measured by the North Dakota State University Soil Testing Lab included organic matter (OM), nitrogen (N), phosphorus (P), potassium (K), electrical conductivity (EC), and pH.

The data were analyzed as a mixed model design with the GLIMMIX procedure in SAS software for Windows version 9.2 (SAS Institute, 2008). Effects of age, distance, and season were tested with a split-plot, repeated measures model. Random effects included road nested within age and subject of the repeated measures design was site nested within distance. Repeated measures analysis was used to identify Season effects since samples were taken from the same sites at approximately one-month intervals. Repeated measures accounts for the assumed correlation between the two sampling times at the same site (von Ende, 1993). An unstructured co-variance structure was used during the repeated measures analysis. Age and distance effects

were tested using a traditional two-way ANOVA with a split plot design. All two-way interactions were included in the model. All soil properties and biochemical properties were analyzed with the procedure described above. The SAS procedure is published in Appendix A.

Microbial community structure was analyzed using nonmetric multidimensional scaling (NMS). Nonmetric multidimensional scaling is an ordination technique designed to reduce a complex data set with multiple variables into a simplified data set with 2-3 axes explaining the most possible variation. In the raw data, each variable represents a dimension creating a complex multidimensional space. Humans are not able to identify gradients within the information contained in so many dimensions. Ordination techniques take multiple variables and compute a solution that explains the most amount of variation between sites. This variation is explained by axes, which are linear combinations of data in the first matrix. Individual sampling units are given a score for each axis. The solution is a new data matrix of two or three new variables representing the individual axes as columns and sampling units as the rows of the matrix. Site scores are plotted along computed axes resulting in a graphic representing the sample units in species space. Distance between the site scores in the graphic represents the similarity of the sites. Sites that group close together are more alike than sites that group farther apart.

The percent of variation in the original distance matrix can be calculated after the initial NMS analysis. The new data matrix can be used to calculate a new distance matrix, which explains the amount of variation in the data. The variance of the new data is divided by the variance of the original data. The percent of variability from the original data can be calculated for each axis as well. The sum is the amount of variability accounted for from the original data. Explanation of the graph is aided by correlating environmental variables with the linear

combinations of the species data. An environmental variable that is highly correlated with one of the axes represents how the species are changing along a gradient explained by that variable.

Data analyzed using NMS was all PLFA signature data as the "species data". Species data was analyzed as relative abundance data. Relative abundance was calculated dividing individual signatures by the sum of all PLFA's with 20 carbons or less within each site.

Axes are scaled in units of beta-diversity, which is a helpful consequence of the detrending process. Beta-diversity is the amount of species turnover or changes in community composition across an axis. A DCA axis with a large scale gives a practical interpretation of how much plant communities are changing over a given gradient. Correlation vectors of environmental data with the axes can be created in a similar fashion as NMS.

Species relative abundance data were analyzed using DCA the in PC-ORD (version 5.0). Low ranked species were down weighted during DCA in PC-ORD. Axes were rotated in a similar fashion as they were after the NMS analysis so soil and biochemical properties with the greatest correlation to Axis 1 lined up with the axis. Correlations of axes with the main matrix and the secondary matrix were calculated as well as the percent variance recovered from the original data. The p-values for correlations of main and secondary matrices were calculated with the F-dist function in Excel, which requires an *x* variable. The *x* variable was calculated from the following formula

$$x = \frac{r^2}{((1 - r^2)/(n - 2))}$$

Where r^2 is the square of the correlation coefficient, and *n* is the number of sample units included in the analysis. Significance testing was performed on correlations from both NMS and DCA procedures.

Ordination procedures calculate new "site scores" for each sampling unit on each axis calculated during the procedure. The numerical values of the sample units allow each site to be plotted on the ordination axes. Statistical analyses can be performed on the sit scores from the ordination analsysis. Multi-response permutation procedure (MRPP) is a non-parametric, multivariate hypothesis testing procedure that does not require meeting assumptions of normality and independence as in parametric statistics. The MRPP program re-arranges the data many times to determine the probability of attaining the original data set by chance. Site scores from both NMS and DCA were tested for effects of Age, Distance, and Time using MRPP analysis in PC-ORD (version 5.0). The output of the MRPP program gives the test statistic (T), the chance-corrected within-group agreement (A), and the p-value. The chance-corrected within-group agreement (A) is a measure of the "effect size," which is a measure of the variability within the group. If "A" is approaching zero, then the effect size is small due to high heterogeneity within the group. Interpretation of significant results is a combination of the p-value and A-value. A significant effect at the $P<0.05$ level with a small A-value could be the result of a large sample size, but the ecological significance of the result may be modest.

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APPENDIX B. SAS CODE FOR UNIVARIATE ANALYSIS OF SOIL AND BIOCHEMICAL PROPERTIES

Question 1: What effects do time since reclamation, season, or distance have on the soil microbial community of reclaimed roads?

