

SPATIAL STRUCTURES OF SOIL MICROBIAL COMMUNITIES AND THEIR
CONTROLLING FACTORS

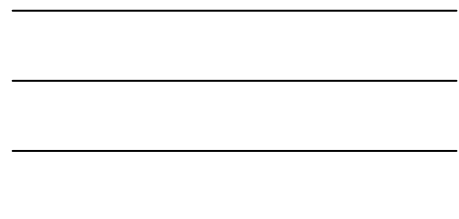
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ABSTRACT

The goal of this research was to quantify the relationship between soil microbial community structures and relating factors, such as plant cover type and soil nutrients. A meadow at the Blandy Experimental Farm was prepared with different degrees of grass-species diversity. Different environmental dimensions were employed for the study: temporal changes (succession), and vertical and horizontal spatial gradient analyses. The succession study was conducted using the entire field with samples collected for 2 years. For the vertical and horizontal dimensional studies, a single 5 m × 5 m experimental field was prepared at the border of an area in the field with a monoculture of *Andropogon gerardii* immediately adjacent to a second area containing three plant species (*Andropogon virginicus*, *Schizachyrium scoparium* and *Bouteloua cultipendula*). Vertical profiles were studied in two 25-cm deep trenches with different plant cover. A combination of multi-scale sampling (0.05 m – 1 m) and the regional sampling was conducted for the horizontal dimension study, and geostatistics were applied for statistical inference.

The most appropriate sample sizes to best represent microbial communities using the various analytical techniques were investigated. A systematic comparison of results from several sample sizes of soil indicated that the abundance and community structures of soil microorganisms obtained varied with the sample size. The best samples sizes for comparing soil microbial community structure with both the analytical technique used and the environmental dimension to which the techniques were applied. In thus study, the optimum sample sizes were 0.1 – 1.0 g for direct count, 0.25 – 10.0 g for bacterial community and 0.1 – 1.0 g for fungal community. Based on these results, it is

recommended that a reconnaissance survey be done to determine the best sized sample to use at the onset of new projects.

The vertical profiles of soil characteristics were highly correlated between the trenches, while the profiles of microbial community structure were not well correlated. Given that the difference between the trenches was largely in the overlying plant community, the clear difference in the community profiles indicated that plant cover was the dominant factor influencing the vertical profiles of soil microbial community structures. Furthermore, the successional changes in the structure of the soil bacterial community were developed in parallel with changes in the plant communities for the first 2 years of secondary succession.

Soil characteristics and total microbial abundance were well spatially structured, but the soil microbial community structures were autocorrelated with less spatial dependence than were the soil characteristics. For all measurements, smaller scales (with higher resolution) possessed higher spatial dependence. The clear separation of both bacterial and fungal communities between different plant settings suggests the dominant influence of plant composition. Among soil characteristics, the best spatial correlation with both bacterial and fungal community structures was found with soil nutrients (total carbon and nitrogen content). Causal modeling indicated that distance (space) had an important role in the differentiation of microbial community structures. Soil characteristics also had a causal relationship with bacterial communities, but not for fungal communities. Thus, plant identity is the dominant controlling factor on bacterial and fungal community structure in the soil of the experimental field for all aspects examined.

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1. INTRODUCTION

Everything in the universe exists as they interact with their surroundings.

Ecology deals with living organisms and their interactions with other living organisms and their environments. What Microbial Ecology studies is microbial activities with other same and different types of microorganisms, macro-organisms and their environments. The question the researcher had for the dissertation project was also about the relationships in more comprehensive and quantitative sense. Some known facts about the relating components and the contents of their relationships are first discussed (1.1). Then the major challenges in research of Microbial Ecology are mentioned afterward (1.2).

1.1. SOIL MICROBIAL COMMUNITY ECOLOGY

1.1.1. Soil as an active habitat for microorganisms

Soil accommodates one of the most complex and dynamic microbial assemblies in the entire biosphere (Curtis *et al.*, 2002). Different sizes of soil particles and aggregates in infinite combination result in a highly diverse physical environment with heterogeneity readily displayed at very fine scale (Heijnen *et al.*, 1993; Grundmann, and Debouzie, 2000; Ranjard, and Richaume, 2001). For example, anaerobic denitrification can occur under apparently aerobic conditions if there are anaerobic microsites made available by compartmentalization of soil particles (Hutchinson, and Mosier, 1979). Another good example is the existence of hot spot for denitrification activity (Hojberg *et al.*, 1994) and methanogenesis (Schuetz *et al.*, 2003) at various scales from μm to cm. The diversity of physical characteristics of soil can harbor a large diversity of microorganisms in close proximity due to the minute size microorganisms. Furthermore, the relationships between microorganisms and soil particles are fully interactive: while

soil particles control the survival and biological activity of microorganisms (Chenu, and Stotzky, 2002), soil microorganisms influence soil structure by modifying the soil particles' arrangement or providing cohesive materials to help form and stabilize aggregates (Kiem, and Kandeler, 1997; Bossuyt *et al.*, 2001).

The chemical composition of soil is also highly heterogeneous in both vertical and horizontal dimensions (Yang *et al.*, 1995; Dighton *et al.*, 1997; Gallardo *et al.*, 2000; Bird *et al.*, 2002). An exception to the "rule" of heterogeneity might be observed for some trace elements which may be heterogeneous in distribution but are adequate for microbial need in most situations (Tate, 2000), such that their distribution has little effect on the distribution of microbial community structure. Leaching of soluble nutrients and variations in cation exchange capacity across different soil textures are two important expressions of chemical heterogeneity (Brady, and Weil, 1999). Those sundry properties of soil may partially, if not entirely, explain the abundance and diversity of soil microorganisms. There are simply a very large number of niches contained within tiny spaces.

1.1.2. Relationship between plant and soil microorganisms

Plants exhibit a wide variety of interactions with microorganisms in both physical and chemical aspects (Rovira, 1965; Kaye, and Hart, 1997; Shabana *et al.*, 1997; Burke *et al.*, 2002; Kiers *et al.*, 2003; Ryu *et al.*, 2003; Deniel *et al.*, 2004). Perhaps the most well documented interaction between plants and soil microorganisms is the symbiosis between leguminous plants and nitrogen fixing-bacteria such as *Rhizobium*. Nitrogen from the huge inert atmospheric pool is fixed by bacteria residing inside of the plant roots. The ammonia produced by the bacteria is readily used by the plants, and the bacteria are

concomitantly provided with optimal conditions for nitrogen fixation. High concentrations of organic matters and nutrients are supplied by the metabolism of the plant, and anaerobic conditions are maintained with oxygen provided for bacterial cell respiration by the high oxygen affinity of the O₂ transport compound, leg-hemoglobin, found in the root nodules of leguminous plants. For those nodule-inducing, nitrogen-fixing bacteria, fixation cannot occur without the host legume.

Another example of a close plant-microorganism relationship is that of mycorrhizae, which literally means “root fungus.” The majority of terrestrial plants develop mycorrhizal relationships with specific fungi that often enhance the uptake of mineral nutrients. These associations probably represent many typical relationships between plants and soil microorganisms, i.e., beneficial proto-cooperations in which each member provides different types of nutrients for the other. At the global scale, microorganisms have the main responsibility for biogeochemical cycling of the most important nutrient elements. A main role of the dominant heterotrophic microorganisms in soil is the decomposition of organic matter coupled with the release of bound nutrients which are then available for all the organisms including the plants. A study with *Arabidopsis* showed that plant growth-promoting rhizobacteria (PGPR) released volatile organic compounds (VOCs, in this study, 2,3-butanediol) into the air and that release promoted the growth of the plants (Ryu *et al.*, 2003).

Conversely, heterotrophic soil microorganisms often rely on organics released from the plant roots as exudates (Barber, and Martin, 1976; Yoshitomi, and Shann, 2001; Marschner *et al.*, 2002) and root debris (Miya, and Firestone, 2001). This is clearly indicated by the drastic decrease of microbial abundance as one moves away from the

root surface, that is, out of the rhizosphere (Fenwick, 1973; Sposito, and Reginato, 1992; Buyer *et al.*, 2002), and that decrease in abundance is accompanied by distinct differences in microbial community structure between the rhizosphere and bulk soil (Steer, and Harris, 2000; Smalla *et al.*, 2001; Kowalchuk *et al.*, 2002; Gomes *et al.*, 2003).

There are also plant-microorganism interactions between the two domains that are not mutually beneficial. There are many competitive relations for certain types of nutrient elements, particularly for nitrogen (Newman, 1985; Zak *et al.*, 1990; Owen, and Jones, 2001). Soil microorganisms may control the growth of the plants by limiting the available inorganic nitrogen in the rhizosphere from immobilization during organic matter decomposition (Kaye, and Hart, 1997). Despite some information that suggests otherwise in limited situations (Schimel, and Chapin, 1996; Kaye, and Hart, 1997), soil microorganisms outcompete plants in uptake of free amino acids, which represent readily available dissolved organic nitrogen (DON) constituents of soil (Owen, and Jones, 2001). A more obvious example of a deleterious interaction is phytopathogenesis: various types of microorganisms, particularly fungi, attack plants in a variety of ways in order to utilize the living plant materials for their growth and reproduction (Shabana *et al.*, 1997; Shabana, and Ragab, 1997; Atlas, and Bartha, 1998). The result, of course, is that the plants are harmed, often with death as the final outcome, whether or not the consequence was what microorganisms originally intended for or not.

1.1.3. Relationship between soil microorganisms and soil

When survival and growth of microorganisms are considered, one immediately thinks of two fundamental and significant laws: Liebig's law of the minimum and Shelford's law of tolerance (Atlas, and Bartha, 1998). Liebig's law states that a nutrient

with minimum concentration among all required nutrients determines the survival and growth of organisms, while Shelford's law states that the range of physicochemical conditions to which organisms are exposed results in survival or death, according to their tolerance to the conditions. In reality, both laws simultaneously work on determination of survival and growth of particular organisms (Odum, 1971). Here are brief discussions about the importance of the soil factors examined in the current project with consideration of two laws.

1.1.3.1. Particles and aggregates

Soil microorganisms found in soil pores can account for significant portion of total soil volume (Ranjard, and Richaume, 2001). Soil microorganisms live in the pores existing between soil particles and aggregates, the latter of which are somewhat random combinations of soil particles and binding agents. Size, amount, and interconnectedness of pores are very important factors determining survival and growth of soil microorganisms. Soil pores act as channels for aqueous and gaseous phases that contain compounds that can be either beneficial (nutrients) or harmful (toxic chemical) to soil microorganisms.

Among soil particles, clay (< 0.002 mm) is the most significant in terms of the effect on soil microorganisms (Alexander, 1977; England *et al.*, 1993). Due to their minute size, the surface area of clay particles is substantially more than those of silt and sand in a unit volume or equivalent mass. Differences in surface area between clay particles and sand can be from 20 fold to many orders of magnitude (Brady, and Weil, 1999; Tate, 2000; Chenu, and Stotzky, 2002). One might expect a positive correlation between the proportion of clay particles in the soil and microbial abundance because of

the high reactivity associated with the particle surfaces. Ionic association of clay particles and other particulate components including microorganisms and nutrients is a key feature in assessing the importance of surface area. Negatively charged surfaces of clay particles attract many positively charged biologically produced compounds (Prosser, 1997). Strong sorption of those compounds onto clay particles generally make them unavailable for most soil organisms (Dashman, and Stotzky, 1982; Dashman, and Stotzky, 1984; Knezovich *et al.*, 1987; Bosma *et al.*, 1997; Brooks *et al.*, 1997). Another point to note about clay particles is that different types of clay particles possesses very different properties, including size, surface area and cation exchange capacity (Atlas, and Bartha, 1998), and there are normally several different types of clay available in most soil systems.

Soil provides highly heterogeneous habitats for microorganisms because of not only different types and sizes of particles but also different arrangements of primary particles into aggregates. Soil aggregates comprise soil particles and the chemical or physical components that bind them together. Based on size, aggregates are classified as macroaggregates ($> 250 \mu\text{m}$ mean diameter) or microaggregates ($< 250 \mu\text{m}$ mean diameter) (Tisdall, and Oades, 1982). Microaggregates are relatively stable because they comprise clay particles and a biodegradation-resistant binding agent, microbial external polysaccharide (EPS) (Chenu, and Stotzky, 2002). Macroaggregates are combinations of microaggregates and larger particles that are bound together mainly by the entanglement of fungal hyphae or fine plant roots. Macroaggregates tend to be less stable in structure, and they are easily disturbed by change in the soil environment due, for example, to soil management practices such as tilling (Tate, 2000). Soil pores, the spaces between

particals and aggregates, are classified according to IUPAC (International Union of Pure and Applied Chemistry): micropores (< 2 nm), mesopores (2 – 50 nm) and macropores (50 – 2000 nm) (Guggenberger, and Haider, 2002). However, in soil science, the same terms are used for much larger sizes. The Soil Science Society of America used the same classification to describe pores that are 5 – 30 μm , 30 – 80 μm and 80 – 5000 μm , respectively (Brady, and Weil, 1999). Conventionally, 10 μm is the criterion to distinguish between macropores and micropores (Jocteur Monrozier *et al.*, 1991; Ranjard, and Richaume, 2001). It is known that bacteria and fungi preferentially reside between micropores and macropores (Foster, 1988). This is partially because of microorganisms' size difference, and accessibility of nutrients, toxic materials, and grazers are also very important. Water retention in micropores is high because of high capillary pressure heads such that the environment in micropores is more favorable for microorganisms under dry conditions (van Gestel *et al.*, 1996). The high capillary pressure heads also make it difficult to circulate water out of micropores, and that can be either beneficial by protecting from contact with dissolved toxic chemicals or disadvantageous by hampering the diffusion of dissolved nutrient and limiting the movement of microorganisms dependent upon the locations of microorganisms (Hattori, and Hattori, 1976; Hassink *et al.*, 1993). Micropores are also known to contain the majority of endogenous organic material in soil (Christensen, 1992). Therefore, micropores are thought to contain primarily bacteria as their size often excludes fungi.

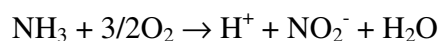
1.1.3.2. Water and pH

Water is essential for life; the major portion of the body weight of any organism is water, and all of the chemical reactions associated with living organisms occur in solution.

The important roles of soil water for microorganisms can be listed: 1) essential material for chemical and enzymatic reactions, 2) transport medium, 3) buffer for soil temperature and 4) the physical growth medium for microbial colonies (Tate, 2000). Bacteria metabolize and reproduce only when immersed in water. Among several forces that affect the potential energy of soil water, matric force (capillary pressure) is the most relevant for microorganisms. Matric force is responsible for adsorption and tension of water in association with particle surfaces, and is the fundamental force that maintains the water film coating soil particles (Brady, and Weil, 1999). Typically, microbes can tolerate a fairly large range of moisture regime; measured microbial respiration rates were fairly high even at water potentials -30 bar in a study by Wilson and Griffin (1975). In their study, microbial respiration of natural soil became unnoticeable at about -50 bar, while soil with single microbial population decreased so rapidly that became negligible at about -5 bar. This phenomenon of high tolerance is due to the high diversity of the microbial community, and hyperdiversity can extend the community's tolerance range for different environmental gradients as well. For example, diverse microorganisms, including facultative anaerobes, have important roles under anaerobic or anoxic conditions induced by saturation of pores during soil flooding.

Microorganisms have a very wide tolerance range for pH. There are microbes that grow at pH 1 and others that grow at pH 11. (Tate, 2000). The optimal pH varies among different reactions and taxa. For example, the sulfur oxidizing autotroph *Thiobacillus thiooxidans* grows optimally at the range of pH 2 to 4, whereas *T. thioparus* grows at about pH 7 (Brock, and Madigan, 1991). However, in general, fungi are dominant in acidic condition because of low tolerance of heterotrophic bacteria to the toxicity of

organic acids at lower pH (Tate, 2000). The mechanisms controlling soil pH over long periods of time are mainly biological, and are mediated by plants and microorganisms (Huang, and Germida, 2002). Release and re-adsorption of H^+ and HCO_3^- , release of CO_2 by root respiration and release of organic acids as root exudates are plant processes affecting pH. Reactions that generate or consume (primarily) cations, which mediated by both plants and microorganisms also have important roles in pH change. Additionally, processes unique to prokaryotes play a major role in altering the soil pH, e.g., nitrification decreases pH through the release of hydrogen ions.



1.1.3.3. Carbon and nitrogen

Carbon and nitrogen are the third and the fourth most abundant atoms found in biological systems, although their existence in the general environment is less significant (Nester *et al.*, 1998). Carbon has a major role in biological system in that organic compounds, which, by definition, contain reduced carbon, are building blocks of various macromolecules. Carbon accounts for about 25 % of all atoms in cells. The oxidation of reduced carbon provides an energy source for heterotrophic microorganisms, which are the dominant components of soil microbial communities. A few organic compounds also serve as terminal electron acceptors for some anaerobic microorganisms. Compared to carbon, the amount of nitrogen in cells is much less significant (about 0.5 % of the dry weight); however, the importance of nitrogen cannot be ignored, since nitrogen is a critical component for functional (protein) and genetic (nucleic acid) macromolecules.

Organic material containing carbon and nitrogen does not just serve as a growth substrate for microorganisms, but it also may modify microbially relevant environmental

conditions in the soil. For example, water retention capacity of organic material is the highest among all soil constituents; mineral soils with high concentrations of organic material (e.g., 10%) provide favorable environments for microorganisms in terms of both energy/mass substrates and optimal moisture content.

The list of four most important macronutrients for microorganisms includes carbon, nitrogen, phosphorus, and sulfur. Carbon and nitrogen in soil are derived mainly from plants – biomass and exudates, but phosphorus and sulfur are available mainly from mineral constituents of soil that phosphorus and sulfur are not usually limiting for microbial growth in soil. There is fierce competition for nitrogen between plants and soil microorganisms, and microorganisms generally outcompete plants such that plant production in most terrestrial systems is nitrogen limited (Kaye, and Hart, 1997; Owen, and Jones, 2001). Because organic carbon availability generally limits microbial production, it is often the main controlling factor in soil microbial community development (Schnurer *et al.*, 1985; van Gestel *et al.*, 1996).

Soil carbon and nitrogen are often expressed as total carbon and total nitrogen (the case for the current project), but not all the compounds are available to the soil microorganisms. Many studies show that quality of substrates is equally, if not more, important than quantity in promoting changes in the soil microbial community (Baath *et al.*, 1995; Bending *et al.*, 2002; Pennanen *et al.*, 2004). Fresh plant biomass is more readily available than materials high in refractory lignin or humic acids. Within the microbial community are diverse populations that metabolize a wide range of nitrogen compounds, including those with different oxidation state, molecular weight, solubility and structure. The large, inert pool of N₂ is fixed to ammonium by nitrogen fixing

bacteria (*Rhizobium*, *Azotobacter*, etc.), and ammonium can either be assimilated by many organisms or oxidized by ammonium oxidizing bacteria (*Nitrosomonas*) and nitrite oxidizing bacteria (*Nitrobacter*).

1.2. APPROACHES TO STUDYING MICROBIAL COMMUNITIES

1.2.1. Appropriate sampling strategy

In environmental research, sampling is usually required to investigate environmental phenomena. The main purpose of sampling is appropriate representation of the environment in a body of a manageable size. Unlike other widely discussed issues of proper sampling strategies for better representation of the environment under study (e.g., replication and randomness of samples), inherent limitations of the process of sampling itself are rarely discussed. Sampling is required when the study of the environment in its entirety is not possible. However, when samples are collected from the environment, the samples themselves constitute an isolated small system which may have a separate character from the rest of the environment from which the sample was extracted. This problem is even greater in microbial ecology in which microorganisms are very physiologically flexible and are quickly responsive to changes in the environment in which they reside. It is not too hard to imagine that neighboring microbial communities with similar pathways in the native soil might diverge substantially due to the different conditions in the sampling containers compared with the original environment. Contamination of foreign microorganisms is another issue in proper representation of the environment, although it is less serious in soil system due to high density and diversity of microorganisms. Sample-storage procedure is a very critical issue

in preservation of samples as they were at the moment of collecting. Freezing or refrigeration to minimize metabolic and reproductive activities of microorganisms is common. By doing this, researchers can obtain snapshots of microbial communities at the moment of sample collection. A view equivalent to a motion picture would be very difficult to obtain; however, series of well preserved samples in temporal fashion could work as kind of the magic lantern or Zoetrope.

Both size and number of samples are important issues in sample collection. Parsimony applied to the issue of sample size would suggest that smaller numbers of smaller sized samples would benefit the procedures of collection, storage, and analysis in terms of both effort and cost. An attempt to determine optimal sample size for measurement of soil bulk density was weighted more toward effort and cost to provide a conclusion (Terry *et al.*, 1981). However, results of measurement should be considered seriously that there should be threshold of minimum amount of sample to represent the environment with acceptable reproducibility. That is, effort and cost minimization is wasteful if the samples do not adequately represent the environment from which they were extracted. Existence of patches, virtually guaranteed in the distribution of soil microbial communities, invokes the rule that sample sizes should be selected so that among replicate variance from variable sample size is minimized (Green, 1979). Green (1979) also argued that the intuitive assumption of the benefit of larger sample size is not necessarily correct. The idea of larger sample size is preferred by many environmental biologists is simply because larger sample size allows obtaining more individuals per sample. He disproved this assumption by simple simulation to show that sample size makes no difference in randomness, and that smaller sample sizes could actually increase

the precision of the estimation. In consideration of 'fundamental error', which is the inherent minimal amount of variation among samples of a mixture of particles, the argument of larger sample size indeed was legitimate (Lame, and Defize, 1993). However, the 'fundamental error' argument is more significant for the analysis on soil itself than microbial portion of soil. Sample size had a significant effect on the measurement of soil microbial biomass (Shristie, and Beattie, 1987). More recent studies on genetic fingerprinting techniques for microbial communities also indicated that there were clear beneficial effects of increasing sample size on the results in terms of better representation (Ellingsoe, and Johnsen, 2002; Ranjard *et al.*, 2003). Therefore, determination of appropriate sample size should be one of the first steps in any quantitative microbial ecological study to make the results more reliable and increase investigator confidence in when comparing to results from other studies.

Unlike the sample size issue, the effect of sample number is quite clear: the more the better. This, of course, follows directly from the Central Limit Theorem which states that means of n samples from any type of distribution approach a normal distribution as n increases. A larger number of samples; therefore, is desirable whenever possible to allow the use of parametric statistics for analyses. A simulation on randomly sampled datasets with the probability of their means located within 95% confidence interval found that 40 samples were required if the samples were drawn from a highly skewed distribution (Barrett, and Goldsmith, 1976). The overall sample number inflates as the number of replicates increases; however, a large number of replicates does not always guarantee more powerful statistical analysis because of the possibility of pseudoreplication from an improper experimental design (Hurlbert, 1984).

1.2.2. Using DNA methods as tools for microbial community structure studies

One of the most difficult obstacles to overcome in studies of microbial ecology is the methodological limitation that arises from the minute size of microorganisms. Unlike studies of macro-organisms, there are not readily available methods to identify and survey directly individuals, populations, and communities of microorganisms. As morphological differences have been applied in macro-biology, morphology (of colonies on agar media and of cells under high magnification) was a primary method of identification of microorganisms in the earlier era of microbiology. Recently, however, the use of genetic material has become a common tool for identifying and characterizing microbial populations and communities. Small-subunit ribosomal RNA (ssu rRNA) of bacteria is highly conserved throughout the evolutionary process and common to all bacteria, so that change in the sequence of the 16S rRNA gene is considered to represent evolutionary change. That assumption then makes possible measurement of phylogenetic differences among bacteria (Woese, and Fox, 1977). The accumulating database of 16S rRNA gene sequences (the Ribosomal Data Base Project, <http://rdp.cme.msu.edu/html/>) is making possible association of determined sequences with known and named bacteria, as well as with organisms that are as yet uncultured, uncharacterized, and unnamed. Because differences in ssu rDNA imply evolutionary distinction, it is possible to analyze microbial communities using ssu rDNA to characterize the communities based on the taxonomy of the microbial populations therein. Some reported the existence of multiple gene copies of ribosomal genes (Fogel *et al.*, 1999; Ueda *et al.*, 1999) and that could lead to misread of the results, because multiple bands could represent either multiple organisms and a single organisms with multiple copies of ssu rDNA. Thus, there have

been attempts to utilize other marker genes than ssu rDNA as well (Morse *et al.*, 1996; Ludwig, and Schleifer, 1999; Dahllof *et al.*, 2000; Peixoto *et al.*, 2002). Dahllof *et al.* (2000) used the gene for the β unit of the RNA polymerase, *rpoB*, in place of 16S rDNA that *rpoB* gene is both common to all bacteria and has conserved as well. They compared band patterns of PCR-DGGE between 16S rDNA and *rpoB* using nine isolates of *Delisea pulchra* separately and in a mixture. Five out of nine isolates showed more than one band from 16S rDNA analysis and only one isolate had two bands from *rpoB* analysis. The *D. pulchra* community of nine isolates was better represented by *rpoB* PCR-DGGE that four isolates were identified while only two isolates were revealed from 16S rDNA analysis.

In practice, nearly all methods for community analysis are based on amplification with the polymerase chain reaction (PCR). There are some well-known PCR related biases (Wintzingerode *et al.*, 1997; Head *et al.*, 1998; Becker *et al.*, 2000; Bruggermann *et al.*, 2000). PCR amplification could happen between templates of similar sequences but from different origins and generate heteroduplex molecules in complex template mixtures like ones from soil microbial communities (Jensen, and Straus, 1993; Judo *et al.*, 1998; Speksnijder *et al.*, 2001). Mixtures of templates might have various efficiencies in priming and DNA polymerase activity, especially when universal primers are used (Polz, and Cavanaugh, 1998; Kanagawa, 2003). These two biases are most significant in PCR-based soil microbial community analysis that the results are not necessary reflect the mixture of templates. Despite the limitations of the method, it has concluded that PCR-DGGE is reliable method providing acceptable view of differences and similarities in the dominating populations of microbial communities (Lindstrom, 2001), thus these PCR-based community analysis approaches have been widely used in microbial community

(Marsh *et al.*, 1998; Buckley, and Schmidt, 2001; Clegg *et al.*, 2003) and microbial diversity (Ovreas, and Torsvik, 1998; Smit *et al.*, 2001; Yi *et al.*, 2003) analyses.

1.2.3. Geostatistics for spatial structure of microbial communities

Studies that employ well-designed environmental sampling can provide understanding of variation on either temporal or spatial scales, or both. By analyzing the collected data, the relationships among variables can be revealed. However, there is chance that the relationship might be generated from some common temporal or spatial structures rather than their true interactions (Legendre, and Troussellier, 1988), i.e., the apparent relationships among variables may be due to factors other than the variables being examined. Spatial autocorrelation, which is inherited property in terrestrial ecosystem for most variables, might hamper to use tests of statistical significance due to the spatial dependence of variables measured in proximal samples, because conventional inferential statistics assume independence of samples. Standard statistical methods such as correlation analysis, therefore, might not capture the true relationships among variables in the soil ecosystem. The geostatistical approach, which takes into account spatial autocorrelation, is the chosen analytical tool for the questions that will be presented for the current project (Webster, and Oliver, 2001). Another reason geostatistical analyses are desirable for the environmental samples is that traditional statistics lose the information which spatially distributed samples would represent. For example, mean total microbial abundance (especially with sizable amount of standard deviation) of a field would not be very useful to provide total microbial abundance in certain region of the field.

1.3. RESEARCH MOTIVATION

Once thought to be pure geochemical processes are now becoming more and more understood as microbiologically mediated processes, so it is critical to understand the structures and functions of microorganisms as a community along with interactions with other constituents of the ecosystems. It is obvious that they are interacting in various ways, and the researches on the mechanisms and the tendency of interactions have great potential in several ways: field status indication, field restoration designing and so on. When soil microbial communities are considered, most obvious interactions are those with plants and soil environment.

Kowalchuk *et al.* (2002) examined how plant species composition and diversity influence soil microbial community composition. Their experimental setting resembles that of the current project in that several plant species were sown in different combination while allowing invasive weeds. They showed convincing plant effect on soil microbial composition is only found within rhizosphere. Even if the experimental portion of the study was very nicely done, their results were mainly qualitatively obtained from the band patterns. Smalla *et al.* (2001) analyzed bacterial community associated with three plant species: *Fragaria ananassa*, *Brassica napus* and *Solanum tuberosum*. The authors tried to determine the degree of the rhizosphere effect in plant dependency and whether this effect would be increased by growing the same crops in two consecutive years. Although the DGGE fingerprints showed plant-dependent shifts in the relative abundance of bacterial populations in the rhizosphere which became more pronounced in the second year, quantitative statistical analysis was lacking again. Girvan *et al.* (2003) analyzed soil bacterial communities using two 16S rDNA based genetic fingerprinting techniques

(DGGE and T-RFLP) and BIOLOG[®] in agricultural fields. Microbial communities represented by BIOLOG[®] and DGGE were clustered by soil types on PC fields. They determined the controlling factor using multivariate statistics, but their field was not appropriate to measure any plant effects. These three studies are well representing examples with questions on the relationships that most of them either were lack of appropriate quantitative measurements or did not have experimental setting to answer comprehensively considering both plants and soil.

Things being closer together in both spatial and temporal distance are likely to be similar to each other than things are farther apart (Cliff, and Ord, 1981). Spatially distributed samples, which are the format of sampling design in many ecological studies, are spatially positively autocorrelated. Autocorrelation causes problem in statistical inference that it violates conventional statistics' fundamental assumption of the independence of observations. Conventionally, one sample accounts for one degree of freedom, but autocorrelation makes it impossible to count degree of freedom from the number of samples. Positive autocorrelation decreases the degree of freedom, so analysis of autocorrelated samples with conventional statistics inflates the degree of freedom (Liebhold, and Sharov, 1998). Autocorrelation decreases the effective sample size, and it would decrease the statistical power $(1-\beta)$, unless the effective sample size is over the critical point which stabilizes statistical power regardless of sample size. This has confirmed by Monte Carlo simulation (Pyper, and Peterman, 1998). Therefore, statistical inference on spatially autocorrelated samples using conventional statistics is simply not acceptable. Geostatistics is required for proper statistical inference out of the autocorrelated samples in both temporal and spatial frames that it can determine

minimum distance (range) for independence of samples. Along with sample distance, sample size is another important but rarely asked question that how much samples is nice balance between better representation of the samples and resource effectiveness. It is also important issue in consideration of Brock's suggestion (1987) that most relevant results are obtained from the microbial ecology researches conducted in a scale and extend of microorganisms (Paerl *et al.*, 2003).

Grayston *et al.* (2001) examined soil samples from three fields with different soil types and vegetation cover to determine the factors regulate soil microbial community organization. They showed that upland grassland soil microbial communities were influenced by both vegetation types and soil types based on PLFA and BIOLOG[®]. However, they did not concern the distance among their samples that they were located far enough to each other to be independent. Two recent studies by Franklin and Mills (2003) and Ritz *et al.* (2004) were published while the current project was still conducted, and shared the basic idea of spatial autocorrelation into actual research of microbial communities. Franklin and Mills had very sophisticated systematic sampling design to reveal spatial structure of bacterial community structures and soil characteristics in multiple scales (Franklin, 2004). Although, their usage of geostatistics was bit unconventional in terms of semivariogram analysis, for example, by exaggerating graph portion for better visual fitting, and vegetation effect was not answered because the experimental field was active wheat field, their experimental approach was very creative and cleaver. The study by Ritz *et al.* was very extensive dealing with field ecology, molecular analysis, lipid analysis, physiological analysis, soil analysis and geostatistics. Their setting was limited to show vegetation effect in general, because they tried to have

sampling field as uniform as possible in vegetation and other apparent physical variables, so their vegetation effect was measured in three species they found in sample cores. Their results were basically confirming that ecological samples were spatially dependent, and the degree of dependence was varying with the measurements.

1.4.

1.5. HYPOTHESES

Hypothesis 1.

- The results of the abundance and microbial community structure are dependent on the size of the collected samples.

Hypothesis 2.

- Depth of soil influences on microbial community structure.

Hypothesis 3.

- The change of the plant community influence on the change of the soil bacterial community structure.

Hypothesis 4.

- Soil characteristics and the soil microbial community (DNA fingerprints and composition of dominant organisms) are spatially correlated.

Hypothesis 5.

- The distribution of microbial community structure is dependent on the chemical and physical properties of the soil and plant community composition from which the microorganisms are collected.

2. MATERIALS and METHODS

2.1. DESCRIPTION OF STUDY SITE

The study was conducted on a field of approximately 14 ha at Blandy Experimental Farm (BEF) in the northern Shenandoah Valley of Clarke County, Virginia (78°06 W, 39°06 N, Figure 2.1) where a meadow comprising native temperate tallgrass species, as a part of the collection of the Orland E. White Arboretum, was being established. The average temperature range (mean 24 hour temperature) is -6 °C in January and 30 °C in July, and the average annual precipitation is 972.1 mm. The average slope of the designated research field is approximately 3.7% from north to south, with an elevation difference of 8 m over the 210 m distance. Soils at the crest of the hill tend to be moderately droughty while the soils at the base are often wet; a swampy area extends into the lower part of the field. Given these conditions, the elevation gradient was considered to represent an analog of a soil moisture gradient with the soils at the top termed xeric, those in the middle, mesic, and those at the base, hydric.

Soils at the site are dominated by the Poplimento silt loam series of Fine, mixed, mesic Ultic Hapludults, with an area composed of the Timberville silt loam (Clayey, mixed, mesic Typic Hapludults) in the southeast corner (in areas H1 and H4, Figure 2.2) (NRCS, 1982). Both of these soils are well drained, but the Poplimento is generally found on side slopes and hillcrests, whereas the Timberville is more characteristic of narrow to moderately broad drainage ways. Neither of these series is considered a hydric soil; the descriptors used here are only to separate the perceived relative soil moisture content of the three portions of the research field.

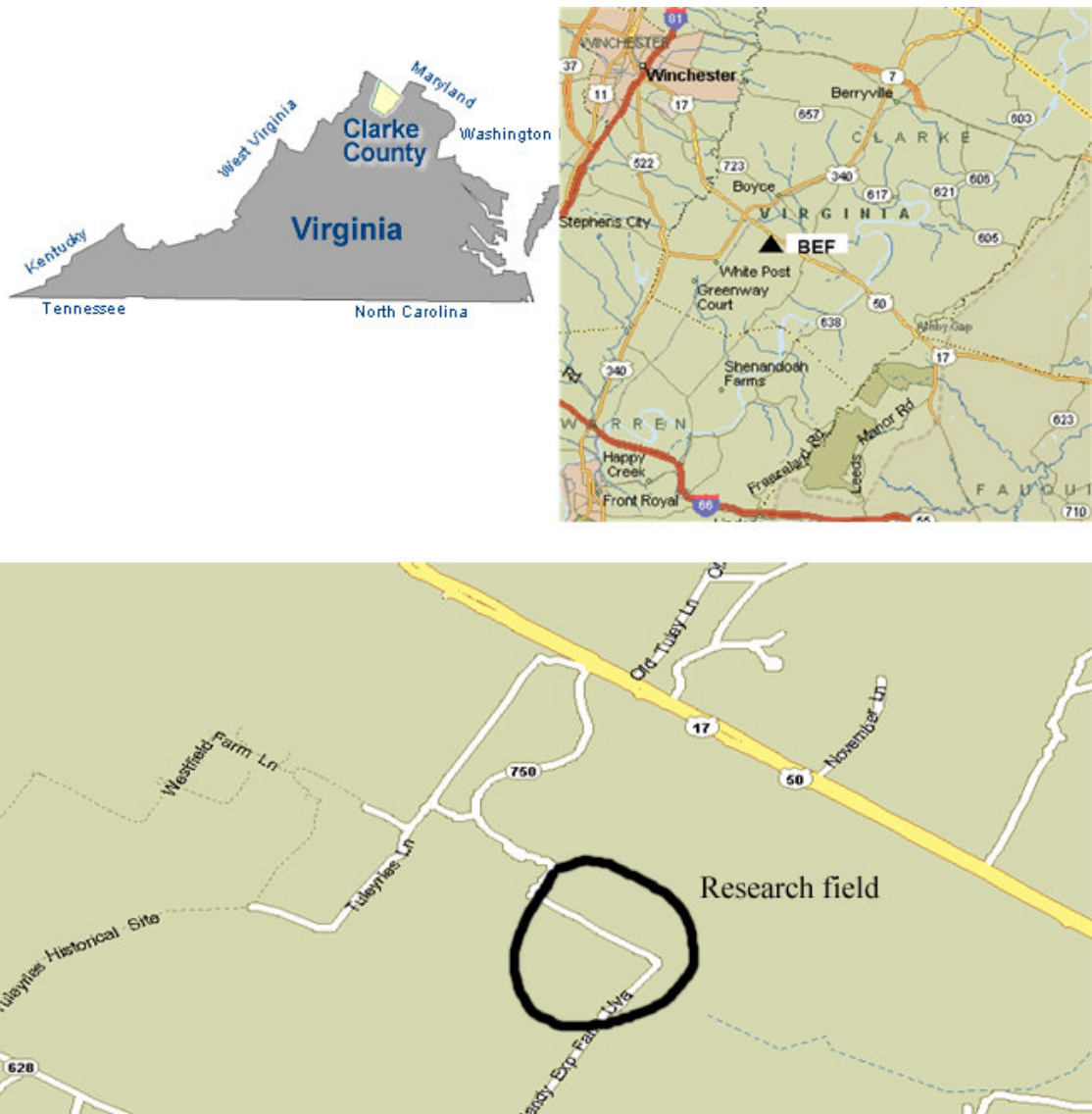


Figure 2.1. Location map for Blandly Experimental Farm (BEF) and research field.

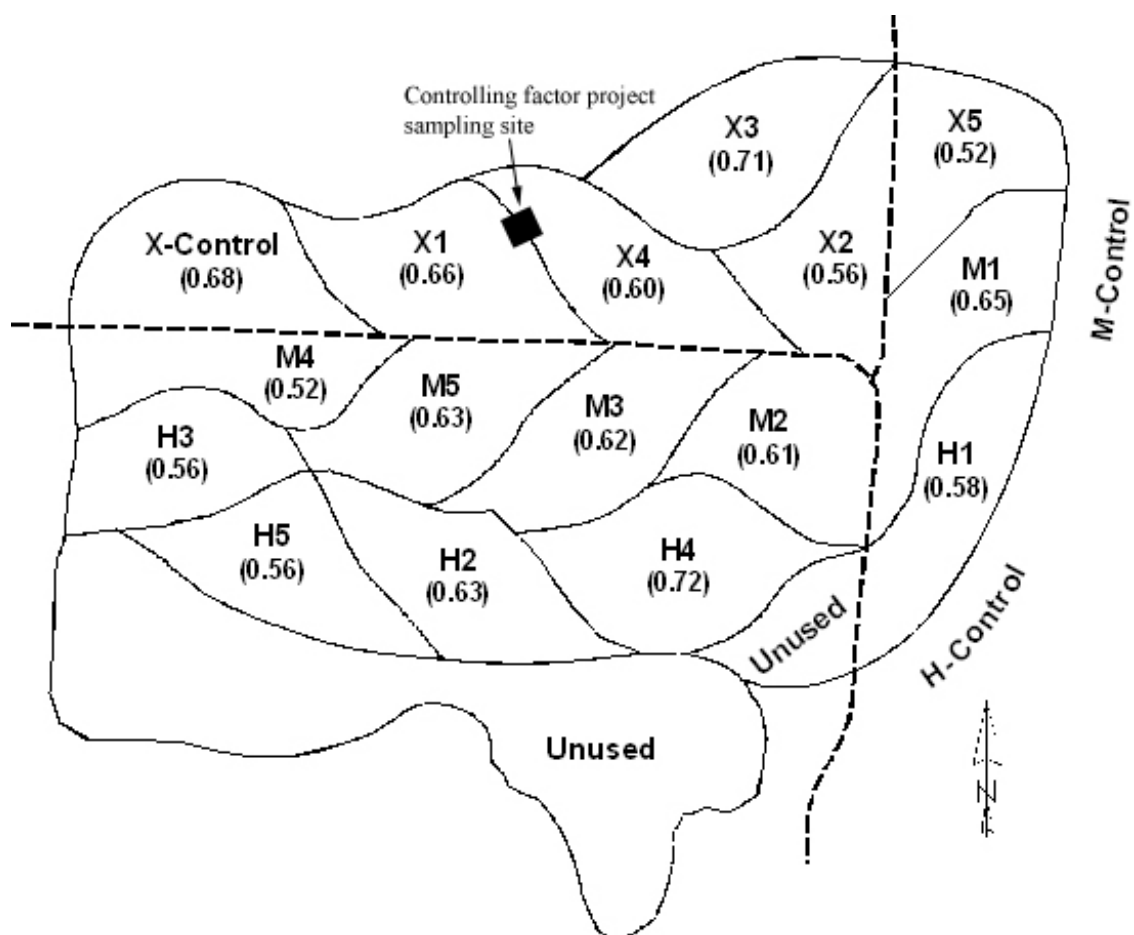


Figure 2.2. Map of experimental site showing the layout of vegetation and moisture blocks as described in Table 2.1. The dotted line indicates unpaved road through the field. The highest (driest) points in the field are at the most northern points, and the lowest (wettest) areas are at the most southern parts. Numbers in parentheses in each each plot represent the area of the plot in ha.

2.2. EXPERIMENTAL DESIGN & SAMPLING PROCEDURE

Five hypotheses were answered throughout the dissertation research and overall description of analytic approaches for each hypothesis is presented and experimental designs are followed.

Experiment 1.

To test the hypothesis that the values for bacterial abundance and microbial community structure are dependent on the size of the collected samples, a series of differently sized samples were collected from the bulk soil which was obtained at X1 plot. Three samples each of 0.01 g, 0.1g, 0.25 g, 1.0 g, and 10.0 g were obtained from homogenized bulk soil sample. Abundance was measured on each sample using Acridine Orange direct counts as described below. Genetic fingerprinting was conducted on the DNA of the microbial community DNA extracted and analyzed as described below. In addition, growth curves were examined by the CFU on 10-fold diluted R2A media. Both means (centroid values for multidimensional ordinations) and variances were tested by ANOVA and the Hartley F_{\max} test ($\alpha = 0.05$) to arrive at a quantitative determination of appropriate sample sizes for each type of analysis.

Experiment 2.

To determine the effect of depth in the soil on the microbial community structure, soil samples were collected at different depths from two 25-cm deep trenches. Soil microbial community structure, total abundance and soil characteristics (pH, moisture content, organic matter content, particle size distribution (texture), and total carbon & nitrogen contents) were measured at each depth. Plant identities and depth of the root systems were also incorporated into the analyses. ANOVA was used to determine

structural difference of microbial communities in vertical gradient. The Mantel test (described in detail below) was used to determine the overall correlation between depth profiles of soil microbial community structure and soil characteristics at corresponding depths.

Experiment 3.

It was hypothesized that the overlying plant community would strongly influence the structure of the microbial community; differences in plant communities would be accompanied by similarly scaled differences in the soil bacterial community structure. The field housing the native grass restoration project was first devegetated and then revegetated with different combinations of plant diversity (details below). Triplicate soil samples collected from each plant diversity treatment (total 18 treatment) for 10 times during first 2 years of the project. Soil bacterial community structures were analyzed using two different genetic fingerprinting analyses (RAPD and DGGE). Total abundance was also measured. Overall temporal trends of soil bacterial community structure development along with seasonal change were measured using the Mantel test. Spatial distribution was analyzed using discriminant function analysis (DFA).

Experiments 4 & 5.

These hypotheses examined the relationship of the overlying plant community composition to the soil microbial community (DNA fingerprints and composition of dominant organisms); by suggesting they are spatially correlated. Furthermore, the extent to which the distribution of microbial community structure is dependent on the chemical and physical properties of the soil from which the microorganisms are collected was examined in a similar correlational analysis. For this portion of the study a sampling grid

was superimposed on the section of the field where distinct vegetation development zones were apparent (e.g., monoculture vs. diverse community) as a result of the revegetation effort. The structure of the soil fungal community and bacterial community was examined separately using PCR-DGGE with “universal” small subunit ribosomal DNA primers. Total abundance was measured by epifluorescence microscopy with Acridine Orange staining (Bottomley, 1994). At each sampling location, several soil characteristics were also measured including pH, moisture content, particle distribution and total carbon & nitrogen content. The cover vegetation was identified at each sampling point. Geostatistical analyses were applied to reveal spatial structure: semivariogram, kriging and stochastic simulation (for hypothesis 4). Mantel test and causal modeling were utilized to identify factors controlling microbial community structure (hypothesis 5).

2.2.1. Native grass restoration project

Although the field shown in Figures 2.1 and 2.2 has long been maintained as a meadow by mowing and occasional prescribed burnings, as with most meadows in Virginia, the grasses in the field were dominated by exotic species (primarily fescue, *Gelium vera*, and Japanese Honeysuckle, *Lonicera japonica*). As part of the activities associated with Blandy Farm’s role as the State Arboretum of Virginia, the 14-ha field shown in Figs. 2.1 and 2.2 was dedicated to a plot-scale restoration of native temperate tallgrass species for both arboretum (display) purposes and research. The restoration was initiated by an herbicide treatment (Roundup®) in October, 1999. In April, 2000, the field was treated with another application of herbicide, followed shortly by a prescribed burn. After the devegetation treatment, the field was sown with 6 species of native temperate

tallgrass species and total of 44 species of forbs (*Coreopsis lanceolata*, *Lupinus perennis* and so on, Appendix C.) arranged in a pattern that included plots of different plant species arranged in combinations of different numbers to allow the examination of effects of differing plant diversity on the restoration. The diversity effect was also examined in the microbiology studies.

The plants were sown in a randomized, complete block design (Figure 2.2). Three blocks were assigned to the field by topographic position corresponding to the assumed soil moisture gradient with the blocks referred to as xeric, mesic and hydric to differentiate among them. Five treatments were randomly assigned within each block (Table 2.1). Experimental plots varied in size from 0.5 ha to 0.7 ha (mean = 0.6 ha). A control plot was also assigned to each block; the controls were neither treated with herbicide nor burned, and thus represented the preexisting community of exotic plants. Six species representing two plant types, tall turf-forming species (big bluestem) and small-statured bunch grasses (e.g., side-oats grama) were chosen, and five combinations of different species and different numbers were assigned as treatments of different plant diversity within each block. The forb mixture was sown uniformly across all treated plots.

Soil samples were taken ten times over a two-year period (Table 2.2). Initial samples were collected immediately prior to the devegetation procedure, 1 day after the burn, and shortly after replanting. Additional samples were collected periodically during the growing season and once in winter for the first year. The second-year sampling schedule was identical to that of the first year except that the mid-winter (non-growing season) sampling was not done. At each sampling time, soil was collected from three randomly chosen locations within each of the plots. Approximately 100g of soil was

Table 2.1. Experimental design of vegetation planting. Treatments represented not only different species composition, but also different diversity levels with the number of grass species sown being 1, 3, or 6. Each treatment comprised three blocks, one of which was in each of the three assumed moisture regimes.

Vegetation Treatments ^a	AREA (ha) ^b	<i>Andropogon gerardii</i> (big bluestem)	<i>Andropogon virginicus</i> (broom sedge)	<i>Schizachyrium scoparium</i> (little bluestem)	<i>Bouteloua cultipendula</i> (side oats)	<i>Panicum virgatum</i> (switch grass)	<i>Sorghastrum nutans</i> (Indian grass)
1	2.27	+					
2	1.89				+		
3	1.91	+	+	+			
4	1.96		+	+	+		
5	1.79	+	+	+	+	+	+
Untreated ^c							

^aEach vegetation and diversity treatment was replicated in each of the three presumed moisture zones (See Figure 2.2).

^bSum of three blocks for each treatment.

^cPlots neither treated with herbicide nor burned.

Table 2.2. Sampling schedule.

Sampling	Date	Comment
1	4.13.00	Immediately after herbicide treatment
2	5. 4.00	1 day after burning
3	5.18.00	Field seeded on 5.10.00
4	6. 1.00	
5	6.22.00	
6	7.20.00	
7	1.11.01	Soil frozen
8	5. 3.01	
9	6.13.01	
10	7.18.01	

taken with a small shovel from the 5- to 10-cm depth interval, placed in polyethylene Ziploc[®] sampling bags, and stored at -20°C until analysis. Obvious plant root debris was removed, but strict discrimination between rhizosphere and bulk soil was not done. The samples were analyzed individually as within-treatment replicates. Most samples were analyzed within a week after collection.

2.2.2. Controlling factors of microbial community structure project

To determine the relative importance of the overlying vegetation vs. physical and chemical soil variable in determining the structure of the resident microbial community, a setting with a clear boundary between two plant communities was necessary. As discussed in the Introduction, the relationship between plants and soil microorganisms is very intimate. Separating its effects from those of other environmental variables is not always a simple task. During summer of 2003 (the fourth growing season after revegetation), several candidate sites in the field were chosen, and the one was selected that showed the most clear boundary between developing plant communities.

The sampling plot was placed between X1 and X4 (Figure 2.2), where a rather sharp distinction between a nearly monospecific stand of *Andropogon gerardii* and an area with 3 dominant species (*Andropogon virginicus*, *Bouteloua curtipendula*, *Schizachyrium scoparium*) occurs. A spatially hierarchical design in which soil samples were collected at 3 different distance increments (0.05m, 0.25m and 1m) was used to reveal spatial structure (Figure 2.3). Each node indicated as a black dot represents a single sampling point. In the Level 1 grid, the nodes were at 1-m intervals so that 36 samples were collected. Each node was named following a column-row (CR) naming rule beginning with 00 at the upper-left corner, moving to 50 in the upper right, 05 in the

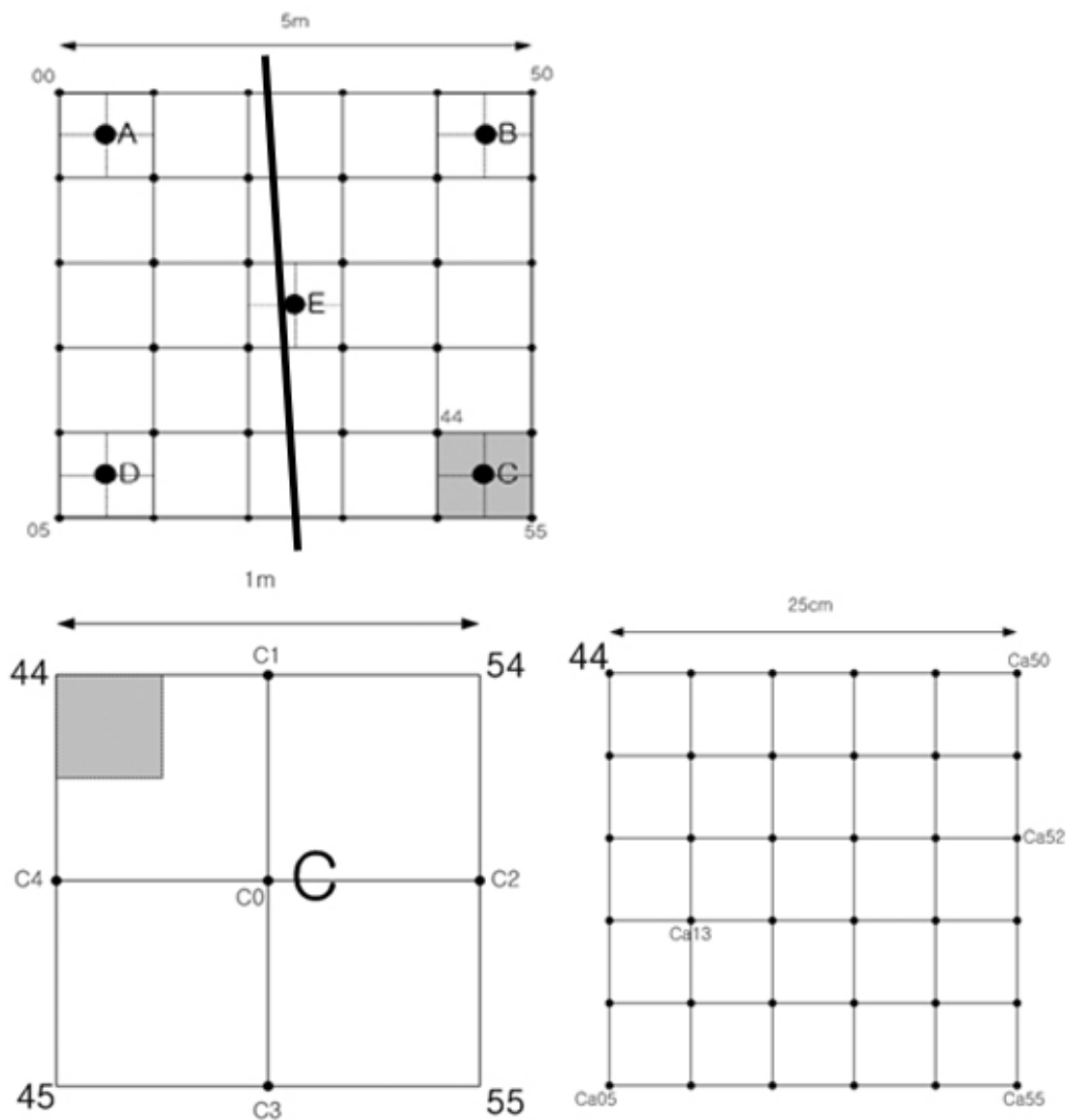


Figure 2.3. Sampling design used to assess spatial structure of soil microbial community. Level II and III are nested within level I and II sampling design, respectively. The vertical line in level I plot indicates the separation between X1 plot (*A. gerardii* monoculture) and X4 plot (mixture of several species).

lower left, and finishing with 55 at bottom right corner. From the 25 Level-1 grids, the 4 corners (indicated as A, B, C and D) and center (E) were chosen for Level-2 sampling. From each grid, 5 samples were collected as shown in the diagram at 0.5m-intervals for a total of 25 samples at Level 2. Sampling points were named by using the grid id and number and the indicating sampling position within the grid beginning with 1 at the top and rotating clockwise; 0 indicated the center of the grid. From each Level-2 corner grid (A, B, C and D), a 25-cm by 25-cm square located closest to the center of the Level-1 plot was chosen for Level-3 sampling. Similar to the Level-1 design, a total of 36 sampling points were established at 0.05-cm intervals. One point from each grid had already been collected during the Level-1 sample (for example, Ca00 from Level 3 is the same point as 44 in the Level 1 matrix). Thus, the total number of samples at Level 3 (including those sampled at Level 1) was 144.

Sampling points were named following rules of Level 1 and 2, i.e., using both grid identity and number, and nodes were numbered the same way as in the Level-1 design. Using this design, spatial autocorrelation of the soil microbial community structure, the relationship of the communities with different overlying plant species, and the influence of soil characteristics on the microbial community structure can be analyzed simultaneously. A total of 199 samples were collected on July 16, 2003. Samples were obtained in approximately 2-cm diameter and 7-cm depth by using a small spatula (Scoopula[®]), because the available soil corer was too large for the Level 3 sampling grids (0.05 m separation). Plants species within a 1.5-cm radius of the sampling point were recorded at the time of the sampling.

On the same day as the extensive sampling of the grid, two of the sampling locations that were separated diagonally on the grid (41 and 14 from Level 1 and named B and D respectively) were chosen for examination of depth profiles of microbial community structure. At each of the sites, approximately 30-40 g of samples were taken from the surface down to 25 cm depth in 2-cm intervals for the top 10 cm, and at 5-cm intervals from 10 to 25 cm depth e.g., the 4-6-cm sample was collected from 4 cm to 6 cm below the surface. The samples thus collected were homogenized before analysis. Plant species and depth of the root zone were also recorded at each of the sites used to examine the depth profiles.