- Answer 1: Use GLIMMIX to identify significant main effects
    - GLIMMIX is a mixed model approach to general linear models
- Tukey-Kramer HSD for mean separation
    - Tukey-Kramer HSD is a conservative method of identifying significance between levels within a class
- Identify specific mean comparisons with the "slicediff=" command
- The syntax for the analysis was:
    - proc glimmix;
    - classes age distance road time point;
    - model Variable = age distance time age*distance age*time distance*time ;
    - random road(age);
    - random time / subject=point(distance) type=un residual;
    - lsmeans age distance time/ pdiff adjust=tukey alpha=0.05;
    - lsmeans age*distance/slicediff=age pdiff adjust=tukey alpha=0.05;
    - lsmeans age*distance/slicediff=distance pdiff adjust=tukey alpha=0.05;
    - lsmeans distance*time/slicediff=distance pdiff adjust=tukey alpha=0.05;
    - lsmeans age*time/slicediff=time pdiff adjust=tukey alpha=0.05;
    - run;
- The experimental design was a split plot

- "Age" was tested with a different error term than distance and time

- The "random time" statement is the repeated statement in this program
  - We needed a repeated statement since we measured microbial indices at the same sites each time rather than different sites through time
- Values in tables were taken from the Age*Distance "Slicediff by Age" or "Slicediff by Distance" tab of the results list

Question 2: How is the microbial community affected by the environmental variables measured?

- Perform an NMS analysis on relative abundance values of the PLFA in the main matrix and soil and biochemical variables (including enzymes) in the explanatory matrix.
  - NMS Options
    - Autopilot Option
      - Slow and Thorought
      - Euclidean distance to calculate Distance Matrix
        - Select "Distance Measure" tab
        - Select "Euclidean (Pythagorean)"
      - Matrices are in Stats for Manuscript → Final Analysis → PCORD → PLFA NMS → Matrices → main no 365 453 and second no 365 453.
- Graph the Results
  - Select "Graph" on the Toolbar → select Graph Ordination from the dropdown menu → Select 2D
- Rotated Axes to align with environmental variables in second matrix

    - Rotated with environmental vectors projected on the ordination axes, aligned Gram-negative bacteria with Axis 1
    - Axes rotated using “By Continuous Angle” option in the graphing portion of PC-ORD
        - Rotated a total of 90 degrees
- Calculated Axes Correlations with Main Matrix
    - Option is listed under the “statistics” button on the Toolbar of the
    - Calculated “Percent Variance in Distance Matrix”
    - Option available under “Statistics” button on the dropdown menu of the graphing screen
- Performed MRPP to test effects of Age, Distance, and Season on the NMS Scores
    - Copy and Pasted Site scores from Row file from NMS analysis
        - Matrices are in Stats for Manuscript → Final Analysis → PCORD → PLFA MRPP → Matrices → Main and Second
    - Open Matrices in main Screen of PC-ORD as Main and Second, respectively
        - Select the Groups button on the main menu
        - Select MRPP from the dropdown list
        - Select second matrix as grouping data
        - Select desired effect
        - Hit Go
        - Repeat for each effect

Question 3: How are soil and biochemical properties related to the vegetation community data?

- Perform DCA on relative abundance data

- Open Main Matrix and Secondary Matrix
    - Matrices are located: Stats for Manuscript → Final Analysis → PCORD → Plant DCA → Matrices → Main no 365 and Second no 365
- Perform DCA
    - Click on the "Ordination" button on the Toolbar of PC-ORD and Select DCA
    - Select Downweight rare species, Re-scale axes, 0 for rescaling threshold, and 26 for number of segments
    - Click okay

- Graph the Results
    - Select "Graph" on the Toolbar → select Graph Ordination from the dropdown menu → Select 2D
- Rotated Axes to align with environmental variables in second matrix
    - Rotated with environmental vectors projected on the ordination axes, aligned Gramn bacteria with Axis 1
    - Axes rotated using "By Continuous Angle" option in the graphing portion of PC-ORD
    - Rotated a total of – 20 degrees
- Calculated Axes Correlations with Main Matrix
    - Options is listed under the "statistics" button on the dropdown menu of the graphing screen
- Calculated "Percent Variance in Distance Matrix"

- Option available under “Statistics” button on the dropdown menu of the graphing screen

- Performed MRPP to test effects of Age, Distance, and Season on the NMS Scores
  - Copy and Pasted Site scores from Row file from NMS analysis
    - Matrices are in Stats for Manuscript → Final Analysis → PCORD → Plant MRPP → Matrices → Main and Second
  - Open Matrices in main Screen of PC-ORD as Main and Second, respectively
    - Select the Groups button on the main menu
    - Select MRPP from the dropdown list
    - Select second matrix as grouping data
    - Select desired effect
    - Hit Go
    - Repeat for each effect

All matrices used in the SAS and PC-ORD analyses were constructed from a single Excel file

- Located: Stats for Manuscript → Final Analysis → Final Data Set.xlsx
- Tabs are labeled by the analysis they were performed
  - Species signifies Main Matrix Data
  - Environment signifies Secondary Matrix Data
    - Deleting rows after outlier analysis was performed by
      - Selecting “Modify Data” on the Toolbar
      - Selecting “Delete Rows”
      - Selecting “Both Matrices”
      - Selecting “You Specify”

- Selecting to delete the desired Sampling Units and clicking OK