Due to the limited volume of sample collected (about 2-cm diameter \times 5-cm depth), careful planning for laboratory analyses was required to provide an adequate sample for analysis of soil characteristics which generally require larger amounts of sample than for microbial community analyses. Approximately 15 g of fresh soils were dried for gravimetric moisture content measurement (2.3.1.1.), and the dried samples were then used for particle size analysis (2.3.1.3.) and determination of organic matter content (2.3.1.4.). Approximately 5 g of fresh soil samples were sifted through an approximately 700- μ m sieve and stored at -20 °C until used for DNA extraction (2.3.3.1.), microbial enumeration (2.3.2.1.), and for analysis of total carbon and nitrogen content (2.3.1.4.). Another approximately 5 g of the fresh samples (not sieved) were taken for pH measurement (2.3.1.2.).

2.3. ANALYTICAL PROCEDURES

2.3.1. Soil Characterization

2.3.1.1. Soil moisture content

Gravimetric moisture content (P_w) was measured according to Pepper *et al.* (Pepper *et al.*, 1995). The gravimetric moisture content is a direct measurement of soil water, thus it is the standard for calibration of all other indirect measurements of soil water. However, since it is not usually possible to recover samples after measurement for other analyses, a larger amount of soil was required. Approximately 15 g of fresh soil sample was dried for 24 hours at 105°C in a drying oven, and the P_w was determined by the formula:

$$P_w = \frac{ww - dw}{dw} \times 100, \quad (2.1)$$

where ww is wet weight and dw is oven-dry weight of the soil samples.

2.3.1.2. Soil pH

Soil pH was measured following the protocol presented by Soil Science Society of America (Thomas, 1996). Approximately 5 cm³ of fresh soil sample and about 10 ml of distilled water were placed in a 200-ml beaker. Soil suspensions were well mixed with a glass rod, and pH was measured with Thermo Orion 720A pH meter (Orion Research Inc., Boston, MA) after the soil has settled. Actual reading was obtained when measurements were stable for at least 5 seconds. The pH meter was calibrated with two-point calibration procedure before the initial measurement each day of use. The combination electrode was rinsed with distilled water between each measurement, and care was taken not to touch the mass of soil with the electrode while making the measurement.

2.3.1.3. Particle size distribution (texture)

The hydrometer method for mechanical analysis of particle distribution presented by Gee & Bauder (Gee, and Bauder, 1986) was used here with some modifications to accommodate the limited amount of soil sample available. 10 g of oven-dry soil and 12.5 ml of Clorox[®] were mixed on shaker for 24 hours to remove cementing agents and organic matter. Then 25 ml of dispersant solution (4% sodium hexametaphosphate ((NaPO₃)₆)) and 25 ml of distilled water were added. After 10 minutes of standing with occasional stirrings, the mixture of soil, Clorox[®], dispersant and water was vigorously shaken for 30 minutes for dispersion of soil particles. Well mixed solution was transferred into sedimentation cylinder and hydrometer readings were performed at 30 second (twice) and at 2 hours after mixing in the cylinder. The percentage of soil particles still in suspension was calculated by the formula:

$$P = \frac{\left[\left(\frac{50000}{W} \right) \times G \right] \times (R_c - G_L)}{(G - G_L)}, \quad (2.2)$$

where

W = oven dry weight of sample

G = specific gravity of soil particles (= 2.65)

G_L = specific gravity of liquid (= 1)

R_c = hydrometer reading corrected by the 'composite correction factor' related to temperature. And

$$R_c = R - R_L,$$

where R is the direct hydrometer reading and R_L is the relative hydrometer reading of the solution portion of mixture (the reading from Clorox, dispersant and distilled water minus the reading of distilled water at the same temperature). The percentage of sand is calculated by $100 - P_{30sec}$, and P_{2hr} is the percentage of clay. Thus the percentage of silt is

$P_{30sec} - P_{2hr}$. As it was discussed in the previous chapter, % clay was reported and used for further analyses.

2.3.1.4. Total carbon, nitrogen and organic matter content

Total carbon and nitrogen contents were measured using an elemental analyzer CE EA 1108 CHNS-O (Fisons Instruments, Italy) with accompanying Eager 200 software. Approximately 20 mg of sifted (approximately 700- μ m sieve), air-dried soil sample was prepared in tin cups. Two different standards were used along with soil samples: atropine ($C_{17}H_{23}NO_3$, 70.56% carbon and 4.84% nitrogen) and acetanilide (C_8H_9NO , 71.09% carbon and 10.36% nitrogen). Each soil sample was replicated ($\times 2$) for better accuracy. Total organic matter content (OM) was also measured for samples from two trenches (experiment 2). Dried soil samples were ashed at 450 °C for 24 hours in a muffle furnace (Thermodyne 10500, Dubuque, IA) and organic matter content was calculated by the formula:

$$OM = \frac{dw - aw}{dw} \times 100, \quad (2.3)$$

where aw is the weight of the soil samples after the ashing process.

2.3.2. **Microbial Abundance**

2.3.2.1. Acridine orange direct counting

Total abundance of soil microorganisms was measured using epifluorescence microscopy for direct counting with Acridine Orange (AO) staining of the bacterial cells (Bottomley, 1994). As determined in the experiment 1, 0.1 g of fresh sifted soil sample was added to with 9.9 ml of distilled water (10^{-2} dilution) and then mixed on a vortex mixer. Serial dilution was done with 1 ml of soil suspension, 8 ml of distilled water and 1

ml of formaldehyde (10^{-1} dilution). For experiment 1, 10 ml of initial soil solution was prepared by adding appropriate amount of soil samples and distilled water. Formaldehyde was added to fix microorganisms in the suspension and on the filter to make it easier to count them. Acridine orange solution (0.1 %, w/v) was prepared in 2 % formaldehyde and then filtered through a 0.2- μm pore size filter (Nalgene 115-ml disposable filter unit, Rochester, NY). Soil suspensions and AO solution were mixed and filtered to collect stained cells on a 0.2- μm pore diameter membrane filter (Osmonics, Livermore, CA). Microorganisms were counted with an oil immersion objective (100 \times total magnification) under epifluorescent illumination (Carl Zeiss, Germany). At least 5 fields with 20 or more microorganisms found were counted and counting was continued until the total count reached 200. Total abundance was calculated by the formula:

$$\text{Microorganisms/g of soil} = \frac{\left(\frac{\text{total area}}{\text{area/field}} \right) \times (\text{counts/field})}{(\text{volume filtered}) \times (\text{dilution factor})},$$

where

total area is total stained area of the filter (314 mm^2)

area/field is area of one field as defined by the eyepiece micrometer (0.008649 mm^2)

counts/field is averaged counts.

2.3.2.2. Cultural enumeration

Culturing on 10^{-1} diluted R2A media was performed to investigate growth patterns of microbial communities of different sample sizes (experiment 1). In each sample size, soil samples were diluted serially at four levels and each dilution was inoculated onto 3 replicate plates. A total of 60 plates were incubated at room temperature (ca. 22 $^{\circ}$) for 160 hours and colonies were counted 8 times during that period.

For each counting, the colonies were marked with different color markers so that emerging colonies were easily identified in each period. Only data from plates with the range of 30 to 300 colonies after 160 hours of incubation were included in the analysis.

2.3.3. DNA Preparation & Genetic Fingerprinting

2.3.3.1. Determination of DNA extraction procedure

There are two main groups of environmental DNA extraction protocols: direct lysis of microorganisms and microbial fractionation (Holben, 1997). The direct lysis method releases the DNA into the environmental matrix and then separates it from the environmental matrix later on. The microbial fractionation method first separates microorganisms from the environmental matrix then lysis and DNA extraction from the cell suspension follow. There are advantages and disadvantages with each method. Direct lysis generally recovers more DNA, and as such, has a better chance to represent the entire microbial community. Direct lysis is also less labor-intensive than the microbial fractionation method. However, the microbial fractionation approach tends to yield purer DNA. Therefore, improved approaches, which combine advantages from both methods, are often based on direct lysis with either a subsequent purification step or use of a special reagent to remove environmental impurities is included in these protocols.

UltraClean™ Soil DNA isolation kit (MoBio Laboratories, Inc., Solana Beach, CA) has been used for soil microbial DNA extraction. The actual protocol incorporated an 'alternative lysis method' in which the mechanical lysis is replaced resulting in less sheared DNA. Highly sheared DNA might lead in more frequent chimeric amplicons (Ogram, 1998), and that would increase microbial diversity measurement and would disturb the results of further analysis (Marilley, and Aragno, 1999). Also UltraClean™

Mega Soil DNA isolation kit was used for extraction DNA from larger amount (10.0 g) for soil sample (experiment 1).

The extraction protocol of UltraClean™ soil DNA isolation kit is based on direct lysis method with PCR inhibitor removal solution (IRS) to minimize soil impurities blocking PCR. Each tube contains 0.25 g of soil samples and procedures with additions series of reagents and separations of supernatants by centrifugation result in fairly clean DNA in 10 mM Tris buffer. For determination of DNA extraction yield and required amount of DNA for genetic fingerprinting techniques, final concentration of DNA was measured. PicoGreen® dsDNA Quantification kit (Molecular Probes, Eugene, OR) was used with BioLumin 960 microassay reader (Molecular Dynamics, Piscataway, NJ) by measuring absorbance at 260 nm (A_{260}).

2.3.3.2. Successful PCR

In modern biology, PCR (Polymerase Chain Reaction) is so essential that one compared relationship between PCR and biology to petroleum and transportation (Pray, 2004). PCR is a critical element of the genetic fingerprinting techniques used for revealing microbial communities in the present study. Since the invention of the thermal cycler, PCR has been expanding its usage throughout the entire field of biology, including ecology (molecular ecology). Many aspects of PCR have changed and become more sophisticated, yet the process has been simplified for everyday usage by the development of high-tech instrumentation. However, PCR is not very reliable in terms of technical stability and reproducibility, and it requires expensive facilities. With the exact same conditions and reagents, the PCR amplification succeeds or fails without the technologist understanding the reasons.

The reagent-intensive PCR reaction requires precise chemical balances among its components: primers, nucleotides (dNTPs), divalent cations (e.g., Mg^{2+}), template DNA and thermostable DNA polymerase. All those components have their ideal concentration ranges, but even when the reaction mixture is ideal, in many situations, successful PCR amplification may not occur. Some components' ideal ranges are determined based on a reasonable rationale. For example, the molar concentration of divalent cations must exceed the combined molar concentrations of nucleotides and primers (Sambrook, and Russell, 2001). The ideal concentration of some others, such as nucleotide and template DNA, however, are mainly determined empirically. Therefore, optimization procedures with combinations of different concentrations of components are required.

Other important factors governing successful PCR amplification are temperature and length of each step: denaturation, annealing, and extension. Denaturation and extension steps have optimal ranges for a specific thermostable DNA polymerase, and the annealing temperature is based on the length and base sequence of the primer. For example, *Taq* DNA polymerase has an optimal reaction temperature of 75-80°C and the denaturation temperature is usually lower than 97°C due to the half-life of *Taq* DNA polymerase (9 minutes at 97°C, 40 minutes at 95°C and 130 minutes at 92.5°C) (Fanning, and Gibbs, 1997; Sambrook, and Russell, 2001). However, as in the case of the PCR reaction components, both the temperature and length of each step is optimized by trial and error, especially when PCR problems arise.

Taq DNA polymerase, which is originated from the thermophilic bacterium *Thermus aquaticus*, has a relatively high error rate ($1.1 \times 10^{-4} - 8.9 \times 10^{-5}$ mistakes / bp)

(Tindall, and Kunkel, 1988; Cariello *et al.*, 1991; Ling *et al.*, 1991; Lundberg *et al.*, 1991) due to the lack of a 3' → 5' exonuclease proofreading function. The amplified ssu rDNA fragments used for this study were relatively short, about 400 bp for the fungal community and about 450 bp for the bacterial community. Thus, this error rate means that approximately one mismatch could occur in every 22-25 fragments in each PCR reaction cycle. This error rate could be problematic in specific PCR, which requires precise amplification of the target genes. Consequently, attempts were made to find a high-fidelity alternative thermostable DNA polymerase (e.g., *Pfu* from the hyperthermophilic archaeon *Pyrococcus furiosus* with a proofreading function and error rate of about 1.5×10^{-6} /bp (Lundberg *et al.*, 1991)) or to use multiple thermostable DNA polymerases together (e.g., High Fidelity PCR Enzyme Mix from Fermentas). For genetic fingerprinting techniques such as DGGE or T-RFLP that are based on the ssu rDNA gene, error rate even relatively higher error of *Taq* DNA polymerase would not create a large problem. DGGE separates ssu rDNA fragments of the same length based on their sequence difference. Although DGGE can theoretically distinguish even single base pair sequence differences (Myers *et al.*, 1985), ordinary polyacrylamide gel electrophoresis cannot resolve such small changes. Therefore, *Taq* DNA polymerase from Perkin Elmer (AmpliTaq DNA polymerase, N808-0153), which was used throughout the present study, presented no major problem with its performance in PCR.

2.3.3.3. Randomly Amplified Polymorphic DNA (RAPD)

Samples collected from sampling time 3, 6 and 8 in native grass restoration project (experiment 3) were analyzed by RAPD (Franklin *et al.*, 1999; Wikstrom *et al.*, 1999) along with denaturing gradient gel electrophoresis (DGGE) for more complete

description of microbial community structure. 36 short arbitrary primers were chosen from the QIAGEN Operon (Alameda, CA) catalog and tested for use in this study. Final selection of primers was based on both the total number of polymorphisms and the contribution of new polymorphisms to the accumulated set. Based on the results of the testing, 10 primers were chosen (Table 2.3). The PCR amplifications were performed using the protocol of Franklin et al. (1999) with slight modification. A 5- μ l portion of diluted template DNA (5 to 10 ng) was added to 20 μ l of a reaction mixture containing: 1.5 mM MgCl₂, 0.2 μ M primer, 100 μ M dNTP mixture (Applied Biosystems, Foster City, CA), 20 μ g BSA (bovine serum albumin, New England Biolabs, Beverly, MD), PCR buffer, and 1 unit of AmpliTaq[®] DNA polymerase (Applied Biosystems). The amplification protocol was: an initial denaturation of 1 minute at 94°C, then 45 cycles of 1 minute at 94°C, 1 minute at 36°C and 2 minutes at 72°C, with final elongation for 10 minutes at 72°C. Amplified products were separated by electrophoresis in 1.5 % agarose gels, and visualized with ethidium bromide staining under UV light.

2.3.3.4. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE (Heuer, and Smalla, 1997; Muyzer, and Smalla, 1998) was the main genetic fingerprinting technique to study soil microbial community structure throughout this research. DGGE was originally introduced to detect point mutations in isolates (Fisher, and Lerman, 1979; Myers *et al.*, 1985), but it has been used widely in microbial ecology since Muyzer *et al.* (Muyzer *et al.*, 1993) presented a new application of DGGE for examining the genetic diversity of complex microbial assemblies (Ovreas *et al.*, 1997; Duineveld *et al.*, 1998; El Fantroussi *et al.*, 1999; Smalla *et al.*, 2001; Yang *et al.*, 2001;

LaPara *et al.*, 2002). There are several review papers on the latter technique, its applications and implications of its use (Muyzer *et al.*, 1996; Heuer, and Smalla, 1997; Fromin *et al.*, 2002).

The 16S rDNA fragments were obtained by the amplification of 20 to 40 ng of template DNA with a universal primer set (P63f and P518r) for soil bacteria (Table 2.3) (El Fantroussi *et al.*, 1999). A GC clamp of 40 bases was added to prevent complete separation of double stranded DNA and loss of the sequences from the gel (Sheffield *et al.*, 1989). The PCR reaction mixture contained 2.5 mM MgCl₂ (3.75 mL was used for hypothesis 3), 0.2 μM primers (prepared from QIAGEN Operon), 200 μM dNTP mixture, PCR buffer, and two units of AmpliTaq[®] DNA polymerase. Two PCR additives were added to the mixture for clear and accurate results: 40 μg BSA and 5% DMSO (dimethylsulfoxide) (Smith *et al.*, 1990; Mahbubani, and Bej, 1994; Hengen, 1997; Muyzer, and Smalla, 1998). Amplification was performed as follows: 5 minutes of initial denaturation at 94°C, and 30 cycles of 1 minute at 92°C, 1 minute at 64°C, and 2 minutes at 72°C, with a final elongation of 10 minutes at 72°C. Amplified 16S rDNA fragments were separated on a polyacrylamide gel with a denaturing gradient of 33 to 47% of urea-formamide denaturant, following procedures established by the equipment manufacturer (CBS Scientific Company, Del Mar, CA). The electrophoretic separation was run for 4 hours at 175V, and products were visualized using SYBR Green I staining (Molecular Probes, Eugene, OR) under UV light.

In addition to bacteria, the fungal community was studied by using FF390 and FR1 (Table 2.3) (Vainio, and Hantula, 2000). FF390 and FR1 primer set is designed to amplify 390 bp fragment of 18S rDNA near the 3' end. Evaluation by FastA searches in

Table 2.3. Primers used in microbial community structure analysis.

<i>name</i>	<i>sequence</i>	<i>used in</i>
A7	GAAACGGGTG	RAPD
A8	GTGACGTAGG	RAPD
A12	TCGGCGATAG	RAPD
A19	CAAACGTCGG	RAPD
A20	GTTGCGATCC	RAPD
C4	CCGCATCTAC	RAPD
D18	GAGAGCCAAC	RAPD
F3	CCTGATCACC	RAPD
S14	AAAGGGGTCC	RAPD
T7	GGCAGGCTGT	RAPD
P63f	5'-GC clamp-CAGGCCTAACACATGCAAGTC-3'	DGGE-bacteria
P518r	5'-ATTACCGCGGCTGGCTGG-3'	DGGE-bacteria
FF390	5'-CGATAACGAACGAGACCT-3'	DGGE-fungi
FR1	5'-GC clamp-AICCATTCAATCGGTAIT-3'	DGGE-fungi

the GenBank database revealed the matching of high number of fungi (Vainio, and Hantula, 2000). The PCR reaction mixture contained 0.5 μ M primers, 2.5 mM MgCl₂, 200 μ M dNTP mixture, PCR buffer, and two units of AmpliTaq[®] DNA polymerase. There were also two PCR additives in the PCR reaction mixture for clear and accurate results: 40 μ g BSA and 10% glycerol (Smith *et al.*, 1990; Mahubani, and Bej, 1994; Hengen, 1997; Muyzer, and Smalla, 1998). Amplification was performed as follows: initial denaturation of 8 minutes at 95°C, and 30 cycles of 30 seconds at 95°C, 45 seconds at 50°C, and 2 minutes at 72°C, with a final elongation of 10 minutes at 72°C. Amplified 18S rDNA fragments were separated on a polyacrylamide gel with denaturing gradient of 40 to 55% of urea-formamide denaturant. The electrophoretic separation was run for 4 hours at 175V, and products visualization was same with bacterial analysis. The original protocols suggested much longer running time (16 ~ 18 hours) at lower voltages (50 ~ 75 V), but preliminary running did not provide improved results. The protocols for fungal community PCR and acrylamide gel electrophoresis were empirically optimized based on those presented by Kowalchuk *et al.* (2003) and Vainio and Hantula (2000).

Denaturing gradients were prepared by combinations of 0% and 80% denaturant stocks. 0% stock (100 ml) contains 15 ml 40% acryl/bis solution (Amresco, Solon, OH), 2ml 50X TAE buffer and distilled water. 80% stock (100 ml) was prepared by adding 33ml dionized formamide (Sigma-Aldrich Co., St. Louis, MO) and 33.6 g urea (Amresco) in 0% stock. Denaturants were prepared and stored in refrigerator up to one month before discarding.

Since each gel can hold only 16 samples, multiple gels must be used to obtain results for all samples. In this situation, gel to gel reproducibility is a very important issue

for reliable results. Most variations that lead to differing bands tend to be generated even before the gel electrophoresis (Fromin *et al.*, 2002); but DNA extraction and PCR procedures were performed enough times to be standardized in our laboratory. For gel electrophoresis, the same denaturant gradient and polyacrylamide concentration are used for gel preparation, and running conditions are optimized for best resolution. Additionally, molecular marker (molecular weight standards) was used in each gel so that gels can be matched by comparing the pattern of the sample relative to that of the marker.

Although some have proposed and conducted studies suggesting that band intensity is directly related to relative abundance of the corresponding phylotype from the mixture of DNA template (Demeke, and Adams, 1994; Murray *et al.*, 1996), the potential artificiality of amplification in mixtures from extracts, for example, heteroduplex molecules (Jensen, and Straus, 1993) or chimeric molecules (Shuldiner *et al.*, 1989; Choi *et al.*, 1994), makes extrapolation to highly complex mixtures (such as used here) tenuous at best. For that reason, band intensity information was not included in these analyses.

2.4. STATISTICAL ANALYSIS

2.4.1. Multivariate statistics for microbial community information

For statistical interpretation of genetic fingerprinting analysis (DGGE, denaturant gradient gel electrophoresis, which is the analysis chosen for microbial community structure measurement for the current project), there are things need to be considered first. Each band from the generated band pattern can be considered to represent a certain microbial population, which has same sequence therefore same mobility. Kowalchuck *et al.* (1997) showed that co-migrating bands were generally identical. Theoretically, even

differences in a single base in the sequence can be differentiated by DGGE, but closely related microorganisms are not necessarily always separated by the resolution power provided by general DGGE analysis. A generally known problem of PCR-based techniques is the formation of artificial bands resulting from heteroduplex molecules (Boer, and Ramamoorthy, 1997; Ferris, and Ward, 1997).

The characteristics of data from microbial community ecology are multivariate, because each sample is described by multiple environmental factors and types of organisms (Gauch Jr., 1982). So multivariate statistics are the appropriate statistical approach for understanding the relationship between complex microbial community structure and influencing environmental factors (Haack *et al.*, 2004). Ordination techniques and matrix correlation analysis are used for the current project among various multivariate statistical methods. Discriminant function analysis (DFA) generates new variables by linear combinations of original variables, and new variables provide maximum discrimination among entities (McGarigal *et al.*, 2000). DFA has a benefit that it possesses predictive ability beyond other ordination methods such as principal component analysis (PCA) and factor analysis (FA). Like PCA, DFA also assumes the normality of the data set but binary data from DGGE are not normally distributed. Because the usage of the techniques is for clustering of data to produce similarity plots for visualization of the relationships and not inferring specific information from the extracted factors, these analyses are quite robust with respect to violations of the normality assumption (Legendre, and Legendre, 1998; Dollhopf *et al.*, 2001).

The Mantel test is an extension of Pearson's correlation analysis for comparison of matrices, so it allows comparison of the variables across the entire field simultaneously.

For example, Mantel tests between matrices of environmental variables and distance can be used to reveal spatial structure of that environmental variable (Legendre, and Troussellier, 1988; Tatineni *et al.*, 1996). In another case, two matrices of the same variable measured at different times can provide a view of the temporal trend for the variable (Kang, and Mills, 2004). Dissimilarity matrix of microbial community structure can be used just like any other conventionally constructed matrix (Franklin *et al.*, 2002). The Mantel test is a very simple, but powerful, test dealing with large amounts of data. Legendre and Troussellier (1988) proposed the way of best fitting model using series of questions with correlation coefficients. Causal modeling would supply description of interactions among factors and microbial community structure in the soil.

2.4.2. Analysis of Variance (ANOVA) and correlation analysis

Differences among means of total microbial abundance from each sample plot were compared with one-way ANOVA and post hoc ANOVA using Ryan's Q test (Sokal, and Rohlf, 1995) (experiment 3). One-way ANOVA was used to assess differences in means among different sample sizes for total microbial abundance and DNA extraction yield. Centroids of bacterial and fungal community structure generated from discriminant function analysis (DFA, see 2.4.4.) were also compared by one-way ANOVA (experiment 1). All analyses were done with a confidence level of 0.05 by using SAS 8.2 (SAS Institute Inc., Cary, NC). To identify the relationships among soil characteristics variables, correlation analysis was performed using SAS 8.2 at $\alpha = 0.05$.

2.4.3. Hartley's F_{\max} test

For the examination of the effect of sample of size (experiment 1), a quantitative determination of variances among measurements was desired. The results of ANOVA are

only valid when several assumptions are met. Independence of samples and normality of residuals are manageable in some degree by careful experimental design and transformation of data respectively. The other assumption, homogeneity of variance, can be tested, and failure to meet the assumption, which occurred in this case, requires the analysis to be done with a non-parametric ANOVA. Two statistical tests are used in different situations for testing homogeneity of variance assumption: Bartlett's test and Hartley's F_{\max} test. Bartlett's test is a commonly used test based on X^2 statistics, and the actual computations are quite complicated. ANOVA based on a least squares method in SAS (proc glm) has an option to invoke Bartlett's test in the means statement (hovtest = bartlett). Bartlett's test is very sensitive to departures from normality of the data. In that case, rejection of the null hypothesis indicates non-normality of the data rather than heterogeneity of variance (Sokal, and Rohlf, 1995). Bartlett's test is also usually used when there are unequal degrees of freedom among groups. Hartley's F_{\max} test, on the other hand, is fast and very easy to run. It is based on F_{\max} statistics and is calculated simply by the ratio of maximum and minimum variance among groups. All the tests of variance homogeneity among cases with different sample sizes were performed by both Bartlett's test and Hartley's F_{\max} test, but the results were reported only from the latter since their results were not different.

2.4.4. Discriminant analysis, Mantel tests, and causal modeling

The band patterns on the agarose (RAPD) or acrylamide (DGGE) gel are the raw data representing the microbial community structures. Each position which contained a band in at least one sample was considered a unit character, and the presence or absence of a band in a given sample was recorded in a binary data format as '1' for a band and '0'


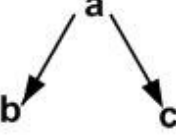
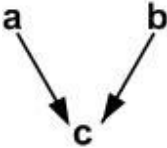
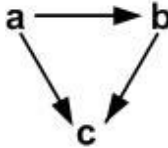
for no band. Then the binary data were fed into analyses of microbial community structure including discriminant function analysis (DFA) and Mantel's test. DFA was used mainly for graphical representation of microbial community structures on discriminant function plots and for validation of sample identities determined by their traits. SPSS v. 11 (SPSS Inc., Chicago, IL) was the software package used for DFA.

For microbial community structure and plant community analysis, similarity matrices were constructed for each community using the Jaccard coefficient,

$$J = \frac{a}{a + c + d}, \quad (2.4)$$

(where a is the number of positive matches (1 and 1), and c and d represent negative matches (1 and 0 or 0 and 1)) (Colwell, and Austin, 1981) to prevent formation of groups based solely on negative data (i.e., to preclude reliance on absence of bands rather than presence) (Lambooy, 1994). For native grass restoration project (experiment 3), a total of 10 similarity matrices of microbial community structure were prepared (1 for each sampling) and used in analysis to reveal temporal change of microbial community structure. For all other experiments except experiment 1, one similarity matrix was constructed for microbial community structure and compared with matrices of other relevant properties. Similarity matrices were converted into dissimilarity ($1-J$) matrices for the Mantel test. Soil characteristics data were summarized into one dissimilarity matrix based on Euclidean-distance calculations for overall causal modeling. Each individual soil characteristics were also used to determine the relationships with soil microbial community structure separately. Distance measurements were also transformed

Figure 2.4. Predictions of the four possible models of causal relationships involving three variables, in terms of expected values of the simple and partial Mantel tests. For simplicity, r_{AB} is noted **AB**, and so on. '**AB** $\neq 0$ ' means that, under the model, the

Model 1	Expectations of the model	Model 2	Expectations of the model
	AB $\neq 0$ BC $\neq 0$ $ AB \geq AC $ $ BC \geq AC $ AB · C $\neq 0$ BC · A $\neq 0$ AC · B = 0 $ AB \cdot C \leq AB $ $ BC \cdot A \leq BC $ AB \times BC \approx AC		AB $\neq 0$ AC $\neq 0$ $ AB \geq BC $ $ AC \geq BC $ AB · C $\neq 0$ AC · B $\neq 0$ BC · A = 0 $ AB \cdot C \leq AB $ $ AC \cdot B \leq AC $ AB \times AC \approx BC
Model 3	Expectations of the model	Model 4	Expectations of the model
	AB = 0 AC $\neq 0$ BC $\neq 0$ AB · C $\neq 0$ BC · A $\neq 0$ AC · B $\neq 0$ $ AC \cdot B \leq AC $ $ BC \cdot A \leq BC $		AB $\neq 0$ AC $\neq 0$ * BC $\neq 0$ * AB · C $\neq 0$ BC · A $\neq 0$ AC · B $\neq 0$

correlation must be significantly different from zero.

* Model 4 holds even if one, but only one, of these two simple correlation coefficients is not significant. Redrawn from Legendre (1993) and Legendre and Legendre (1998).

matrix containing a value for every pair of samples to make a matrix comparable with other factor matrices. The prepared distance matrices were analyzed with the Mantel test using the program R Package v. 4.00 (Casgrain, and Legendre, 2001). The Mantel test was used to reveal temporal trends of microbial community structure in experiment 3. It was also used to test correlation among microbial community structure and other measured environmental factors including separation distance of each community (experiments 2, 4 and 5). Correlation coefficients from the simple and partial Mantel tests were used for causal modeling. Legendre and Legendre (1998) proposed the method of finding best fitting model among variables using series of questions with correlation coefficients from the simple and partial Mantel tests (Figure 2.4). Causal modeling would supply description of interactions among environmental factors and microbial community structure in the soil.

2.5. GEOSTATISTICS

2.5.1. Semivariogram

Geostatistics permit a researcher to incorporate the spatial coordinates of observations into the data set. It then allows describing and modeling spatial patterns, estimating variable values at unsampled locations, and assessing the uncertainty of the estimations. The semivariogram is one of the most popular descriptive first steps in a series of geostatistical procedures that depict and model spatial patterns (spatial continuity of the observations or spatial autocorrelation) (Isaaks, and Srivastava, 1989). The semivariogram is defined as the semivariance of the increment and be formulated:

$$\gamma(\mathbf{h}) = \frac{1}{2N(\mathbf{h})} \sum_{i=1}^{N(\mathbf{h})} [z(\mathbf{u}_i) - z(\mathbf{u}_i + \mathbf{h})]^2, \quad (2.5)$$

where \mathbf{h} is the separation distance between any pair of points, also referred to as the lag distance. The formula can be verbally described as the average dissimilarity between observations separated by a vector \mathbf{h} . From this understanding, semivariograms of microbial community structure were created using the ‘relative dissimilarity’ values calculated from the Jaccard similarity matrix, because DGGE generated multivariate binary data, which cannot be used to create semivariogram in traditional sense (Mackas, 1984; Franklin, and Mills, 2003; Mummey, and Stahl, 2003; Lilleskov *et al.*, 2004).

$$\gamma'(\mathbf{h}) = \frac{1}{2N(\mathbf{h})} \sum_{i=1}^{N(\mathbf{h})} [1 - J] \quad (2.6)$$

It was intended to employ similar approach to analysis plant composition of the field, but the data showed too abundant negative pair that constructing similarity matrix was not successful. Among several different geostatistical analyses, indicator semivariogram and indicator kriging are designed for categorical variable. However, difficulty is that what indicator analyses estimate is proportion of certain ‘0’s and ‘1’s (in case of binary variable). Certainly that could be very useful in some cases, but not particularly useful in dealing with microbial community composition data, because what it matters is patterns of ‘0’s and ‘1’s in each sample not proportions of them in defining identities of each microbial community.

In general, semivariogram models can be categorized into two large groups: bounded and unbounded models (Deutsch, and Journel, 1998; Goovaerts, 1998; Webster, and Oliver, 2001). A pure nugget semivariogram model is a flat line that indicates no spatial structure in the data. A Linear semivariogram model is an unbounded model and indicates linear relationship between lag distance and semivariance: $\gamma(\mathbf{h}) = C_0 + b\mathbf{h}$,

where $\gamma(\mathbf{h})$ is semivariance, C_0 is nugget, b is the slope of the model and \mathbf{h} is the spatial lag distance (Figure 2.5). Here nugget represents the spatial variability which cannot be modeled using a spatial autocorrelation function, i.e., amount of variability at distance = 0. It is generated because of either measurement errors or spatial variability existing at distances shorter than the measurement. The linear model represents the situation in which all observations are autocorrelated at the entire lag distances measured. There are three models that are bounded: spherical, exponential and Gaussian (Figure 2.6). They are termed bounded as the upper limit of the variance is bounded by a maximum value, whereas in the linear model, there is no maximum value of the variance. These component models allow one to model different situations at infinity (bounded and unbounded) and at the origin (linear and quadratic). However, experimental semivariograms are usually constructed by the linear combinations of two or more component models to form a combined model. In such a case, a nugget effect is almost always included. For example, when an exponential model is combined with a nugget effect:

$$\gamma(\mathbf{h}) = C_0 + C \left\{ 1 - \exp\left(-\frac{3\mathbf{h}}{a}\right) \right\}, \quad (2.7)$$

where C is structure variance, the sill is expressed as $C + C_0$. The practical range (a) is defined as the distance at which semivariance is 95% of the sill, and takes the value $3b$. Due to the difference in convention of defining Gaussian model, range from VARIOWIN has to be multiplied by $\sqrt{3}$ before fed into GSLIB. The proportion of model sample variance ($C + C_0$) explained by structural variance (C) is called the normalized measure of spatial dependence (NMSD). If a system's NMSD reaches 1, the system is highly

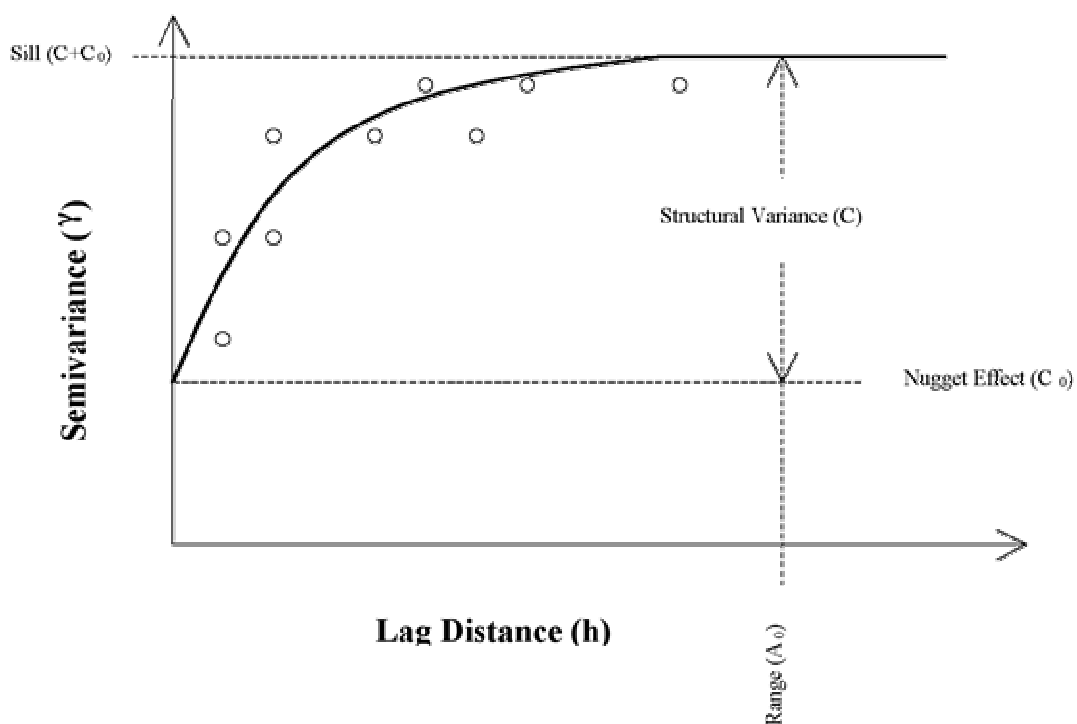


Figure 2.5. Sample semivariogram plot. Nugget (C_0) is the discontinuity at the origin of the semivariogram (y-intercept) and arises from measurement errors or spatial sources of variation at distances smaller than the shortest sampling interval, or both. Sill ($C + C_0$) is semivariance for very large lag distance and same as covariance value at $|h| = 0$. Range (a) is the distance beyond which semivariance can be considered as statistically independent.

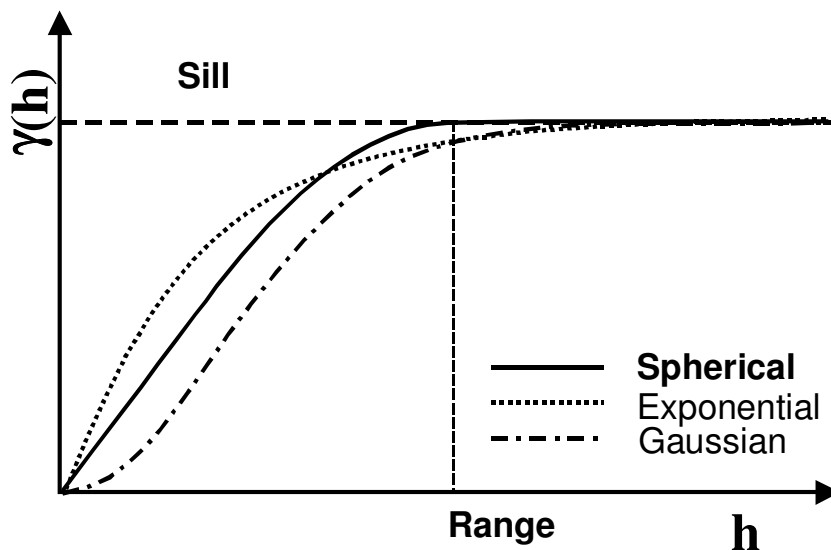


Figure 2.6. Bounded semivariogram models.

Spherical model (sill C , range a)

$$\gamma(h) = \begin{cases} C \left\{ 1.5 \frac{h}{a} - 0.5 \left(\frac{h}{a} \right)^3 \right\}, & \text{if } h < a \\ C & \text{otherwise} \end{cases}$$

Exponential model (distance parameter b , practical range a')

$$\begin{aligned} \gamma(h) &= C \left\{ 1 - \exp\left(-\frac{h}{b}\right) \right\} \\ &= C \left\{ 1 - \exp\left(-\frac{3h}{a'}\right) \right\} \end{aligned}$$

Gaussian model (distance parameter b , practical range a')

$$\begin{aligned} \gamma(h) &= C \left\{ 1 - \exp\left(-\frac{h^2}{b^2}\right) \right\} \\ &= C \left\{ 1 - \exp\left(-\frac{3h^2}{a'^2}\right) \right\} \end{aligned}$$

spatially structured, i.e., a large proportion of total sample variance, s^2 , is spatially dependent. Lower values of NMSD, on the other hand, indicate either high measurement errors or the existence of spatial structure at a scale smaller than the average distance in the first lag.

The experimental field used for this project was anisotropy which is identified by the phenomena of the major change in the range or sill as the direction changes. As the direction changes, only range might change while sill remains unchanged (geometric anisotropy), or sill might change while range remains unchanged (zonal anisotropy). In reality, both range and sill change simultaneously and the experimental field was that case as well. Anisotropy axes were identified by looking at variogram maps and maximum and minimum values of ranges of directional experimental semivariograms. In identification of directional experimental semivariogram, directional tolerance (or angular tolerance) of 30° was used, which meant pairs within 30° range of both side of direction (azimuth) were included for calculation. Therefore, isotropic semivariogram is calculated with directional tolerance of 90° (figure 2.7). In dealing with anisotropy in semivariogram modeling, rotation of coordinate system and rescaling were required to transform anisotropic semivariogram structure into a common (reference) model. Rotation of coordinate systems identifies principal axes (i.e., maximum range direction). In original coordinate system, anisotropic semivariogram structure appears as an ellipsoid with maximum and minimum ranges as long and short diameters and rescaling makes it unit sphere. The multiplication of rotation and rescaling matrices is called transformed matrix. The current data are purely 2D, therefore transformation matrix is way simple, so is the procedures in VARIOWIN. In VARIOWIN, geometric anisotropy was modeled

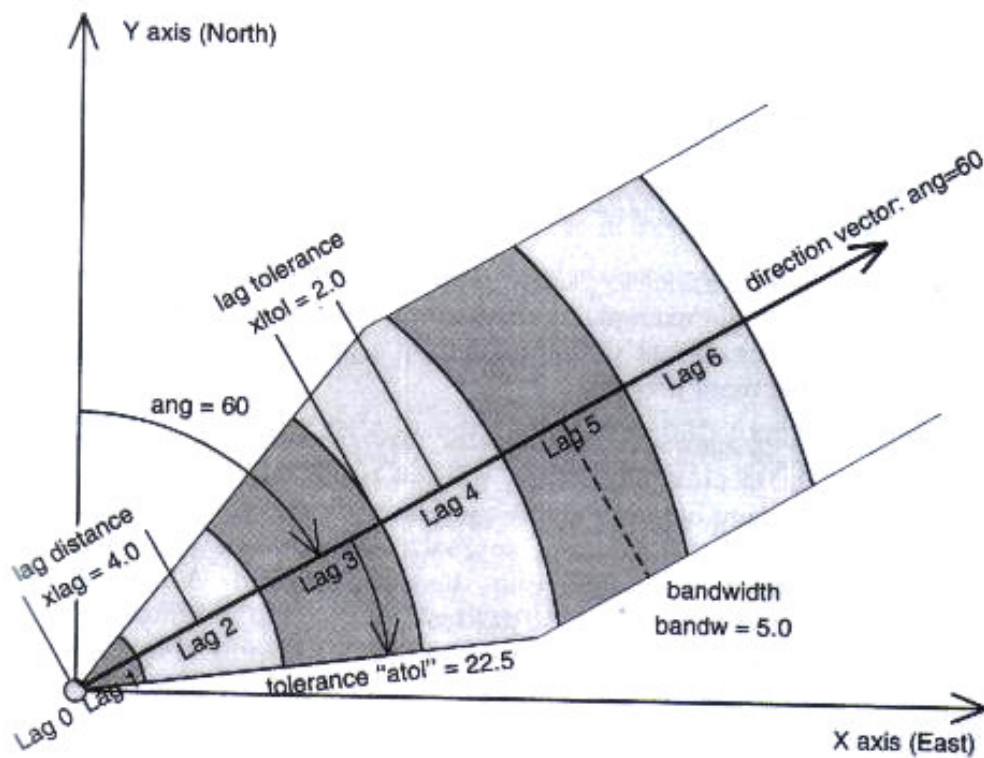


Figure 2.7. Diagram describing lag (distance) and angular (directional) tolerance. All samples located in shaded area are used for the calculation of semivariance ($\gamma(\mathbf{h})$). Redrawn from Pannatier (1996).

using ratio of maximum and minimum ranges (Anis.). Zonal anisotropy was modeled by adding 1000 in Anis. that very large range virtually nullified change in range.

$$R_{Dir.} = \begin{bmatrix} \cos(Dir.) & \sin(Dir.) \\ -\sin(Dir.) & \cos(Dir.) \end{bmatrix} : \text{rotation matrix}$$

$$S_{Anis.} = \begin{bmatrix} 1 & 0 \\ 0 & \frac{1}{Anis.} \end{bmatrix} : \text{rescaling matrix}$$

$$M = S_{Anis.} R_{Dir.} : \text{transformation matrix}$$

2.5.1.1. Analysis design

Semivariogram analysis was performed to understand spatial structures of bacterial and fungal communities for comparison to similar measures for the soil characteristics. There were two main purposes of the analysis. First, the overall spatial structure of the sampled field at different scales could be determined. It is necessary due to the efforts of balance between smooth representations with maximum resolution. Simply it is not easy or very intuitive to run semivariogram modeling analysis for entire field (7.07 m scale) with minimum possible lag distance (0.025 m). Second, spatial structures at different locations within the experimental field were described. This is important because the field had distinct separations in plant community composition. For the first purpose, the entire data set was used for an average portrait of the field and subsets of data structured at different distance scales were analyzed separately. Therefore, 4 different data sets were available: entire field scale (lag distance up to 7.07 m), medium scale (up to 2.5 m), small scale (up to 1 m) and fine scale (up to 0.25 m). For the second purpose, data from different regions, especially where plant development was distinct (left vs. right), were analyzed independently. This approach was used to understand the

effect of plants on the spatial structures of microbial communities. It was also used to reveal possible hidden dominant controllers of the microbial community structure other than plants in the experimental field. In addition to that, four corners with two different scales were also analyzed: fine scale (0.25 m × 0.25 m) and medium scale (2.5 m × 2.5 m). Data were subsampled with new coordinate systems as lower left corner of each grid being origin (0, 0).

2.5.1.2. Analysis procedure

Semivariogram analysis is initiated by determining the lag distance for all pairs of sampling points. Rather than deal with individual values for lag distances, they are usually distributed into groups (bins) of similar values. Binning, the determination of appropriate lag distance groupings, is a critical part of semivariogram analysis in that it decides the number of bins (lags) and resolution of the semivariogram model. The object of binning was, therefore, obtaining more bins with relatively constant pairs of samples in each of bin. Isaaks and Srivastava (1989) suggest that for gridded sampling designs, the grid spacing can form a good basis for bin-size determination. Because a grid was used for the present studies, the grid spacing was used to set the bin size. However, for the entire field scale, multiple experimental semivariograms with several lag distances in the range of largest and smallest grid size to determine best lag distance were desired using Vario2D module of VARIOWIN. In actual analysis, multiplication or division of grid spacing were tested for best representation from all levels of data and one with smaller number of bins possessing all the features was chosen. By looking at the h-scattergrams, some obvious outliers were eliminated for final semivariogram modeling. To minimize

misinterpretation, lags with less than 1% of total pairs were eliminated for semivariogram modeling.

As one can expect not all of the sampling points are located exact lag spacing without strict gridded sampling design. Sampling design for present study employed grid system, but different levels of grid systems in the field make all the sampling locations fall into each lag impossible. To solve this prevalent problem, tolerance in both distance and direction are incorporated in semivariogram analysis (Isaaks, and Srivastava, 1989). Distance tolerance (or lag tolerance) was determined to include statistically meaning number of pairs for calculation. Distance tolerance for the analysis was set following the suggestion of Isaaks and Srivastava (1989) to be half the lag distance (Figure 2.5) (Goovaerts, 1998). Due to distance tolerance, the actual lag distance varied because it was determined by the average of distance of all pairs within the tolerance range.

For semivariogram modeling in this study, the suggestion of Isaaks and Srivastava (1989) along with the procedures from the software manual for VARIOWIN 2.2 (Pannatier, 1996) were used. Modeling began with an omni-directional experimental semivariogram ($\hat{\gamma}(\mathbf{h})$). In VARIOWIN, nugget was first determined and then other component models (nested models in VARIOWIN) were chosen based on the best model fit and the principle of parsimony. A very complex combined model with 4 component models probably does not improve the accuracy of the solution above that which a simpler model meeting the fitting criteria would do.

Modeling with many components model takes more resources without providing significant benefits, and will often generate a model that is more complex than can be explained by the data. Another often forgotten rule in modeling is that the phenomenon

described by the model is important. A model which is contradictory to the processes and structure that actually exist would likely provide spurious results in estimation (kriging) as well as providing a false view of the system being modeled. One nice feature of VARIOWIN is the existence of a numeric parameter to determine goodness of fit: Indicative Goodness of Fit (IGF) (Pannatier, 1996; Goovaerts, 1998).

$$\text{IGF} = \frac{1}{N} \sum_{k=1}^N \sum_{i=0}^{n(k)} \frac{P(i)}{\sum_{j=0}^{n(k)} P(j)} \cdot \frac{D(k)}{d(i)} \cdot \left[\frac{\gamma(i) - \hat{\gamma}(i)}{\sigma^2} \right]^2, \quad (2.8)$$

where N is the number of directional variograms on the plot window,
 $n(k)$ is the number of lags relative to variogram k ,
 $D(k)$ is the maximum distance relative to variogram k ,
 $P(i)$ is the number of pairs for lag i of variogram k ,
 $d(i)$ is the mean pair distance for lag i of variogram k ,
 $\gamma(i)$ is the experimental measure of spatial continuity for lag i ,
 $\hat{\gamma}(i)$ is the modeled measure of spatial continuity for $d(i)$,
 σ^2 is the variance of the data for the variogram and the covariance.

The IGF feature is particularly useful in determining structural variance (C), which is usually more difficult to estimate reliably compared to the other parameters like range (a) and nugget (C_0) (Isaaks, and Srivastava, 1989). The IGF measures sum of squares of the difference between experimental semivariances ($\hat{\gamma}(i)$) and model semivariances ($\gamma(i)$), and replaces traditional visual fits and enables testing of several possible models to find the best fit quantitatively.

VARIOWIN was the main software used for semivariogram analysis; soil characteristics measurements and total microbial abundance. For microbial community structure, .var files (experimental semivariograms) were prepared from experimental semivariogram analysis based on dissimilarity and then modeling was performed Model module of VARIOWIN. Simple program was prepared using Matlab 6.5 for calculating

experimental semivariogram between distance matrix and dissimilarity matrix by a friend/colleague (Joon Jin Song, Dept. Mathematics and Statistics, University of Massachusetts at Amherst). For regional analyses, separate matrices of dissimilarity based on Jaccard coefficient were prepared.

2.5.2. Cross-semivariogram

Semivariograms between two variables are called cross semivariogram and used for the measurement of joint variability. There are two usages in cross-semivariogram: identification of nugget effect and joint variability measurement mainly for cokriging. Using standardized variables by unit variance, the sill of cross-semivariogram directly indicate the magnitude of correlation between variables (Goovaerts, 1997). Cross-semivariogram models are also used to identify nugget effects of semivariogram models between experimental error ($\varepsilon_i(\mathbf{h})$) and microscale variation ($\eta_i(\mathbf{h})$) (Goovaerts, and Chiang, 1993).

The semivariogram ($\gamma_{ii}(\mathbf{h})$) of variable ($Z_i(\mathbf{h})$) is expressed as

$$\gamma_{ii}(\mathbf{h}) = \gamma_{w_i}(\mathbf{h}) + \gamma_{\eta_i}(\mathbf{h}) + \text{Var}[\varepsilon_i(\mathbf{h})], \quad (2.9)$$

where $W_i(\mathbf{h})$ is small scale variation. From this equation, nugget effect is defined as a sum of semivariations of microscale variation and experimental error ($\gamma_{\eta_i}(\mathbf{h}) + \text{Var}[\varepsilon_i(\mathbf{h})]$). The equation could be extended to cross-semivariogram

$$\gamma_{ij}(\mathbf{h}) = \gamma_{w_i w_j}(\mathbf{h}) + \gamma_{\eta_i \eta_j}(\mathbf{h}) + \text{Cov}[\varepsilon_i(\mathbf{h}), \varepsilon_j(\mathbf{h})]. \quad (2.10)$$

It is not too unrealistic to assume that two experimental errors are independent to each other that nugget effect of cross-semivariogram model is cross-semivariance between two microscale variations $\eta_i(\mathbf{h})$ and $\eta_j(\mathbf{h})$. Therefore, if larger nugget effects from

semivariogram models were not found from cross-semivariogram model, nuggets in semivariogram models were generated from experimental error. On the other hand, a larger nugget effect from cross-semivariogram model can indicate major portion of semivariogram model nugget is from the variations less than the measurement unit of choice.

2.5.3. Kriging

Kriging is “a collection of generalized linear regression techniques for minimizing an estimation variance defined from a priori model for a covariance” (Olea, 1991). As the definition indicates, kriging is better suited for working with covariance models, but models from semivariogram analysis have been used instead (Deutsch, and Journel, 1998). Among other interpolation techniques, kriging has a definite advantage in that it accounts for the spatial structure by incorporating the results from semivariogram analysis (Goovaerts, 1998). Ordinary kriging was used here for mapping of all observations including microbial community structure due to the fact that ordinary kriging does not require *a priori* knowledge of a stationary global mean. Ordinary kriging estimates values at unsampled locations by linear interpolation of measured values around with weight λ_i :

$$Z_{OK}^*(\mathbf{u}) = \sum_{i=1}^n \lambda_i \cdot Z(\mathbf{u}_i), \quad (2.11)$$

where \mathbf{u} refers to location, $Z_{OK}^*(\mathbf{u})$ is an estimation at location \mathbf{u} , and there are n data values $Z(\mathbf{u}_i)$, $i = 1, \dots, n$. The important issue here is the choice of weights assigned to each observation. Weights are determined to minimize estimation error by the rule of

unbiased ordinary kriging estimator. In solving the ordinary kriging system, semivariances at different lag distance are required.

Values for ordinary continuous variables (*viz.*, soil characteristics and total microbial abundance) were fed along with parameters obtained from semivariogram modeling into GSLIB (Deutsch, and Journel, 1998) using “kt3d” module for ordinary kriging analysis. The actual analysis was performed through Geostatistics Package (Cyze & Associates, Ltd.) which provides user friendly interface for kriging (kt3d) and stochastic simulation (“sgsim”) analysis provided by GSLIB. Actual computation of kriging involves three steps: 1. creating a grid over the field, 2. performing estimation at all grid nodes, 3. interpolating within grid nodes (Welhan, 2004). The grid size and number were determined from the sampling design that 500 grids with 1 cm grid size were used. During preliminary running to optimize search strategy, however, 100 grids with 5 cm grid size were used to save CPU time. In step 2, several considerations including area of searching data and range of number of data. In determining searching area, both direction (in case of anisotropic semivariogram model) and radii were tested simultaneously. Search radii were began with the range of semivariogram model and in many cases set to be much smaller. However, there is no reason to have search radii longer than the range of semivariogram model, because samples beyond the range are not spatially structured. The maximum number of data was set to 10, and minimum number was tested several times along with search radii within the range of 3 – 5. When there were many points did not satisfy the searching strategy in both number and radii, the kriging maps were not generated for those variables. In choosing map, two methods were

used: visual comparisons with location maps, which were produced by “locmap” module of GSLIB and sum of squared error residuals from cross validation (SSR),

$$SSR = \sum_{i=1}^n \left[\hat{Z}(\mathbf{u}_i) - Z(\mathbf{u}_i) \right]^2. \quad (2.12)$$

However, binary data from the microbial communities were not readily suitable for the analysis. Therefore, numeric values of the first three principal components from principal component analysis (PCA) were used for ordinary kriging analysis.

Semivariogram analyses using VARIOWIN with PCs were performed as would be done for continuous variable data (Franklin, and Mills, 2003). Then an estimation map was constructed by ordinary kriging analysis and stochastic simulation. While doing that, the results of semivariogram analyses with similarity coefficients and PCs were compared to see if there were significant discrepancies between them.

2.5.4. Stochastic simulation

Stochastic simulation is simply defined as Monte Carlo realization of the random variable. Kriging analysis produces the best unbiased estimation of the field, but due to its inherent properties, kriging loses some detail in that it underestimates larger observations and overestimates smaller observations. Another drawback is the smoothing effect is not uniform that smoothing is minimal near the data locations and increases as the estimation points are farther away from the data locations. The estimation map from kriging analysis is best in statistical sense that the estimation minimizes estimation error variance. So kriging analysis produces statistically best point estimation but not necessary best global estimation. Stochastic simulation generates alternative, equally probable joint maps (realizations) of the component random variables from a semivariogram model. The

aim of stochastic simulation is an estimation of the uncertainty of the kriging analysis by producing multiple realizations and production of single improved map. If the purpose is to generate best map using collected samples, therefore, stochastic simulation probably is better choice. The sampling design of present study is not the best one for kriging estimation, because there is large area in the middle of the field with scarce sampling points (Figure 2.3). For those of measured variables with shorter range, kriging estimation of the area where insufficient number of observations is available tends to be close to the mean of the values (Isobel Clark, Personal Communication). As mentioned in the previous section, search radii are not usually beyond the range and shorter range, therefore, indicates smaller area of search for interpolation. When there is not enough number of samples to use in interpolation, kriging estimation is easily biased to the arithmetic mean of available samples. In “kt3d” module, minimum number of samples was set and it produces -999.000 for both estimate and estimation variance. In that case, stochastic simulation is the choice of estimation. Among several available algorithms, sequential Gaussian simulation (“sgsim” module) was used.

Sequential simulation estimate the value of a variable from all the available ($Z(\mathbf{u})$) observations, including original measurement and all previously simulations, of a location (\mathbf{u}). Thus, simulation analysis is sort of the last one to go following semivariogram modeling and kriging estimation. As the name implies, normality and independence of random variables are required for Gaussian simulation. Other than those assumptions, Gaussian simulation is relatively easy and convenient in both theoretical and practical aspects (Welhan, 2004). The actual procedure of sequential Gaussian simulation is rather similar to that of ordinary kriging analysis that begins with grid

identification and follows with estimation at each grid node. Choice of node, however, is random as initial node is determined by random seed and move to next random node by random number generator after estimation is completed. Global random walk never revisit same grid node, though. In case of multiple realizations, seed and global random walk are randomly chosen. Local estimation at each node is conditional estimation that mean and variance are estimated by using kriging analysis with semivariogram model. Estimated mean and variance define cumulative distribution function (cdf) of the variable in the neighbor, and that cdf is conditional to the spatial structure and all the available observations within the search area. Simulated value is drawn from the conditional cdf (ccdf).

2.5.5. Modeling of multivariate categorical data into a single continuous variable

The information of soil microbial community structures was obtained as a multivariate binary format. Although current approach employed here is conceptually acceptable that both semivariance and mean relative dissimilarity measure difference between pairs of samples. The lack of standardized computational approach made each one of researcher who tries to do similar types of work code one for his / her own purpose. Even if it is assumed that all of them understand the algorithms and applied properly, there might still be misunderstanding and / or bugs, because only a couple of colleagues would take look at. Another limitation is that calculation of directional semivariograms was not as easy as it was desired to identify possible existence of anisotropy. There is no way close to produce variogram map for initial investigation of anisotropic axes.

One approach considered was indicator semivariogram which was designed for binary data format. However, in steps of estimation and simulation, the output was

proportion of '0's and '1's. It is not too difficult to imagine that is not the perfect approach for microbial community information, because with same numbers of '1', which means same number of existence of ribotypes of microorganisms, different location of those '1's (different ribotypes) result in different microbial community structure. Another approach was transformation of multivariate binary data into continuous variable by using PCA. It is easy and reliable and used for estimation / simulation purpose in present study, but also has limitation especially for complex soil microbial community data. Each microbial community structure was determined by 59 (bacterial) and 56 (fungal) ribotypes of soil microorganisms. That means PCA produces 58 (bacterial) and 55 (fungal) new continuous variables (PCs) and explainable variance of first three PCs were nowhere close to even half of the total variance: 14.2% for bacterial and 15.8% for fungal community. Since PCs are independent to each other, by definition of PCA, use of several variables for better integrated estimation (cokriging) was not possible either.

It was desired to deal with all those limitation by modeling multivariate binary data into single continuous variable. First of all, simply it is beyond researcher's current capability of mathematics that there have not been such attempts available from the literature research between the researcher and his colleague. Therefore, modeling attempts was not successful in the timeframe of dissertation project; however, it will be pursued in the near future.

3. RESULTS

3.1. SUMMARY OF RESULTS

Appropriate sample size for the best representation of the microbial community of the field for each of the techniques to be used was determined. Within the range of acceptable sample sizes (Table 3.1), one was chosen accordingly: AO (0.1 g), DNA extraction and microbial community structure (0.25 g) (section 3.2.). All measured soil characteristics, with the exception of pH, had similar general vertical profiles in that values were maximum at the surface and decreased downward. The vertical profile of total microbial abundance was similar to those of most soil characteristics. Correlation analysis confirmed the graphical representation. Based on the data for soil characteristics, three groups (0-6 cm, 6-10 cm and 10-25 cm) in trench B and two groups (0-6 cm and 6-25 cm) in trench D were identified. The groupings were well matched with the vertical profiles of both total microbial abundance and microbial community structure (section 3.3.).

The microbial abundance of the field with developing vegetation was relatively constant in both time and space. Discriminant analysis and the Mantel test were used to visualize the quantitative relationships in community similarity revealed a strong temporal trend over the entire two years. Spatial differentiation was clear in the block design of microbial community structure, and distinction was observed between control and treated plots as well. However, the vegetation development in the original field design with different diversity of plant species was still less than successful in first two years, and it was verified by microbial genetic fingerprinting data (section 3.4.).

Spatial structure in soil microbial communities and measured soil variables were identified. Semivariogram models were determined for different scales within the entire

field and different regions of the field were examined at different scales. Information from semivariogram modeling was used for estimation by kriging and stochastic simulation analysis. Moisture content, % clay, pH and total microbial abundance showed anisotropic distribution, and principal (maximum range) and minor (minimum range) axes were identified. Semivariogram models for carbon and nitrogen contents were very similar to each other in each scale and region models. Due to lack of densely sampled area in the middle of the field, kriged maps had some artificial structures, particularly in those sparsely sampled areas of the field. Simulation maps, which were supposed to do better in situations like this with sparse sampling, were used mainly for estimation mapping purposes. Roughly speaking, area near the center of the field at A corner direction had high values in all the soil characteristics, except pH. With variations like proportions of higher, mid-range and lower values and directionality of search sphere / ellipse, carbon, nitrogen, moisture and % clay measurement were relatively well matched to each other in their pattern. Kriged maps with first three PCs for bacterial and fungal communities had similar problem with kriged maps of soil characteristics. Since all PCs were modeled as isotropic semivariogram models, there were apparent vertical lines of search remnants. Except bacteria PC2 and PC3, overall patterns were distinctive in comparison among same domain.

Both bacterial and fungal community structures were well differentiated in level II sampling design (5 regions) by DFA. In both domains, all are relatively well separated in the first function axis but all but C regions were closely clustered in the second function axis. The patterns of microbial community structures in DFA field were distinctive in level III sampling design (4 corners). In bacterial community structure, B

and C corners (right section of the field) were clustered together. In fungal community structure, on the other hand, A and B corners (upper section of the field) were clustered together. There were total of 13 species of plants in the experimental field: 7 species in the X1 plot section and 12 species in the X4 plot section. Overall matching of group membership between two different plot sections (vegetation combination) on bacterial community structure (78.1%), fungal community structure (79.6%) and soil characteristics (66.3%) indicated definite effect of vegetation types on microbial community and soil characteristics. Pairwise simple Mantel and partial Mantel tests indicated that all possible relationships among microbial community structures, soil characteristics and distance were very significant. The causal models based on the results of Mantel tests were somewhat different between bacterial and fungal community. In bacterial community, distance influences on both soil characteristics and bacterial community structure, and bacterial community structure is also controlled by soil characteristics. In fungal community, distance also influences on two other components, but soil characteristics are controlled by fungal community structure.

3.2. EXPERIMENT 1: EFFECT OF SAMPLE SIZE ON THE OBTAINED SOIL MICROBIAL COMMUNITY ECOLOGY

3.2.1. Growth patterns on R2A medium

The viable portion of soil microbial community was obtained by plating on 1/10 diluted R2A medium and incubated for 160 hours (Figure 3.1.A). Means of viable abundance for the different size samples were significantly different by one-way ANOVA ($p = 0.0039$), and the pairwise comparison using Ryan's Q test separated 0.25 g from rest of sample sizes ($\alpha = 0.05$). There was no significant difference when the 0.25 g

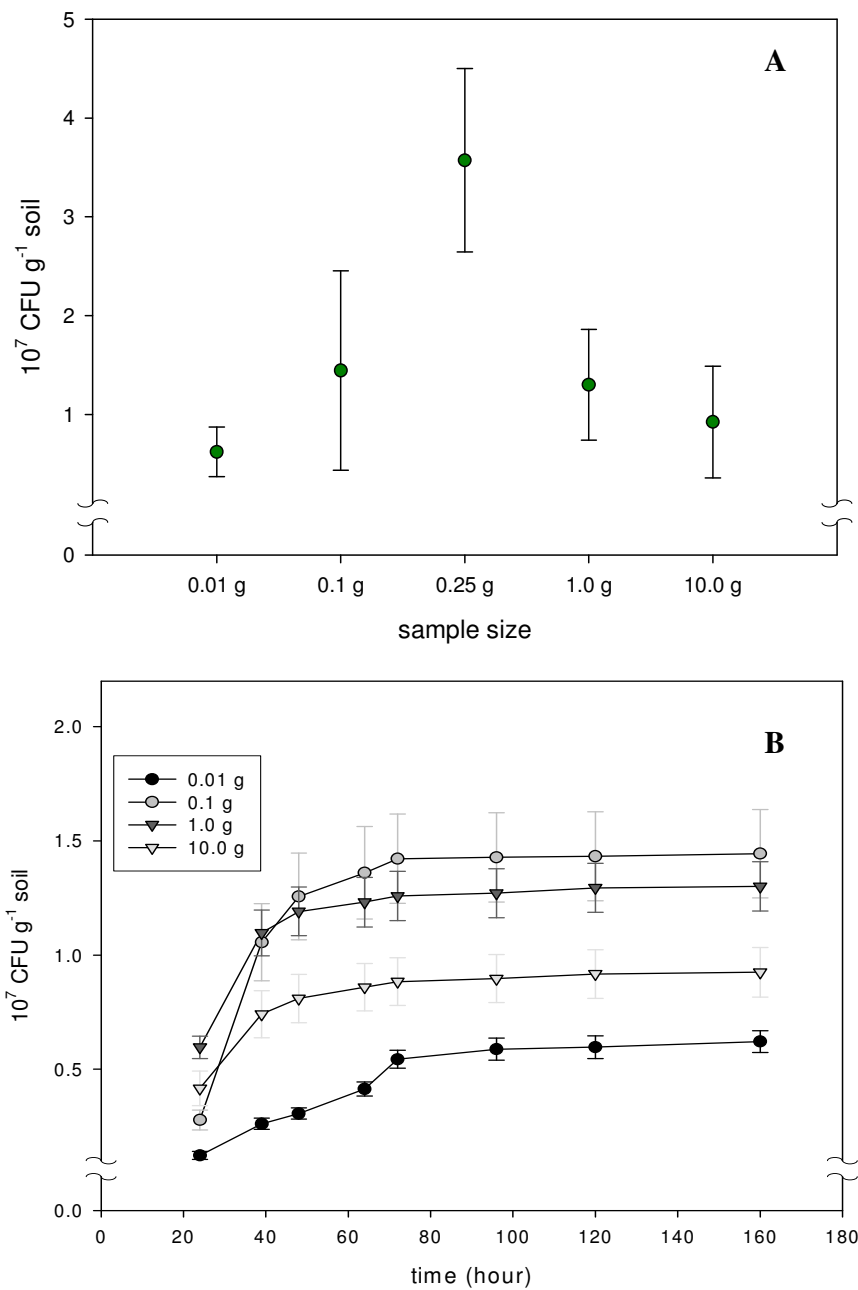


Figure 3.1. Viable abundance (A) and growth curves (B) of different sample size measured on R2A media. Error bars are 1 standard error of the mean. Growth curve of 0.25 g is omitted because it is decided to be outlier.

sample size was excluded ($p = 0.4523$). This trend was similar for sample variances among replicates in that variances were not significantly different among different sample sizes when data from the 0.25 g sample size were excluded ($F_{\max} = 16.23$; $a = 4$ and $v = 2$). Variance among replicates for each sample size was positively, but not significantly correlated with the mean values for viable abundance ($r = 0.81618$, $p = 0.1838$).

The apparent outlier (0.25 g sample size) was excluded from the data set for the growth curve analysis. For all sample sizes, growth curves representing appearance of colonies on R2A followed typical microbial population growth kinetic models with density dependence (logistic model). However, maximum growth rates (μ_{\max}) and universal growth rates (μ) were roughly proportional to the sample sizes. The first measurements were made after 24 hours of incubation when the first colonies became visible. Visual examination of the growth curves suggested differences among sample sizes, and that 0.01-g sample was lowest in its increase in abundance (Figure 3.1.B).

3.2.2. Total abundance measured by AO staining

Total microbial abundance was measured in the various sized samples by epifluorescence microscopy with AO staining (Figure 3.2). The result of one-way ANOVA on all sample size indicate abundance was dependent upon sample sizes ($p = 0.0001$). Pairwise comparison of the mean abundance using Ryan's Q test separated the 10.0-g sample from all of the smaller sample sizes ($\alpha = 0.05$). However, one-way ANOVA still rejected null hypothesis ($p = 0.0249$), even when the 10.0-g data were excluded. Data for the 0.01-g samples were next excluded for the analysis, and when

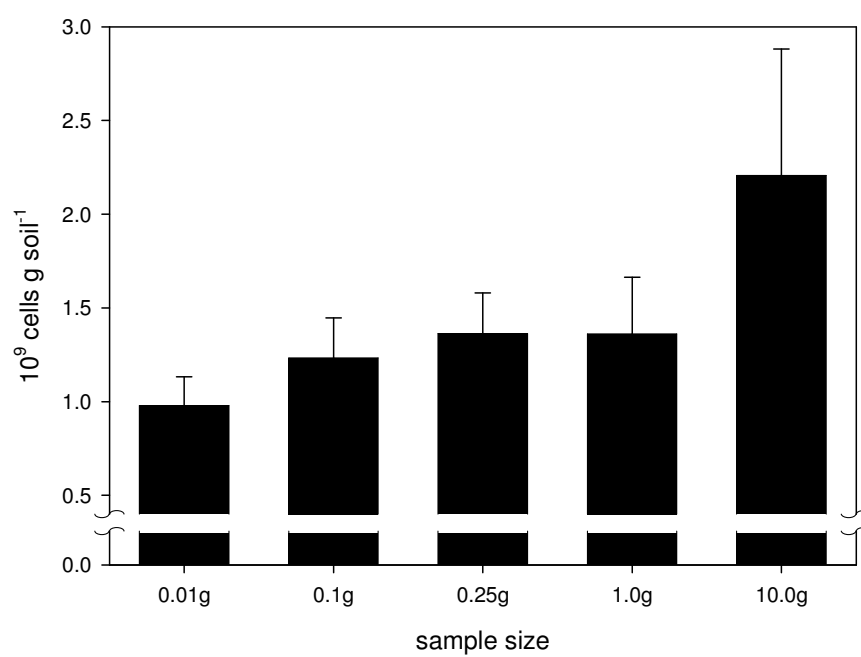


Figure 3.2. Total microbial abundance in different sample sizes of soil. Error bars represent 1 standard deviation.

sample sizes of 0.1 g and 1.0 g were compared, the null hypothesis was accepted ($p = 0.5925$). Hartley's F_{\max} test on all sample sizes rejected null hypothesis ($F_{\max} = 19.13$; $a = 5$ and $v = 5$) of equal variance. F_{\max} test on the samples excluding the 10.0-g sample data accepted the null hypothesis that variances of sample size between 0.01 g and 1.0 g are homogeneous ($F_{\max} = 1.98$).

3.2.3. DNA extraction yield

The concentrations of extracted DNA were compared among different sizes of samples (Figure 3.3). The results of one-way ANOVA on mean yield ($P = 0.0004$) and variance among replicates of all tested sample sizes determined heterogeneity of DNA extraction yield ($F_{\max} = 317.86$; $a = 5$ and $v = 2$). Pairwise comparison of the mean abundance using Ryan's Q test separated the 10.0-g and 0.01-g samples from rest of sample sizes ($\alpha = 0.05$), and data of 0.01-g and 10.0-g samples were chosen for exclusion from the analysis. For sample sizes of 0.1 g, 0.25 g and 1.0 g, means were equal ($p = 0.1322$) and variances were homogeneous ($F_{\max} = 14.03$).

3.2.4. Microbial community structure

Both bacterial and fungal community structure analysis indicated major differences in the communities recovered from different sample sizes (Figure 3.4). Centroids of all sample sizes were significantly different in both DFs ($p < 0.0001$ for DF1 and $p = 0.0001$ for DF2) (Figure 3.4.A). The larger sample sizes (0.25 ~ 10.0 g) yielded similar bacterial community structure and it is roughly confirmed by post-hoc ANOVA using Ryan's Q test on the DF1 axis (0.1 ~ 1.0 g samples were grouped on the DF2 axis). Variances among replicates were not significantly different from each other in both axes

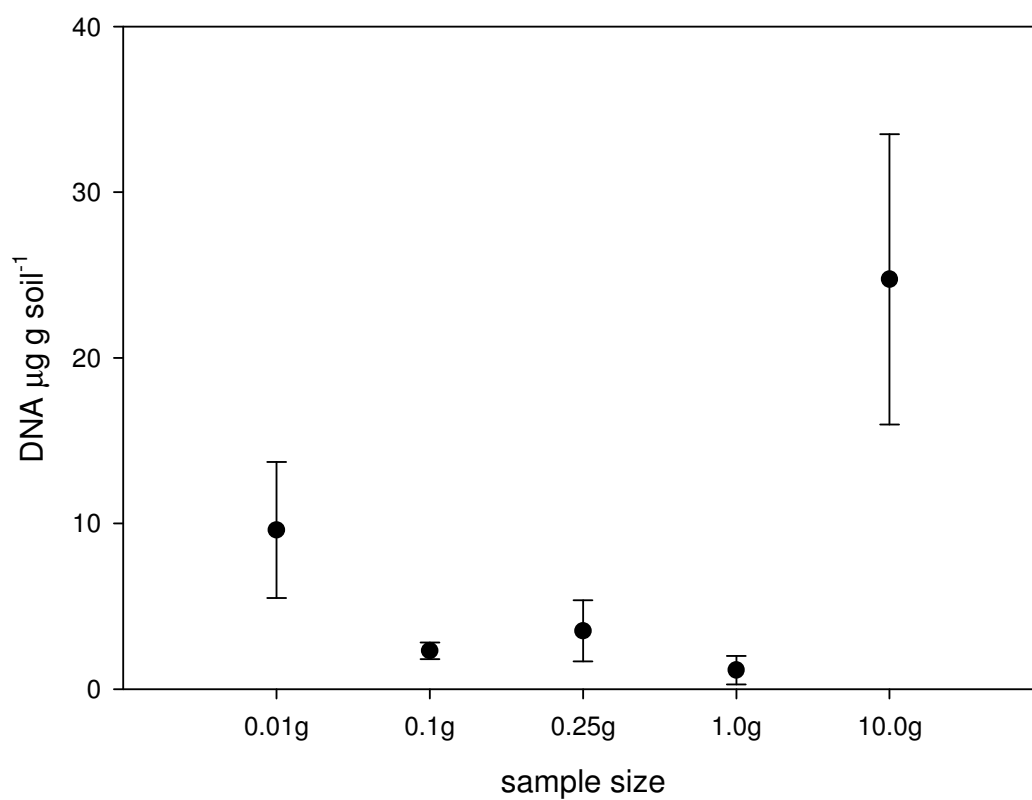


Figure 3.3. DNA extraction yield from different sample sizes. Error bar represents 1 standard deviation.

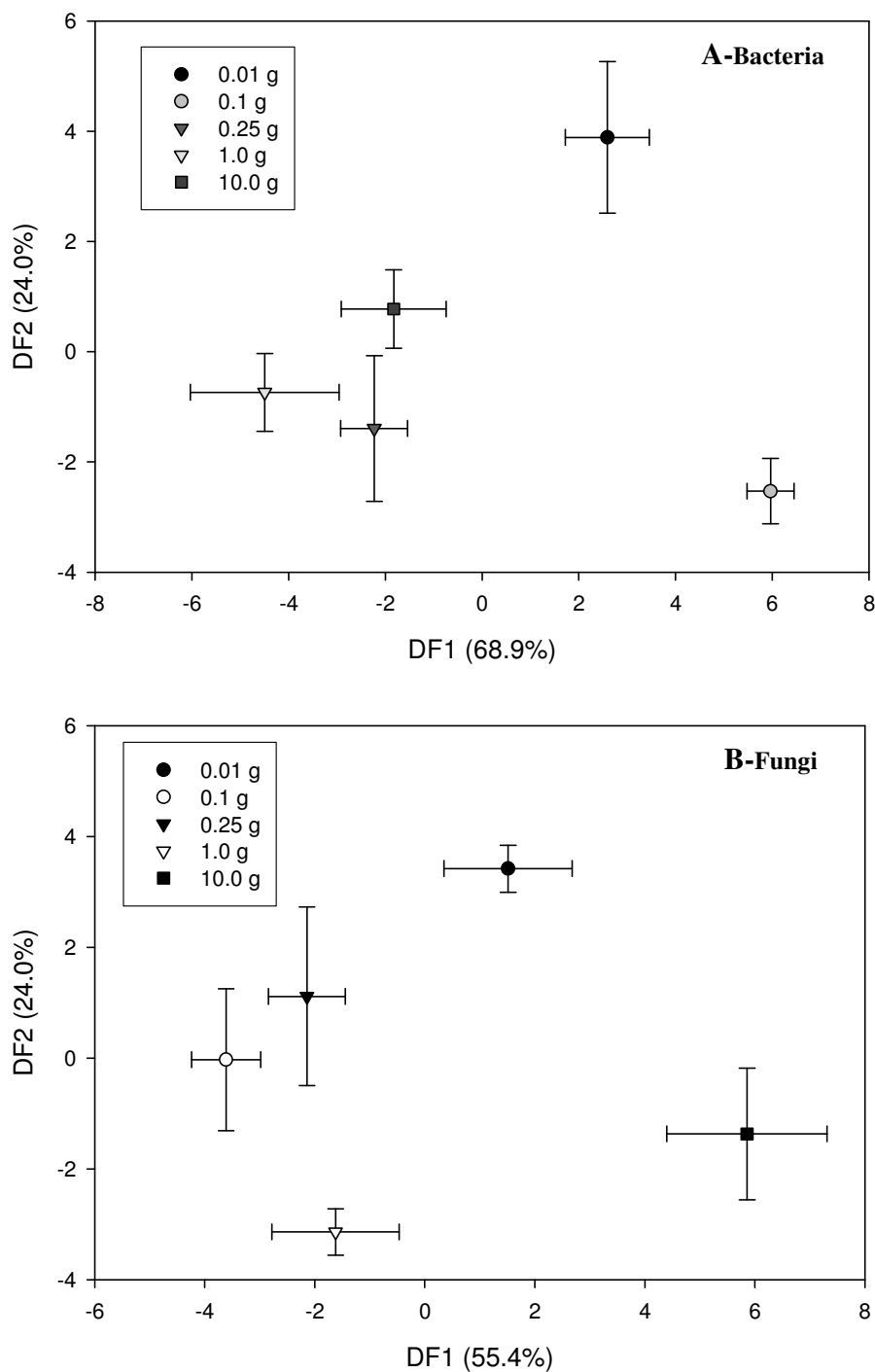


Figure 3.4. Discriminant function plot of bacterial and fungal community structure using first two functions. Major portions of the variances were explained by the first two functions for both the bacterial and fungal community (92.9 % and 79.4% respectively). Points indicate centroids which are means of triplicates and error bars represent 1 standard deviation.

($F_{\max} = 9.986, 5.361$ and 4.859 for DF1, DF2 and sum of two respectively; $a = 5$ and $v = 2$). Overall matching of original and calculated memberships was 86.7% (2 mismatches out of 15). In fungal community structure, all sample sizes were significantly different in both DFs by comparing using the group centroids ($p < 0.0001$ for DF1 and $p = 0.0001$ for DF2) (Figure 3.4.B). Sample sizes of 0.1 g and 0.25 g were well clustered, and the 1.0 -g sample size might be included in the group as well (0.1 g, 0.25 g and 10.0 g were clustered on DF2 axis). That observation is supported by post-hoc ANOVA as well. Overall matching of membership was 93.3% (one mismatch out of 15). The overall patterns of separation of samples for each community type (bacteria vs fungi) were quite similar even though the identity of the community within the individual centroids was different, and the scales at which the centroids were most similar to one another differ between the bacterial and fungal communities.

Acceptable ranges of sample sizes were determined for analytical techniques used for the present study. Except viable count, which was not used in the rest of study, sample sizes were chosen within those acceptable ranges: 0.1 g for total microbial abundance and 0.25 g for microbial community structure (Table 3.1).

3.3. EXPERIMENT 2: VERTICAL PROFILE OF SOIL MICROBIAL COMMUNITY STRUCTURE

Plantago lanciolata and *Rudbeckia herta* were identified at the B trench and the root zone reached almost 13 cm deep. At D trench, *A. gerardii* was the only plant found and roots reached about 18 cm deep.

Table 3.1. Summary for sample size study.

	<i>means</i>	<i>variances</i>	<i>Sample size chosen</i>
AO	0.1 – 1.0 g	0.1 – 1.0 g	0.1 g
R2A	0.01 – 0.1 g, 1.0 – 10.0 g	0.01 – 10.0 g	
DNA yield	0.1 – 1.0 g	0.1 – 1.0 g	0.25 g
	<i>centroids</i>		
Bacterial comm.	0.25 – 10.0 g	all	0.25 g
Fungal comm.	0.1 – 1.0 g	all	0.25 g

3.3.1. Vertical profiles of soil characteristics

Several soil chemical and physical properties were measured to characterize the vertical profile of soil (Figure 3.5, 3.6, and 3.7). The overall shapes of decreasing to reach stable point were very similar among different soil characteristics, except pH whose shape was rather opposite from the rest. Volumetric moisture content of the soil were high in the surface (25.5% in B and 26.1% in D, average from 0-4 cm), and continuously decreased to about 10% less (14.7 % in B and 14.9% in D, average from 20-25 cm) from the surface (Figure 3.5.A). Both trenches were relatively consistent in general changing pattern, but B trench reached minimal moisture content earlier (10-15 cm range) than D trench. pH increased down to 15 cm then either held constant or decreased slightly thereafter (Figure 3.5.B). In general, B trench was more acidic than D trench through the entire 25-cm depth profile, and the difference was largest at the surface (0.38 pH units) and smallest at the bottom of the trenches (0.17 pH units).

The general vertical profile of organic matter was very similar to that of volumetric moisture content in both trenches (Figure 3.6.A). As expected, organic matter content was highest at the surface (9.2% in B and 8.4% in D, average from 0-4 cm) and gradually decreased to reach about 3% in both trenches at 15-25 cm. As in the moisture content profile, the minimum organic matter content was found at around 15 cm from the surface in both trenches; that matched the fact that most of roots were observed in the upper 15 cm or so in both trenches. Because of a limited amount of soil sample, data for % clay from the particle size analysis were not available for the D trench. The vertical profile of % clay in B trench was also following the general profile trend (Figure 3.6.B).

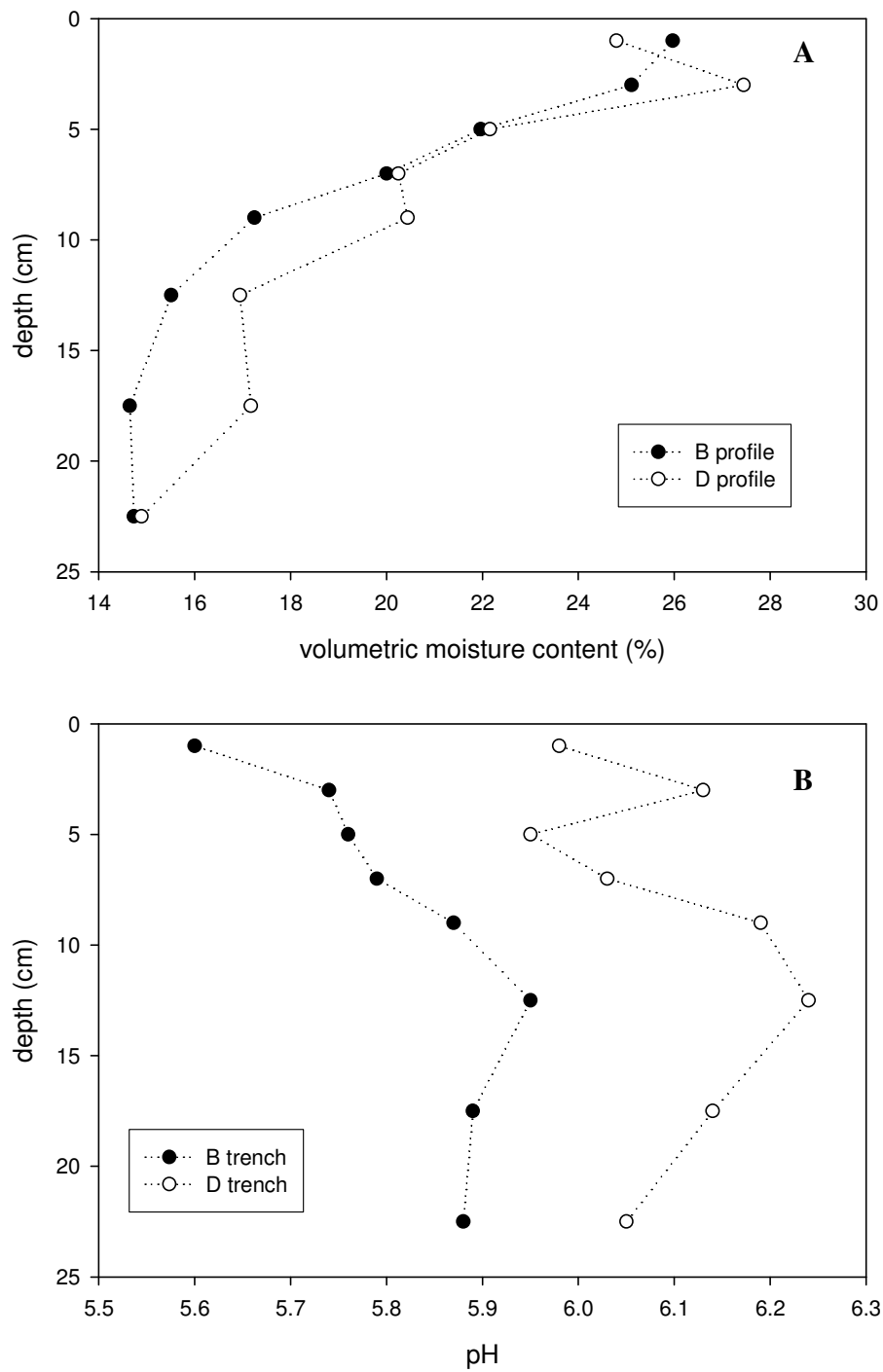


Figure 3.5. Vertical profiles of moisture content (A) and pH (B) of B and D trenches. Depth represents the distance from the surface soil.

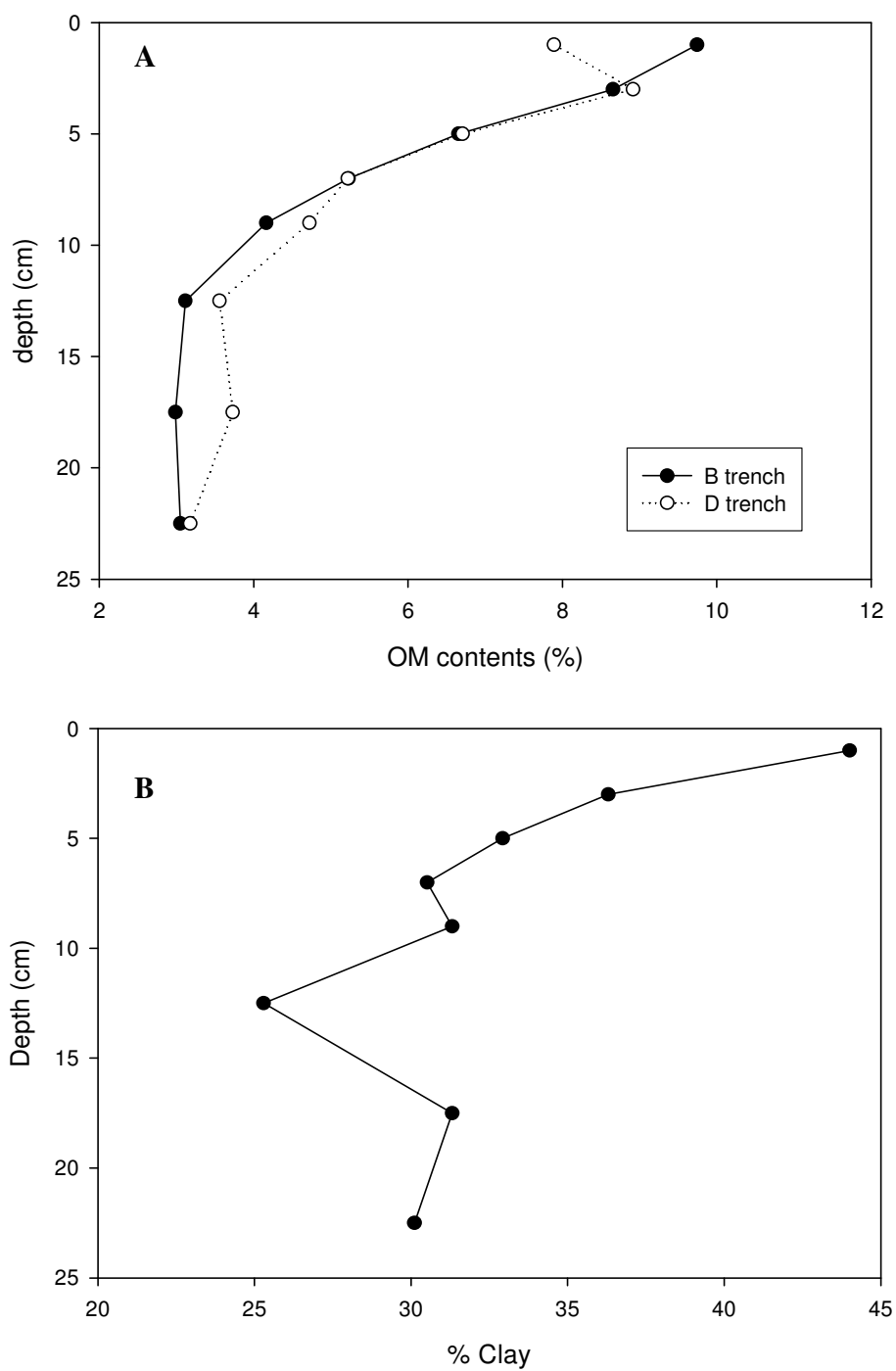
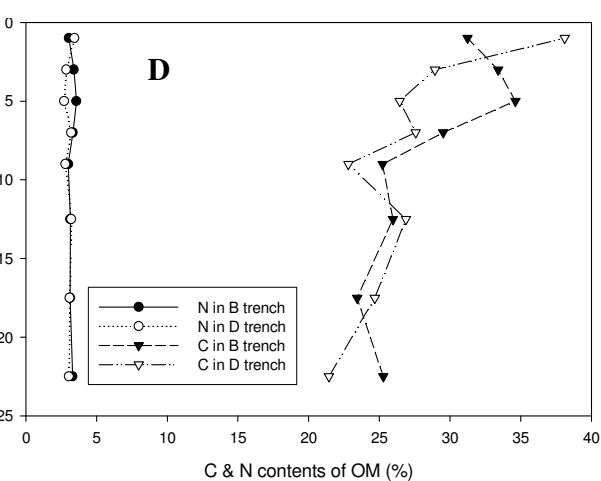
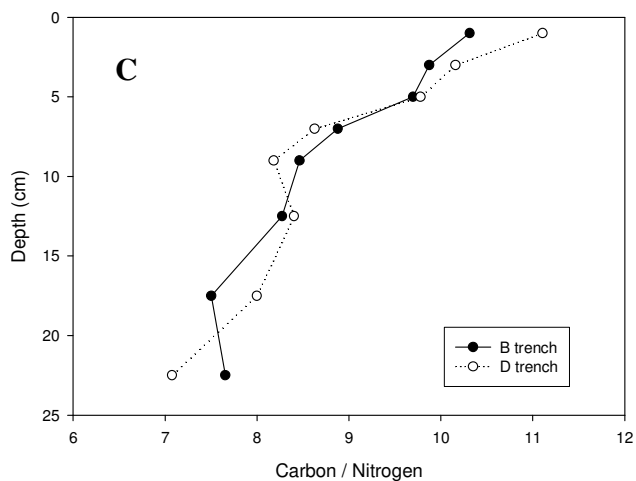
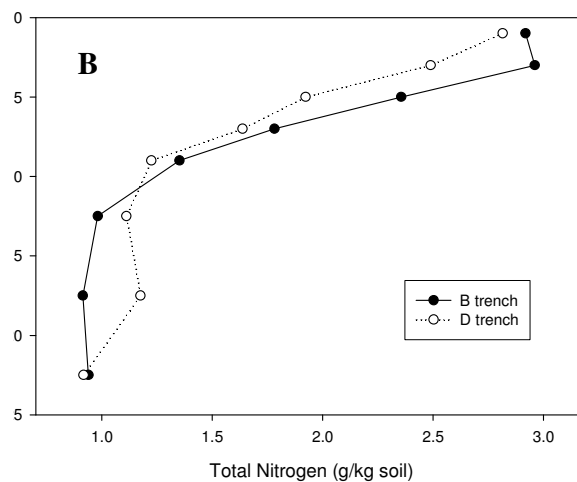
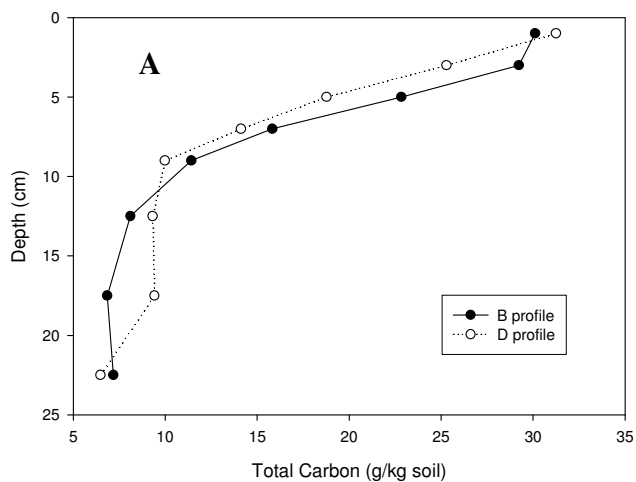


Figure 3.6. Vertical profiles of organic matter content (A) for both trenches and % clay (B) for B trench. Depth represents the distance from the surface soil.

It was highest at the surface (44.0%) and stabilized at around 30% at the bottom of the trench. The only noticeable difference was the significantly smaller % clay at 10-15 cm interval (25.3%).

The vertical profiles of total carbon and nitrogen contents were almost identical (Figure 3.7.A and 3.7.B) and were among those general shapes. Total carbon contents were highest at the surface (30.11 g / kg soil in B and 31.25 g / kg soil in D) and lowest at the bottom (7.19 g / kg soil in B and 6.48 g / kg in D). Total nitrogen contents were also highest at the surface (2.90 g / kg soil in B and 2.8 g / kg soil in D) and lowest at the bottom (0.94 g / kg soil in B and 0.92 g / kg soil in D). For both total carbon and nitrogen contents, minima were identified at the 10-15 cm range. From the vertical profiles of total carbon and nitrogen content, the vertical profile of C/N ratio was expected to be nearly constant. However, the actual vertical profile of C/N ratio continually decreased from the surface to the bottom of the trenches (Figure 3.7.C). However, the magnitude of the changes was very small (11 to 7), and it is doubtful that the change is meaningful. Vertical profiles of organic matter, total carbon and nitrogen content were very similar, but relative content of total carbon and nitrogen content form organic matter content indicates relative change of carbon content is more drastic than that of nitrogen content (Figure 3.7.D).

The vertical profiles of soil characteristics were grouped based on Euclidean distance measurement using the UPGMA fusion method (SPSS v. 11). The groupings were similar between trenches in that the 6-cm depth was a turning point for both trenches. The B trench had three groups (0-6 cm, 6-10 cm and 10-25 cm) while the D trench had two groups (0-6 cm and 6-25 cm). Cluster analysis (CA) was used before



discriminate function analysis (DFA) to determine groups (McGarigal *et al.*, 2000).

Those clusters were used in DFA as group membership and the results of DFA confirmed the grouping very well: 100% matching for both trenches. However, another candidate grouping (within and below the root zone) was also tested by DFA and the results were also 100% matching of group membership.

3.3.2. Vertical profiles of soil microbial communities

Since all measured soil characteristics were chosen based on the presumed close relationships with soil microorganisms, it was expected that the vertical profiles of soil microbial community would be similar to those of the soil characteristics. Although the data were not congruent, the overall vertical profile of microbial abundance (Figure 3.8) was following general soil characteristics pattern. There was overall significant difference in the vertical profile of B trench ($P = 0.0014$) and the total abundance data were grouped roughly 0-10cm and 10-25cm by pairwise comparison using Ryan's Q test. Total abundance of D trench was also significantly different in overall profile ($P = 0.0001$) and two groups (0-6 cm and 6-25 cm) were identified by pairwise comparison. ANOVA indicated no difference between two trenches ($P = 0.5497$).

An attempts to obtain arbitrary groups in microbial community structure data using cluster analysis as performed with the data of soil characteristics failed due to the existence of many negative bands. Based on the assumption of the close relationships between soil microorganisms and soil characteristics; therefore, DFA was performed with the group membership determined from the soil characteristics data (0-6 cm, 6-10 cm and 10-25 cm in B trench, Figure 3.9, and 0-6cm and 6-25 cm in D trench). For bacterial community structure, the original memberships were matched 87.5% (7 out of 8) for both

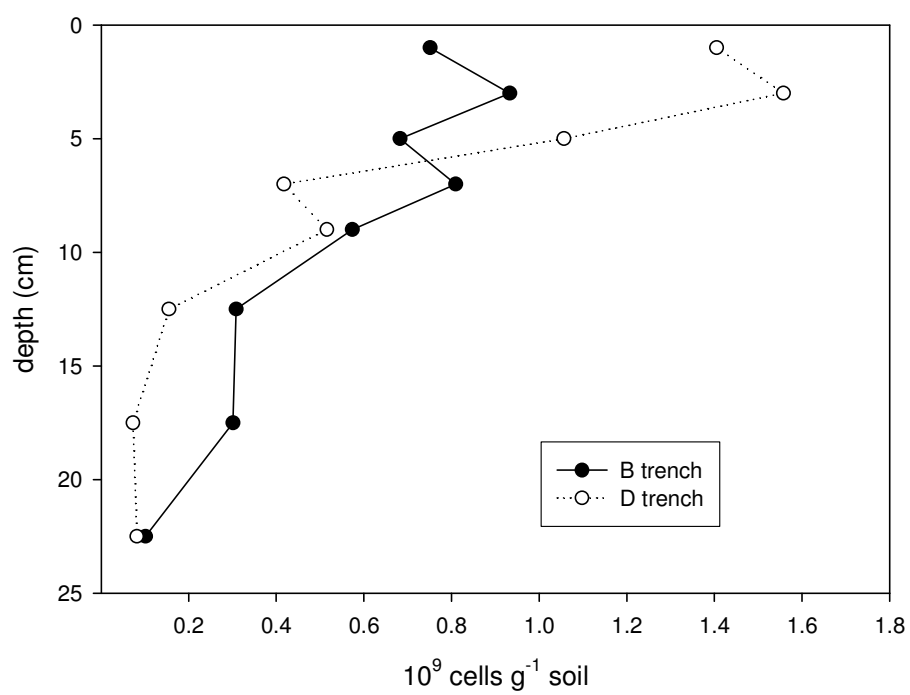


Figure 3.8. Vertical profile of total abundance of microbial communities in B and D trenches.

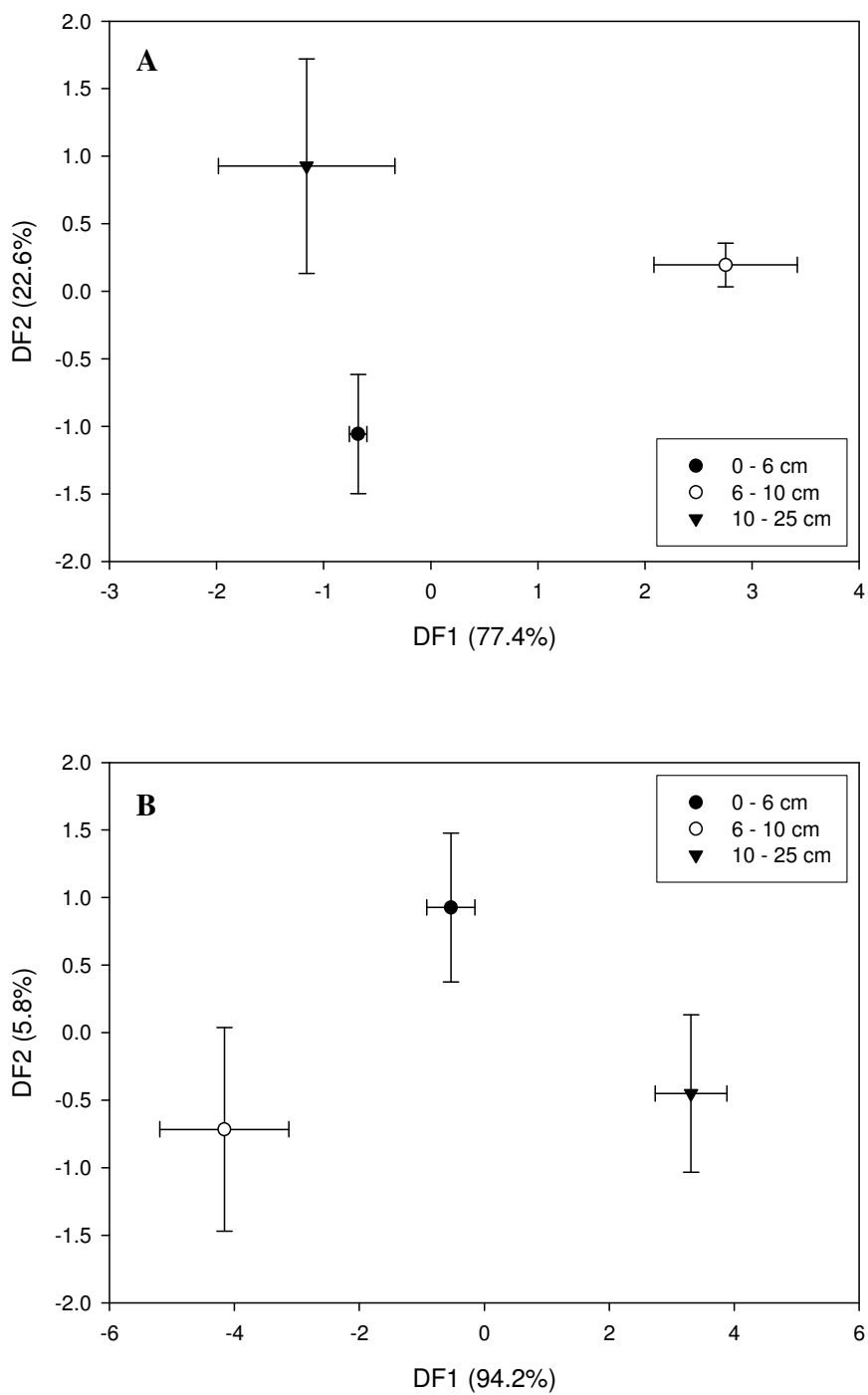


Figure 3.9. DF plot of bacterial (A) and fungal (B) community structures in vertical gradient from B trench. Error bar indicates 1 standard error.

trenches. The same percentage matching was found for fungal community in D trench, and 100% matching was found in B trench. DF plots (Figure 3.9) clearly show the separations among groups, and distinctive trends along the vertical gradient. Both had same counter-clockwise direction of trajectory, but actual trajectories from the surface to the bottom of trench between bacterial and fungal community structures were different. Comparisons between two trenches were somewhat different between bacterial and fungal community structures. Both were not significantly associated, but bacterial community structures were relatively well associated between trenches ($r = 0.244$, $P = 0.051$). Between-trench fungal community structure association was weak negative ($r = -0.119$, $P = 0.296$).

3.3.3. Relationship between soil microbial community structure and soil characteristics in vertical gradient

The vertical profiles of the structures of bacterial and fungal communities were not correlated between trenches (Table 3.2). Well correlation of soil characteristics between trenches; thus indicate that soil characteristics were not the controlling factor on soil bacterial and fungal community structures. Only bacterial community structure in B trench was significantly associated with soil characteristics (Table 3.3).

The correlation analysis was done with the total microbial abundance data and each soil characteristic measurement (organic matter content, pH, moisture content, total carbon content, total nitrogen content, C/N ratio and % clay (only for B trench)) (data not presented). As suggested from the graphs representations of vertical profiles, pH was the least correlated with other variables especially in the D trench (all negative Pearson's

Table 3.2. Comparisons between B and D trenches.

	Correlation analysis	Mantel test
Bacterial community	—	0.2441 $P = 0.051$
Fungal community	—	-0.1191 $P = 0.296$
Abundance	0.8218 $P = 0.0123$	0.5066 $P = 0.023$
Soil characteristics	—	0.8970 $P = 0.001$
Carbon	0.9701 $P = <.0001$	0.9072 $P = 0.001$
Nitrogen	0.9716 $P = <.0001$	0.9091 $P = 0.001$
C/N ratio	0.9551 $P = 0.0002$	0.8565 $P = 0.001$
Moisture	0.9455 $P = 0.0004$	0.8270 $P = 0.001$
Organic matter	0.9660 $P = <.0001$	0.9124 $P = 0.001$
pH	0.7116 $P = 0.0478$	0.0345 $P = 0.381$

Table 3.3. Results of the Mantel tests comparing the community structures of bacteria and fungi, soil characteristics and distance on vertical gradient. The significant test was based on 1000 permutations.

<i>B trench</i>	Fungi	Soil characteristics	Distance
Bacteria	0.2058	0.4973	0.3085
<i>P</i> =	0.143	<i>P</i> = 0.012*	<i>P</i> = 0.057
Fungi	—	0.2112	0.2400
		<i>P</i> = 0.138	<i>P</i> = 0.141
Soil	—	—	0.6580
			<i>P</i> = 0.007*

<i>D trench</i>	Fungi	Soil characteristics	Distance
Bacteria	0.1516	-0.1058	-0.0185
<i>P</i> =	0.196	<i>P</i> = 0.308	<i>P</i> = 0.437
Fungi	—	0.3699	-0.0674
		<i>P</i> = 0.101	<i>P</i> = 0.373
Soil	—	—	0.5678
			<i>P</i> = 0.026*

* Mantel test was significant at probability level of 0.05.

correlation coefficients). Moisture content was correlated with other variables better than any other variables ($r > 0.80$ in B trench and $r > 0.89$ (except with pH) in D trench). All measurement of soil characteristics were well correlated to each other except pH ($r > 0.75$ in B trench and $r > 0.89$ in D trench). % clay was well correlated with organic matter (C, N and C/N ratio) and moisture, but poorly correlated with total abundance ($r = 0.57$ and $P = 0.1403$) and pH ($r = 0.47$ and $P = 0.2371$).

Associations among variables including microbial community structures were analyzed using Mantel test. The overall pattern among soil characteristics was similar to that from correlation analysis, even if specific values of coefficients and probabilities were different in many cases. As summarized in Table 3.3, only bacterial community structures in B trench were significantly correlated with any of soil characteristics. The pairwise Mantel tests of individual soil characteristics confirm it; bacterial community structures in B trenches were significantly correlated with all soil characteristics except pH ($P < 0.05$). All three other microbial community structures were not significantly correlated with any of soil characteristics, except total nitrogen content with fungal community in B trench ($r = 0.370$, $P = 0.036$).

The changes in soil microbial community structure in the soil profiles were more complex. The Mantel test results among bacterial and fungal community structures, soil characteristics and the average distance between the samples suggested complex relationships among them with inconsistency between the trenches. The overall assumption of close relationships between microbial community structure and soil characteristics (including physical characters and not just soil nutrients) was weakly confirmed (bacterial community in B trench). Some of the measurements of the soil

characteristics might have had less influence on soil microorganisms in the profile (for example, moisture content (Sait *et al.*, 2002)) than has been seen in more general settings (van Gestel *et al.*, 1996; Corre *et al.*, 2002). Lag distances (vertical) between the samples were almost equal in influence on the vertical profiles of microbial community structure in the B trench, but the effect of distance was virtually negligible in D trench. This discrepancy between trenches is confirmed by the Mantel test that both bacterial and fungal community structures from two trenches were weakly associated (Table 3.3).

3.4. EXPERIMENT 3: EFFECT OF PLANT COMMUNITY ON SOIL BACTERIAL COMMUNITY STRUCTURE

3.4.1. Genetic fingerprinting technique

By using 10 RAPD primers, 88 genetic polymorphisms were generated from the 3 samplings to which the approach was applied (sampling 3, 6, and 8). There was a larger total number of bands obtained with RAPD (88 vs 56) and a larger proportion of rare polymorphisms obtained with RAPD as compared with DGGE. Simultaneous comparison of the two genetic fingerprinting techniques for those samples on which both approaches were employed (sampling 3, 6, and 8) with the Mantel test indicated that there was no correlation between them ($r = 0.003$, $P = 0.128$), i.e., the two genetic fingerprinting techniques examine the community genome in different ways (RAPD examines the frequency of arbitrary sequences in the entire genome, whereas DGGE examines the sequence information of the most dominant ribotypes). However, for that limited sample set, DFA of both temporal trends and plants differences yielded 98.1 % (DGGE) and 100 % (RAPD) matching of original group membership, suggesting that,

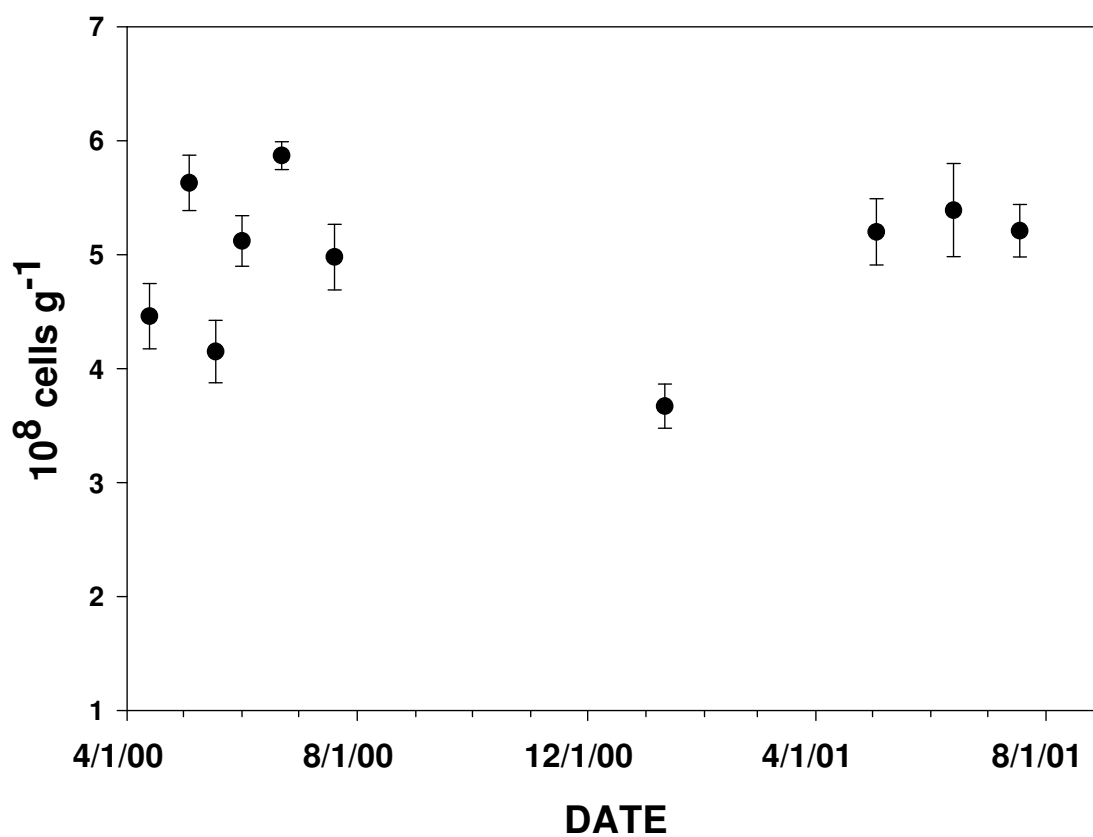


Figure 3.10. Average bacterial abundance at each sampling during the 2 years of study. Error bars are 1 standard error of the mean.

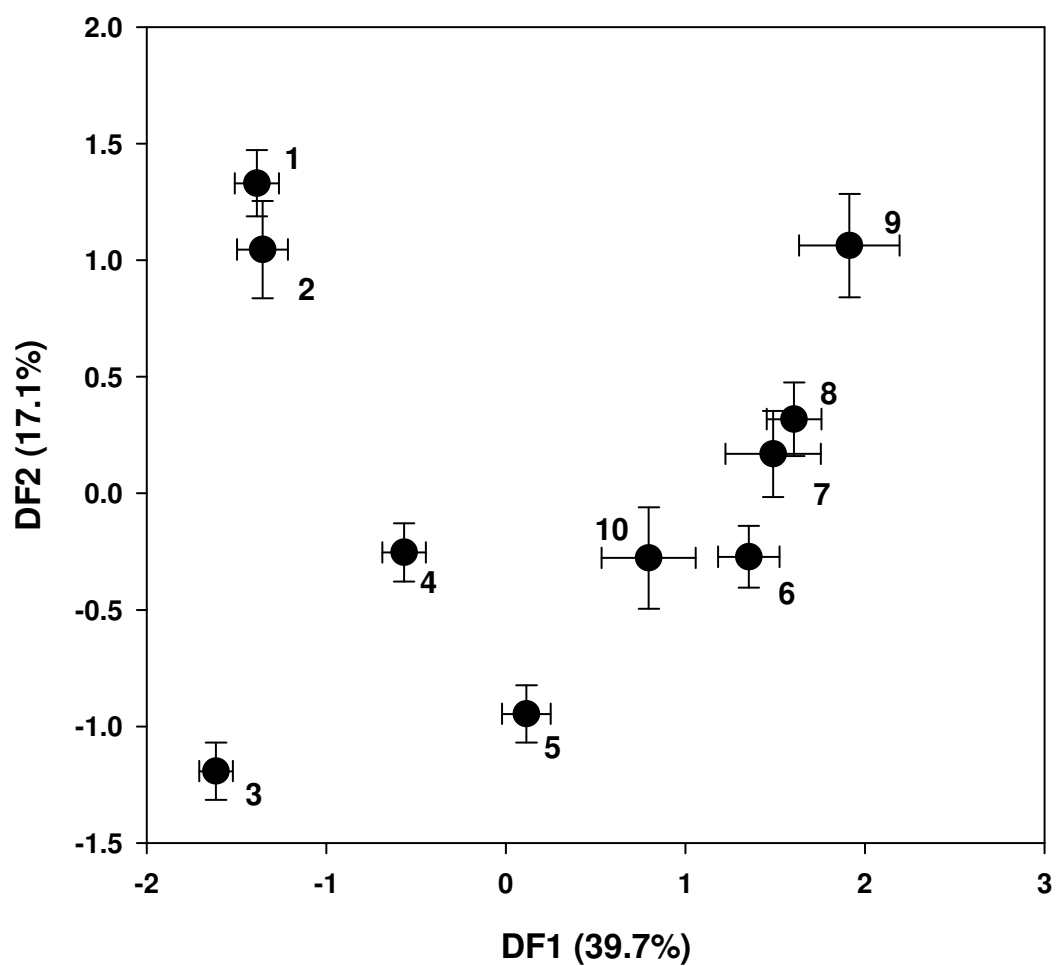


Figure 3.11. DF plot of DGGE data according to the group membership of sampling time from sampling 1 to 10. Points indicates centroids with 1 standard error. Number in parentheses indicates the amount of variance explained by that function.

Table 3.4 Results of Pairwise Mantel tests of sampling times.

2	<i>r</i>	0.081								
	<i>P</i>	0.001								
3	<i>r</i>	0.142	0.121							
	<i>P</i>	0.001	0.001							
4	<i>r</i>	0.096	0.050	0.059						
	<i>P</i>	0.001	0.007	0.001						
5	<i>r</i>	0.187	0.083	0.140	0.107					
	<i>P</i>	0.001	0.003	0.001	0.001					
6	<i>r</i>	0.336 ^b	0.157	0.228	0.129	0.065				
	<i>P</i>	0.001	0.001	0.001	0.001	0.001				
7	<i>r</i>	0.211	0.324 ^b	0.380 ^b	0.208	0.063	0.121			
	<i>P</i>	0.001	0.001	0.001	0.001	0.043	0.007			
8	<i>r</i>	0.233	0.148	0.234	0.108	0.104	0.053	0.149		
	<i>P</i>	0.001	0.001	0.001	0.001	0.001	0.001	0.002		
9	<i>r</i>	0.245	0.257	0.310	0.140	0.019	-0.005	0.008	0.022	
	<i>P</i>	0.001	0.001	0.001	0.001	0.292	0.529	0.322	0.309	
10	<i>r</i>	0.121	0.195	0.248	0.084	0.008	0.020	0.048	0.015	0.046
	<i>P</i>	0.001	0.001	0.001	0.039	0.384	0.320	0.082	0.345	0.060
		1	2	3	4	5	6	7	8	9

^a *r* : standardized Mantel Statistics, *P* = probability after 1000 permutations

^b Relatively highly correlated to each other; $\alpha = 0.05$

even though they work differently, the two genetic fingerprinting techniques were equally effective in detecting differences in the bacterial communities.

3.4.2. Effect of herbicide treatment and burning

The field was burned on May 3, 2000, about 20 days after the second herbicide treatment. Although the burning lasted the entire day, there was a portion of field that was less intensively burned than rest of the field, especially the lower southeast part that was near the road (M2) and that had dense perennial vegetation (H4). As shown in Figure 3.10, the abundance of microorganisms was not decreased by the burning, in contrast to what has been frequently observed elsewhere (Ahlgren, and Ahlgren, 1960; Borchers, and Perry, 1990; Stendell *et al.*, 1999). Indeed there was an apparent increase in bacterial abundance shortly after the burning. As the data suggest that the intensity of the burning was not sufficient to cause a major decrease in bacterial abundance, neither was bacterial community structure changed greatly immediately after the fire (Figure 3.11 and Table 3.4). However, samples obtained two weeks after the fire showed drastic change in bacterial community structure.

3.4.3. Effect of different plant community on bacterial community structure

The three blocks established on the presumed moisture regime were separated by both RAPD and DGGE data as seen in the results of the DFA (Figure 3.12). The overall matching of the samples to the groups to which they were assigned in the experimental design was 58.9 % for all groups. The large number of samples allowed a territory map to be generated as a part of the DFA (overlaid on the DF plot, Figure 3.12) by the statistical software (SPSS v. 11), and that map indicated separation of the samples into three distinct groups. However, bacterial abundance among the locations on the hillslope did

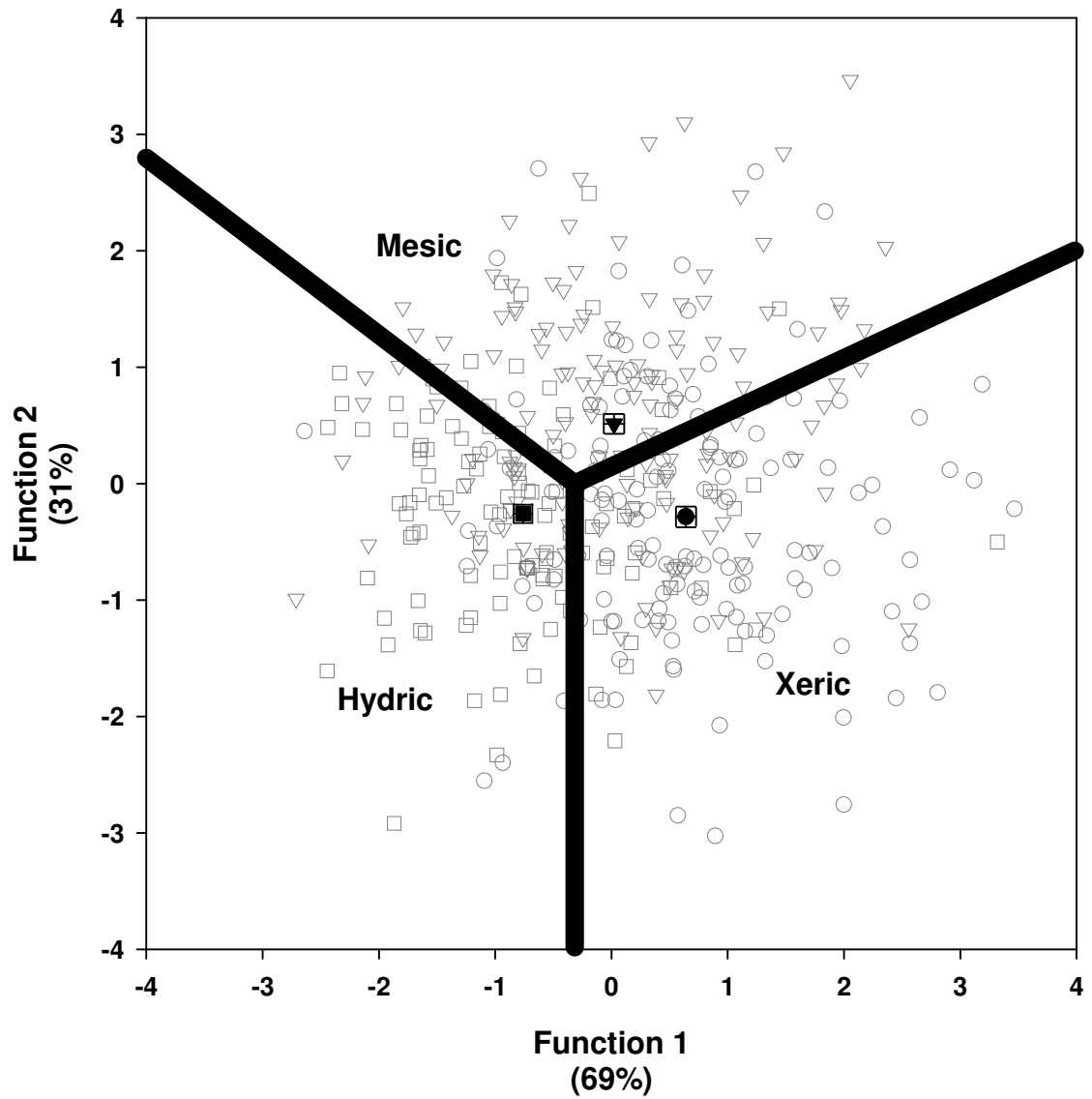


Figure 3.12. DF plot of bacterial community structures according to the group membership in the presumed moisture-regime block. The overlapped territorial map (heavy lines represent territory boundaries) clarifies the data separation. The dark points indicate group centroids with 1 standard error bars. Symbol ●, ▼, and ■ represent the “xeric”, “mesic” and “hydric” blocks, respectively.

not show segregation similar to that of the community structure. The only exception was found right after the burning, where a significant difference ($P = 0.0424$) was found between the xeric and hydric block samples using Ryan's Q test (Sokal, and Rohlf, 1995).

ANOVA ($\alpha = 0.05$) of the abundance data did not discriminate among the different vegetation treatments overall, at any time during the 2-year study. Fingerprints of the communities from the different vegetation treatments obtained with RAPD were well separated by DFA (not shown), but this observation may be a result of the analysis being performed on only 54 samples (18 from each of sample times 3, 6 and 8). DGGE fingerprints from the entire 414 samples represented the total bacterial community from the field much better (including over time). The vegetation-diversity treatment obtained by grouping treatments 1 and 2 (1 grass), 3 and 4 (3 grasses), and 5 (6 grasses) could not be well separated by DFA (Figure 3.13); however, differences between all the treated plots (treatments 1-5) taken as a group were distinguished from the controls by DFA with 73.6% group membership for samples from the "Treated" plots and 60.9% membership for samples from the "Control" plots. Although substantial differences were found among many of the treatment plots taken individually, no systematic differences (such as diversity or species composition treatments) were observed.

3.4.4. Temporal change along with plant community development

The 10 sampling times were analyzed using both DFA and the Mantel test to examine overall trends of bacterial community structure change. The overall matching of the samples to the appropriate group (DFA, times 1-10) was 61.6 %, a high value considering there were 10 different groups with 414 samples to be assigned to them. As shown in Figure 3.11, the sampling times clustered into two distinct groups with the

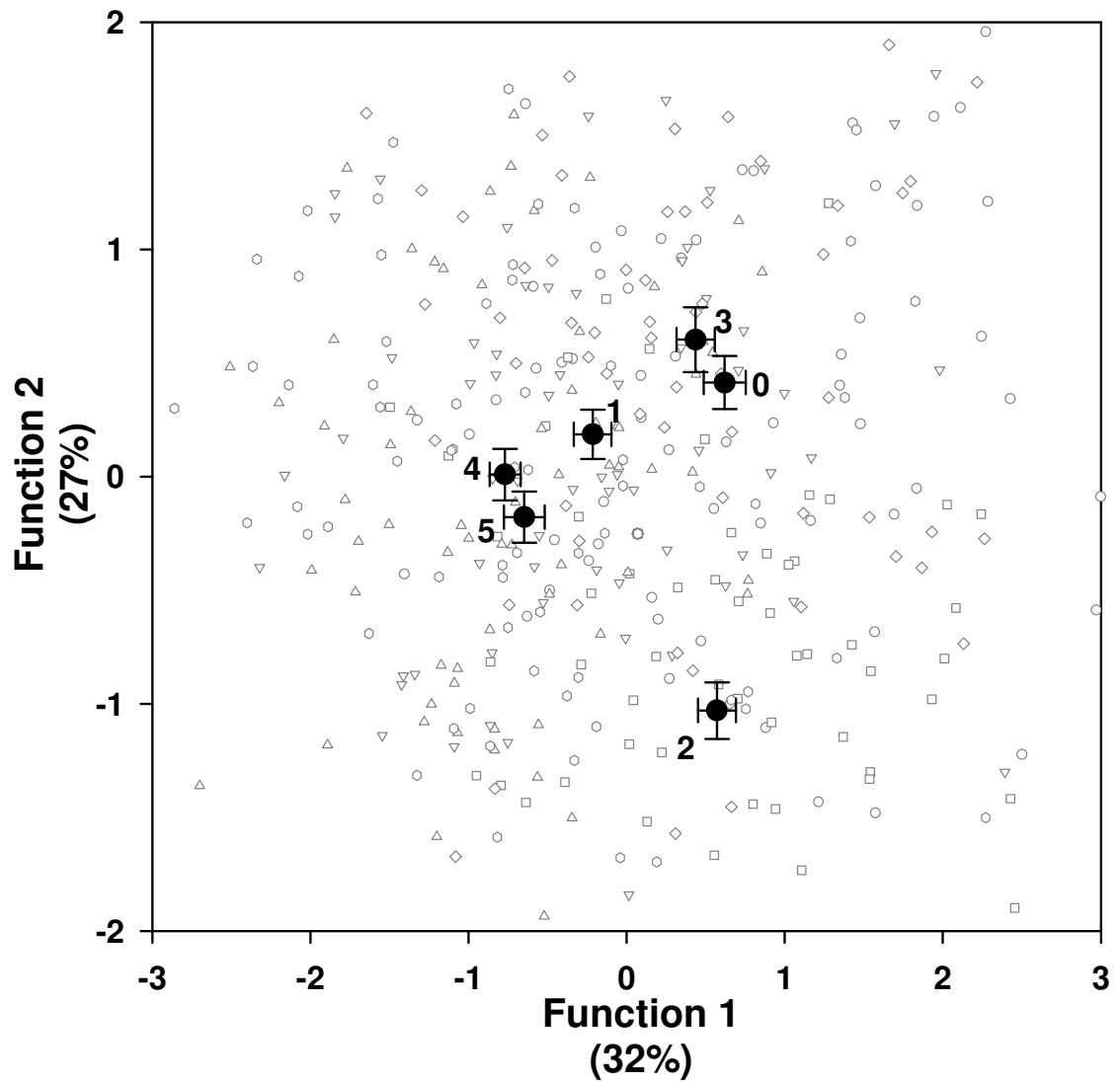


Figure 3.13. DF plot based on the vegetation treatment. Black points indicate group centroids with 1 standard error bars indicated. Designations 1 through 5 represent the vegetation treatment assigned in Table 2.1., and 0 is the unmanipulated control.

communities recovered in the first two samplings being decidedly different from all other samplings. In addition, there was also a strong directionality in the shift of bacterial community structure in the multivariate space, suggesting a successional change away from the initial condition. Only at time 10 did the progression not follow the directional pattern. The pairwise Mantel test (Table 3.4) indicated that the overall bacterial community structures of change was significant ($r = 0.093$, $P = 0.001$); only 4 of the 45 pairwise comparisons had meaningful correlation between them, and in each case the r value was between 0.31 and 0.38. This result corroborates the DFA results that the bacterial community structure never returned to its original state.

The data were also analyzed for seasonal change and annual patterns. Group membership of non-growing season (sampling 1-3, and 7) and growing season samples (all others) by DFA showed agreement of 83.3% and 77.8%, respectively. Additionally, the distinction between the first and second year communities was very clear, with 87.3 % and 76.9% agreement for the samples from the first and second years, respectively, an observation consistent with the observed difference in vegetation in those years.

3.5. EXPERIMENT 4: SPATIAL STRUCTURE OF SOIL MICROBIAL COMMUNITY

3.5.1. Geostatistical analysis of soil characteristics

3.5.1.1. Semivariogram modeling analysis

All measured and derived factors defining soil characteristics were analyzed for identifying spatial structure. The exploratory descriptive statistics was first conducted to better designing for geostatistical analyses (Table 3.5). The overall structure of the entire

Table 3.5 Exploratory data analyses of soil characteristics measurements and total microbial abundance.

	Min	Mean	Max	SD	CV (%)
Carbon (g/kg soil)	14.61	23.15	37.22	3.89	16.80
Nitrogen (g/kg soil)	1.33	2.23	3.15	0.34	15.25
C/N ratio	8.71	10.38	12.26	0.55	5.30
Moisture (%)	21.26	26.16	31.17	1.76	6.73
% clay	22.48	32.52	50.02	4.23	13.01
pH	5.53	6.31	7.10	0.29	4.60
Abundance	2.52E+8	1.06E+9	2.21E+9	4.62E+8	43.58

experimental field was first studied (field scale model), and then nested scales from the entire field (medium, small and fine scale models) and combinations of nested scale along with regions of the field (region model) were subsequently analyzed. The data were binned factor by factor for optimum results among several experimental semivariograms. Since a 1% cut-off rule was employed, the largest lags in field scale were not included for the analysis, and resulted in maximum lag distance of about 6 m rather than 7 m. The number of pairs used in calculating semivariance was 39402 (field scale), 12470 (medium scale), 7580 (small scale) and 4713 (fine scale). The presence of nested semivariogram models was not always apparent mainly due to the difference of resolution in different scale. Larger scale analysis was done with much coarser lags such that the largest possible lag distance used at the fine scale was not even reached by the smallest lag distance measured in field scale. Number of pairs used in calculating regional semivariance were 4900 (medium scale) and 1296 (fine scale). Number of pairs in each lag was relatively constant and the minimum number of pairs used was at least 10% of maximum number of pairs in each semivariogram analysis. Models were chosen by the process of best fitting based on the values of indicative goodness of fit (IGF value) and visual examination, but some data (e.g., C corner in medium scale for most factors) were simply too exotic to allow fitting by one of five models with acceptable IGF value. Results of each factor are presented following this order: experimental semivariograms, semivariogram models for entire field and semivariogram models for different regions of the field.

Table 3.6 Summary of results from semivariogram analysis of soil characteristics. For variables with strong anisotropy, major and minor directional semivariogram models were presented.

Factor	Scale	Corner	Model ¹	IGF ²	Nugget (C ₀)	Sill (C ₁)	Range (a) ³	NMSD ⁴		
Carbon	Field	Entire	E	0.0631	6324	3255	64.9	0.34		
		Medium	Entire	S	0.0624	4556.7	5579.7	37.5	0.55	
	Small	Entire	A	S	0.152	1898	7300	36.8	0.79	
			B	G	0.189	4956	4130	> 250	0.45	
			C	P	0.761	6660	112	1.03		
			D	P	0.422	12220	42.9	0.68		
			Entire	S	0.0452	4835.7	6602.7	47	0.58	
		Fine	Entire	S	0.00164	558	8091	15.6	0.94	
			A	S	0.0313	1116	6200	21.58	0.85	
			B	N	0.0858	5076				
			C	S	0.00306	2197.8	21998.5	18.98	0.91	
			D	G	0.0119	2520	12000	17.42	0.83	
	Nitrogen	Field	Entire	S	0.0310	41.9	34.1	84	0.45	
			Medium	Entire	S	0.163	23.43	60.35	47.5	0.72
Small		Entire	A	E	0.225	18.9	44	112.7	0.70	
			B	G	0.236	32.9	20.0	> 250	0.38	
			C	P	1.46	33	1.22	1.05		
			D	N	0.115	83.6				
			Entire	S	0.139	22.7	71	52	0.76	
		Fine	Entire	S	0.00273	2.84	53.3	16.38	0.95	
			A	S	0.0168	7.48	34	22.62	0.82	
			B	N	0.0684	33.17				
			C	E	0.0177	25.2	180	26	0.88	
			D	G	0.0115	18.8	75	18.46	0.80	
C/N ratio		Field	Entire	S	0.195	0	0.348	49.5	1.00	
			Medium	Entire	S	0.223	0.044	0.352	70	0.89
	Small	Entire	A	G	0.147	0.15	0.255	> 250	0.63	
			B	G	0.300	0.07	0.2	193.2	0.74	
			C	P	1.17	0	0.037	1		
			D	P	0.273	0.198	0.001	1		
			Entire	S	0.0550	0.024	0.344	61	0.93	
		Fine	Entire	S	0.00444	0.0040	0.136	13	0.97	
			A	S	0.0270	0.012	0.2	21.06	0.94	
			B	N	0.0244	0.0728				
			C	G	0.0473	1.798	5.8	26	0.76	
			D	S	0.00994	0.026	0.15	19.24	0.85	
	Moisture	Field	Entire	E	0.0369	1.550	1.612	126.5	0.51	
			180° ³	G	0.0114	1.86	2.108	450	0.54	
90°			G	0.0278	1.426	1.674	31.5	0.54		
Medium		Entire	Entire	E	0.0122	0.93	1.829	50	0.66	
			A	N	0.255	2.574				
			B	G	0.0659	1.278	1.152	88.8	0.47	
			C	P	2.35	0	0.288	1.05		
			D	N	0.375	1.958				
		Small	Entire	E	0.00158	0.900	1.953	49.50	0.68	
			Fine	Entire	S	0.00043	0.900	1.55	> 25	0.63
				A	G	0.0124	1.711	2.117	> 25	0.55
				B	E	0.00161	0.63	0.84	17.16	0.57
				C	P	0.0453	9.57	1.3	1	
D		E	0.0254	0.408	2.4	> 25	0.85			

Table 3.6. *Continued.*

Factor	Scale	Corner	Model ¹	IGF ²	Nugget (C ₀)	Sill (C ₁)	Range (a) ³	NMSD ⁴
% clay	Field	Entire	E	0.177	5.4	13.68	95.16	0.72
		135°	E	0.101	8.64	11.88	186	0.58
		45°	E	0.391	4.14	16.02	117.6	0.79
	Medium	Entire	S	0.197	5.94	18	92.5	0.75
		A	S	0.221	11.27	22.31	96	0.66
		B	S	0.376	5.5	25	108.08	0.82
		C	P	1.22	2.6	0.445	1.06	
	Small	Entire	P	0.166	8.03	0.042	0.86	
		Fine	S	0.0558	4.14	11.88	38	0.74
	Fine	Entire	P	0.00236	3.96	0.448	1	
		A	P	0.00492	11.22	0.351	1	
		B	G	0.0189	4.07	11	> 25	0.73
		C	G	0.0650	15.4	57	> 25	0.79
		D	P	0.0511	3.3	0.288	0.87	
pH	Field	Entire	S	0.0945	0.0216	0.0702	71.5	0.76
		45°	S	0.0786	0.0306	0.0891	268.8	0.74
		135°	S	0.0995	0.0378	0.0693	123.2	0.65
	Medium	Entire	E	0.0276	0.0252	0.09	175	0.78
		A	S	0.792	0.0408	0.0712	184	0.64
		B	S	0.259	0.0448	0.08	> 250	0.64
		C	P	3.16	0	0.02	1.04	
	Small	Entire	P	0.257	0.05	0.00016	1.03	
		Fine	S	0.0152	0.0252	0.0783	95	0.76
	Fine	Entire	P	0.00435	0.018	0.00182	1	
		A	G	0.0536	0.0174	0.06	> 25	0.78
		B	N	0.0249	0.0505			
		C	G	0.0679	0.667	2.3	> 25	0.78
		D	S	0.0225	0.0078	0.0201	25	0.72

1. E. Exponential model, G. Gaussian model, N. Pure nugget model, P. Power model, and S. Spherical model. For power model, slope and power are recorded under sill and range, respectively and NMSD was not calculated for power model either. By definition, the range of power model was beyond the limit of each scale.

2. Indicative Goodness of fit (2.5.1.2.).

3. Unit of range is cm.

4. Normalized measure of spatial dependence ($C_1 / (C_1 + C_0)$).

* Direction is measured from East in counter-clockwise following the convention of VARIOWIN.

Directional models were identified separately, but presented as transformed models in Figures 3.17.-19.

CARBON

Determined lag distances of experimental semivariogram field models were 0.5 m (field scale), 0.25 m (medium scale), 0.1 m (small scale) and 0.025 m (fine scale). At the field scale, no strong anisotropy was identified. Lag distances for the regional analysis across different scales used the lag distance from corresponding scales entire field analysis, except medium scales of B (0.3 m) and D (0.2 m) corners which used 0.25 m instead.

Semivariogram models were identified based on IGF value and visual examination (Table 3.6 and Figure 3.14). Models were generally fit the data better at fine scales, but the worst fits were obtained in region models at the medium scale. Field models fit better than region models in both medium and fine scale. 9 of 12 model fit yielded a bounded model: 6 spherical models, 1 exponential model and 2 Gaussian models were obtained. B-corner model at the fine scale was best fit with a pure nugget model. Except for the field scale model (exponential model), all of the other field models were spherical model, and nested structures were evident. The first spatial structure was determined from fine scale model (0.156 m). Since field scale model was very weak, second structure was determined from medium and small scale models (0.4 m). Considering the relationship of nested structures between different scales, those two ranges were the ranges of the field; two nested scale of the spatial structure of carbon content. Spatial dependence increased from field scale to fine scale indicating carbon content was highly spatially structured in smaller scale.

Region models were not always compatible with same scale field model. Only the A corner had the same spherical model as the medium scale field model, and A and C

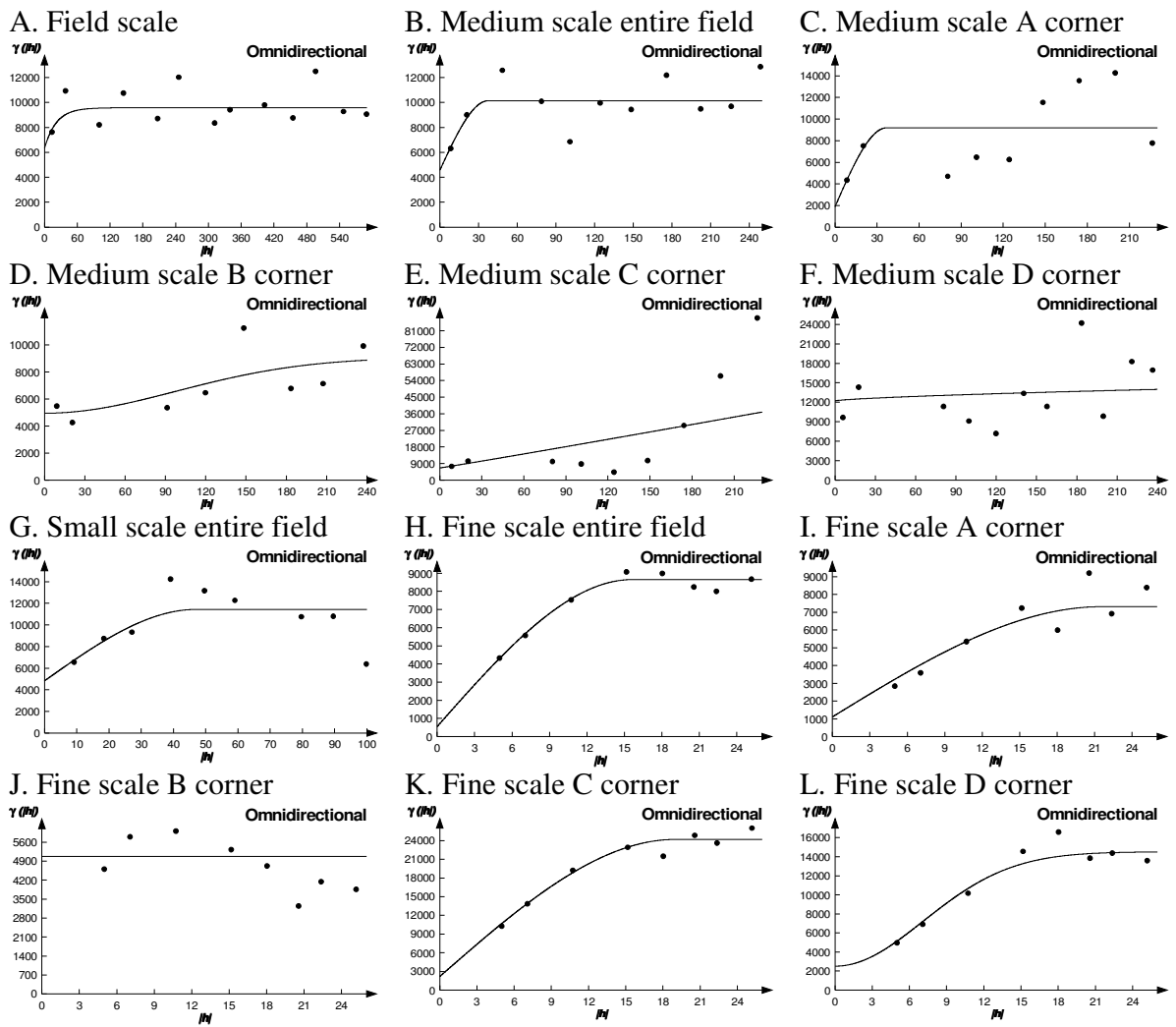


Figure 3.14. Semivariogram models of total carbon content measurement.

corners for the fine scale had same spherical models as the field model. The calculated ranges for the region models were compatible with the field model as the same scale with one exception, a Gaussian model determined from the B corner at medium scale. Spatial dependences of region models were relatively consistent with field models.

NITROGEN

Lag distances of experimental semivariogram field models were determined: 0.7 m (field scale), 0.25 m (medium scale), 0.1 m (small scale) and 0.025 m (fine scale). Anisotropy was not identified at the whole field scale. Lag distances of region models were same as the corresponding field scale except for the B corner at a fine scale for which a lag of 0.02 m was used.

As with carbon content, semivariogram models for the fine scale fit better and region models at the medium scale yielded the poorest fits to the data (Table 3.6 and Figure 3.15). Field models were better fits than region models in both the medium and fine scales. 9 of the 12 models obtained were bounded models: 5 were spherical models, 2 were exponential models and 2 were Gaussian models. There were also pure nugget models: The D-corner model at the medium scale and the B-corner model at the fine scale. All field models displayed nested structure at the larger scales and all were best fit with a spherical model. The range for nitrogen content in the scaling for the entire experimental field was 0.84 m, and the fine scale's range was 0.164 m. Considering the relationship of nested structures between different scales, those two ranges represent the ranges of the entire field. The trend of spatial dependence among different scales was opposite to that of the magnitude of the range in that the highest was found at the fine scale (0.95) and lowest at the field scale (0.45).

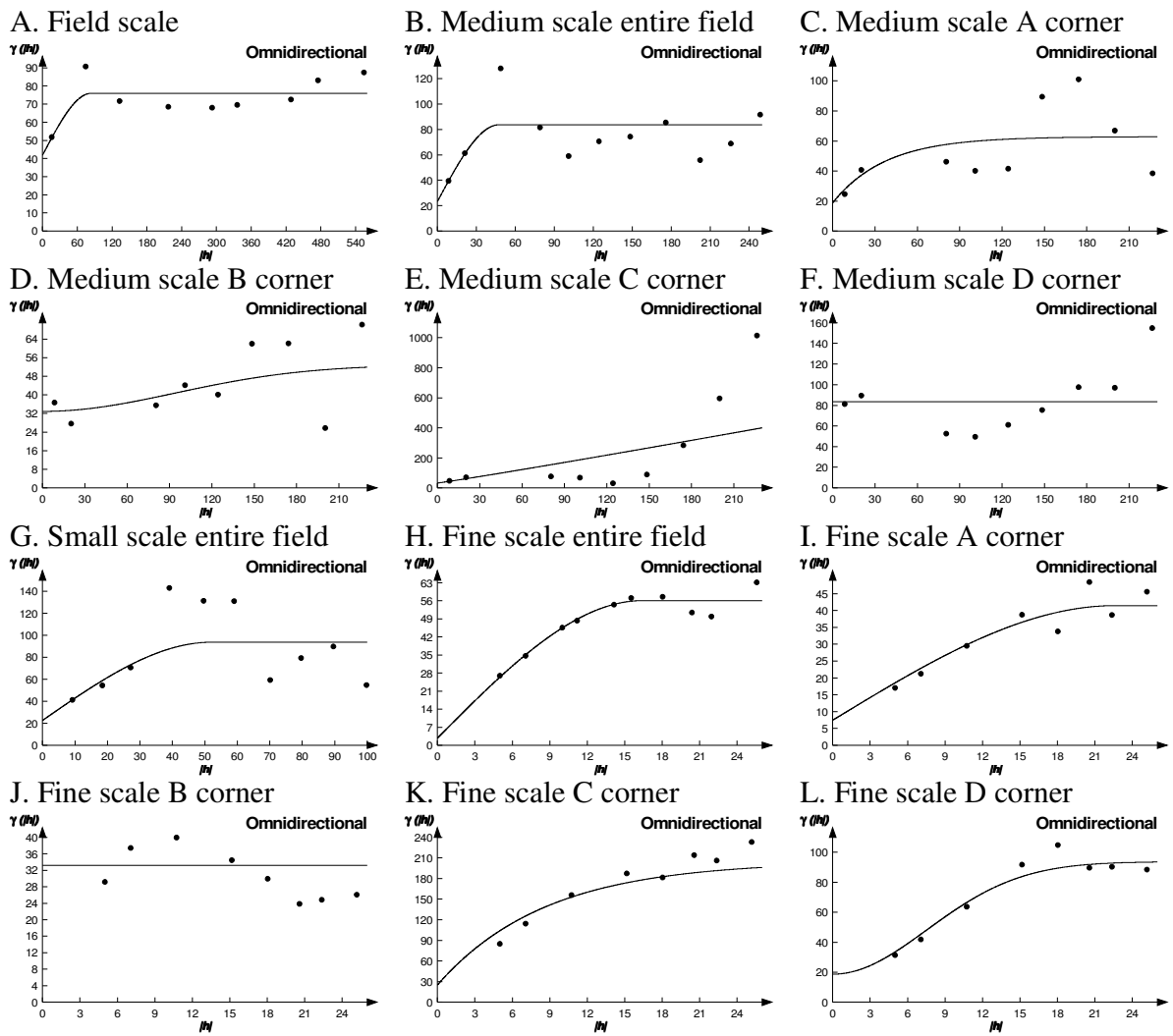


Figure 3.15. Semivariogram models of total nitrogen content measurement.

None of the region models for medium scale was same type of model as was best fit at the field scale (spherical model) and the A-corner model was only similar to the field scale model at fine scale. All of the measured ranges in (bounded models) from both medium and fine scale were longer than those of the corresponding field scale. Spatial dependence of the region models in fine scale was higher than that of models in medium scale.

C/N RATIO

By looking at Figures 3.14 and 3.15, it is obvious that the semivariogram models of carbon and nitrogen contents are very similar to each other, especially at smaller scale. The ratio of carbon and nitrogen contents was derived; because C/N ratio in soil is maintained at around 10 and variation of it could indicate the dynamics of nitrogen mineralization/immobilization. The lag distances used for the experimental semivariogram analysis of models at each scale were 0.5 m (field scale), 0.25 m (medium scale), 0.1 m (small scale) and 0.02 m (fine scale). Like carbon and nitrogen contents, the C/N ratio did not show any anisotropy. Lag distances of all region models for the medium scale were same as for the field scale except for the D-corner model (0.3 m); and all of the fine scale region models had lag distances of 0.025 m.

Models fitted better the data in the smaller scale and the poorest fits were obtained for the region models at the medium scale (Table 3.6 and Figure 3.16). Field models were always better fitted compared to region models at the both medium and fine scale. 9 out of the 12 models obtained were bounded models: 6 were spherical models and 3 were Gaussian models. The B-corner model at the fine scale was a pure nugget model. All field models were spherical, and they were well nested into larger scale models. The

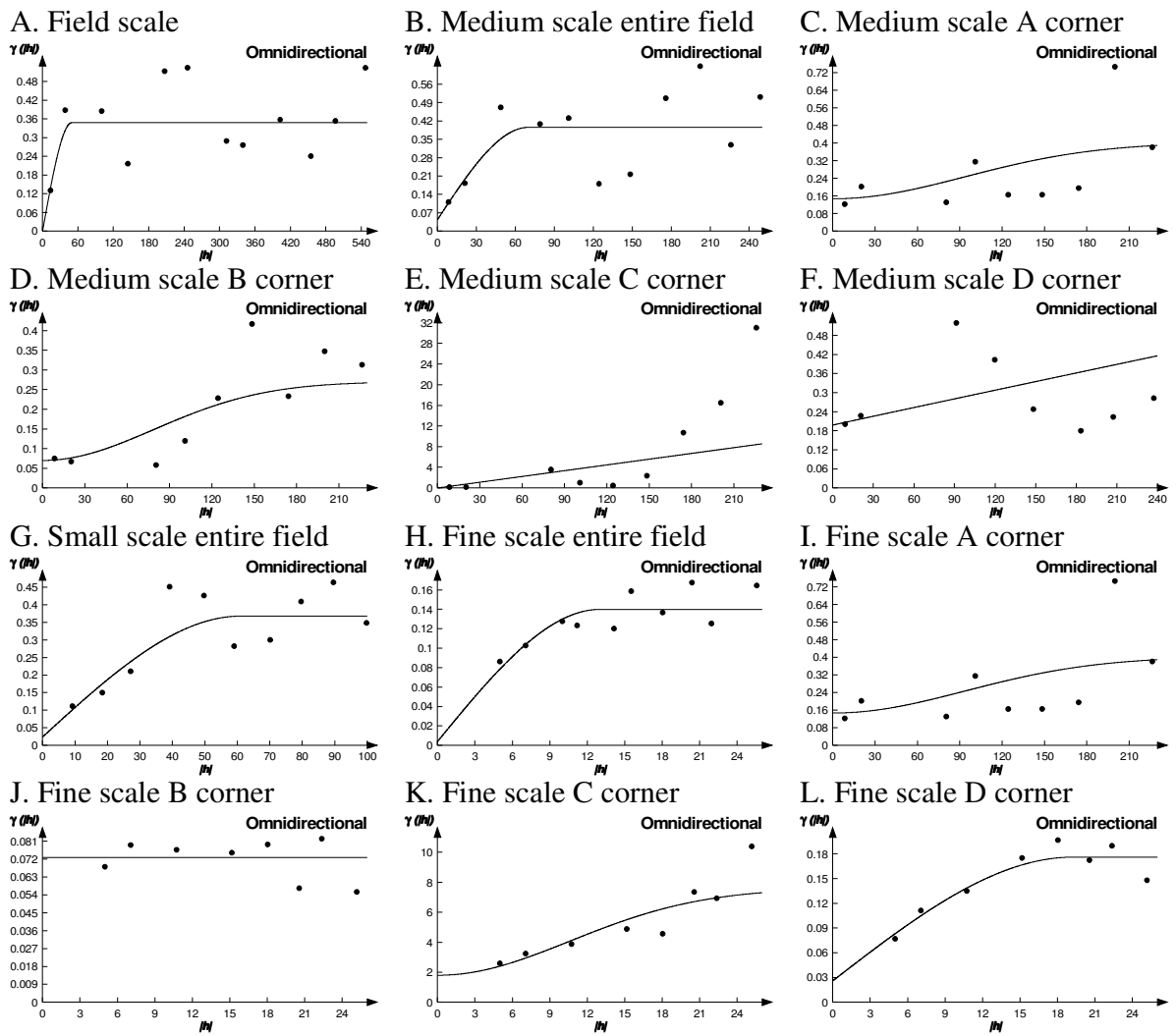


Figure 3.16. Semivariogram models of C/N ratio calculation.

ranges of field, medium and small-scale models were more or less similar to each other especially in the shapes of their diagrams (Figure 3.16.A, B and G). Due to the nested structure among different scales, the ranges of C/N ratio measurement was determined to be 0.6 m, which was determined from the medium and small scale semivariogram models, and 0.13 m, which was determined from the fine scale model. Regardless of scale, C/N ratio was highly spatially structured (NMSD > 0.89).

All medium-scale region-model types were different from the field model at the same scale, but two of the region model types at the fine scale were same as the field model at fine scale (spherical model). Ranges of spatial autocorrelation were longer in region models than in field model from in medium and fine scale. C/N ratio was still well spatially structured in regions where bounded models were determined, but was slightly less than the field models. Spatial dependence was higher at the fine scale than at the medium scale.

MOISTURE

Lag distances for the experimental semivariogram field models were determined: 0.5 m (field scale), 0.25 m (medium scale), 0.15 m (small scale) and 0.025 m (fine scale). Strong zonal and geometric semivariogram anisotropy was identified such that 180° was principal axis (maximum range) and 90° was minor axis (minimum range). Except for the A and B corners at the medium scale (0.3 m); all other lag distances for the region models were the same as the corresponding field model.

Model fits were better at the smaller scale and region models fit less well among all the models (Table 3.6 and Figure 3.17). Fitting of region models was worse than that of the corresponding field models. Directional models were a better fit than the

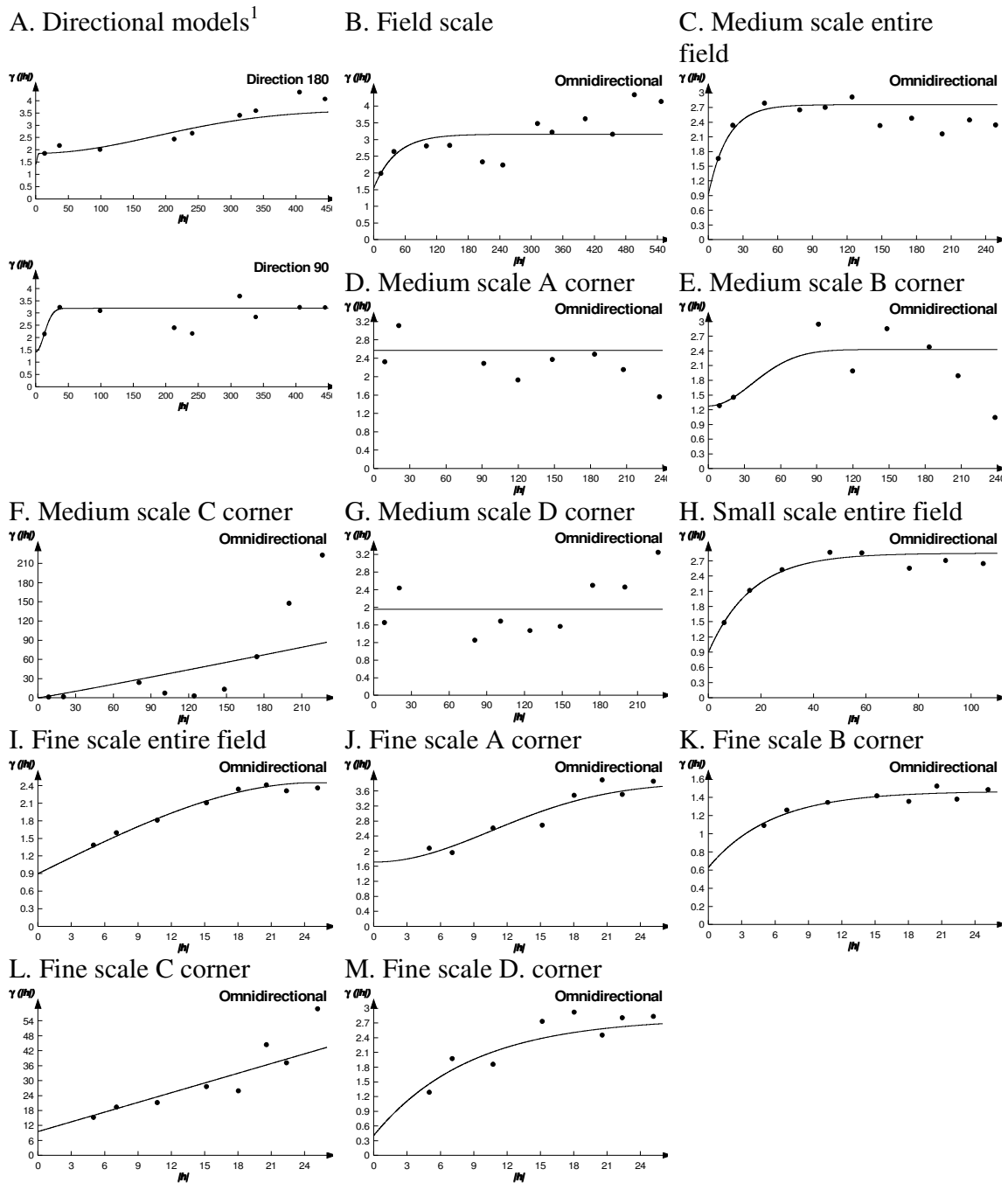


Figure 3.17. Semivariogram models of moisture content measurement. All models are omnidirectional unless specified.

1. Major and minor directional semivariogram models as simultaneously modeled for further analyses.

omnidirectional model, and the transformed reference model was a still better fit than the omnidirectional model (IGF: 0.00247). It is interesting that directional models (Gaussian model) and omnidirectional model (exponential model) fit best in different model types. 10 out of the 14 models (including directional models) were bounded models: 1 were spherical model, 5 were exponential models and 4 were Gaussian models. The A and D corner models at the medium scale were pure nugget models. All omnidirectional field models were exponential except for the fine scale model which was spherical. Nested structures between field models were obvious especially between field and medium scale models that sudden increase of semivariance was found right outside of the scale of medium model resulting in very different nugget and sill between two extended scale models (Figure 3.17). The range of fine scale model was beyond the limit of scale (0.25 m) and the ranges of medium and small scale model were almost same (0.5 m). Decreasing pattern at the end of the scale limit at medium scale model defined another structure from field scale model at around 2.7 m. Spatial dependence of moisture content was relatively constant throughout different scales and regions of the field. None of the region model types were the same with the corresponding field model. Only the B-corner model was bounded, and the range was much longer than the range of the field model.

% CLAY

Lag distances of experimental semivariogram field scale models for % clay measurement were 0.35 m (field scale), 0.25 m (medium scale), 0.1 m (small scale) and 0.02 m (fine scale). Apparent geometric semivariogram with zonal nugget anisotropy was identified at the field scale: 135° (principal axis) and 45° (minor axis). The lag distances

of all medium scale region models were same as that of medium scale field model, except A corner model which used 0.3 m, and the lag distances of all fine scale region models were 0.025 m.

Like other factors of soil characteristics, semivariogram model fit better in smaller scale (Table 3.6 and Figure 3.18). Region models fit less than field model in both medium and fine scale. Omnidirectional and directional models were compatible in the degree of model fit, and transformed reference model were slightly less fitted to data than omnidirectional model (IGF: 0.271). 9 out of the 14 models were bounded model: 4 were spherical models, 3 were exponential models and 2 were Gaussian models. All the field scale models, including directional models, were exponential, and medium and small scale field models were spherical. The fine scale field model was linear model (power = 1). The first range was determined as 0.38 m in small scale as the fine scale field model was beautifully nested within the small scale field model. The second range was determined from the medium scale field model (0.8 m). The range of omnidirectional field scale model was shorter than the minimum range of directional model. Spatial dependence was very constant and high (0.72 ~ 0.75) throughout the semivariogram models for different scales of the field.

The A-corner models in both medium and fine scale were similar to those of corresponding field models, especially in shapes. In addition, the types of the B-corner model at the medium scale and the D-corner model in fine scale were same with the corresponding field models (power). The ranges of all region models and field model of fine scale were not identified within the limit of the fine scale (0.25 m). All available

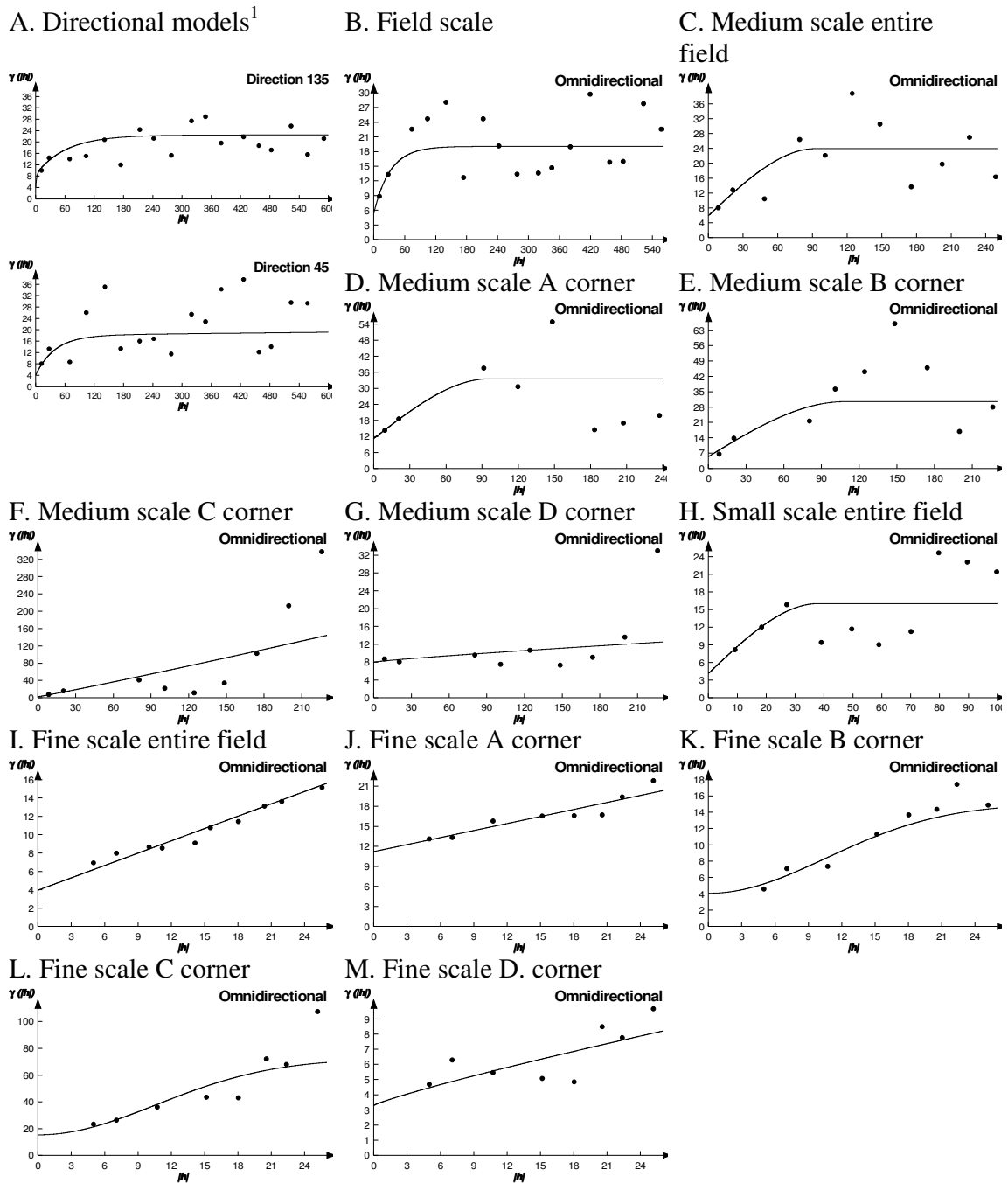


Figure 3.18. Semivariogram models of relative amount of clay particles. All models are omnidirectional unless specified.

1. Major and minor directional semivariogram models as simultaneously modeled for further analyses.

spatial dependence among region models was relatively high and consistent to each other, regardless of scale.

pH

Lag distances of experimental semivariogram field models were determined first: 0.5 m (field scale), 0.25 m (medium scale), 0.1 m (small scale) and 0.02 m (fine scale). Strong geometric semivariogram with zonal nugget anisotropy was identified: 45° (principal axis) and 135° (minor axis). All medium scale region models had same lag distance with field model, but all fine scale region models' lag distances were 0.025 m rather than 0.02 m, which was the field model's lag distance.

Models fit better in smaller scale than larger scales, and models in the medium scale, especially their region models were poorest fitted (Table 3.6 and Figure 3.19). The omnidirectional model and both directional models fit about same degree, and the transformed reference model fitted as good as other field scale models (IGF: 0.0870). 10 out of the 14 models were bounded: 7 were spherical models, 1 was exponential model and 2 were Gaussian models. There was also one pure nugget model (B corner model at the fine scale). The fine scale field model was linear (power = 1), but all other field models were bounded: spherical model (field and small scale) and exponential model (medium scale). The first range of 0.95 m was determined from the fine, small and medium scales. The second structure was identified from the field scale model at around 2.7 m where discrepancy was identified, just like in moisture. The range of the omnidirectional field scale model was shorter than the minimum range of directional model. All spatial dependence of pH was very consistent and high (0.76 or 0.78) regardless of the different scales and regions of the experimental field.

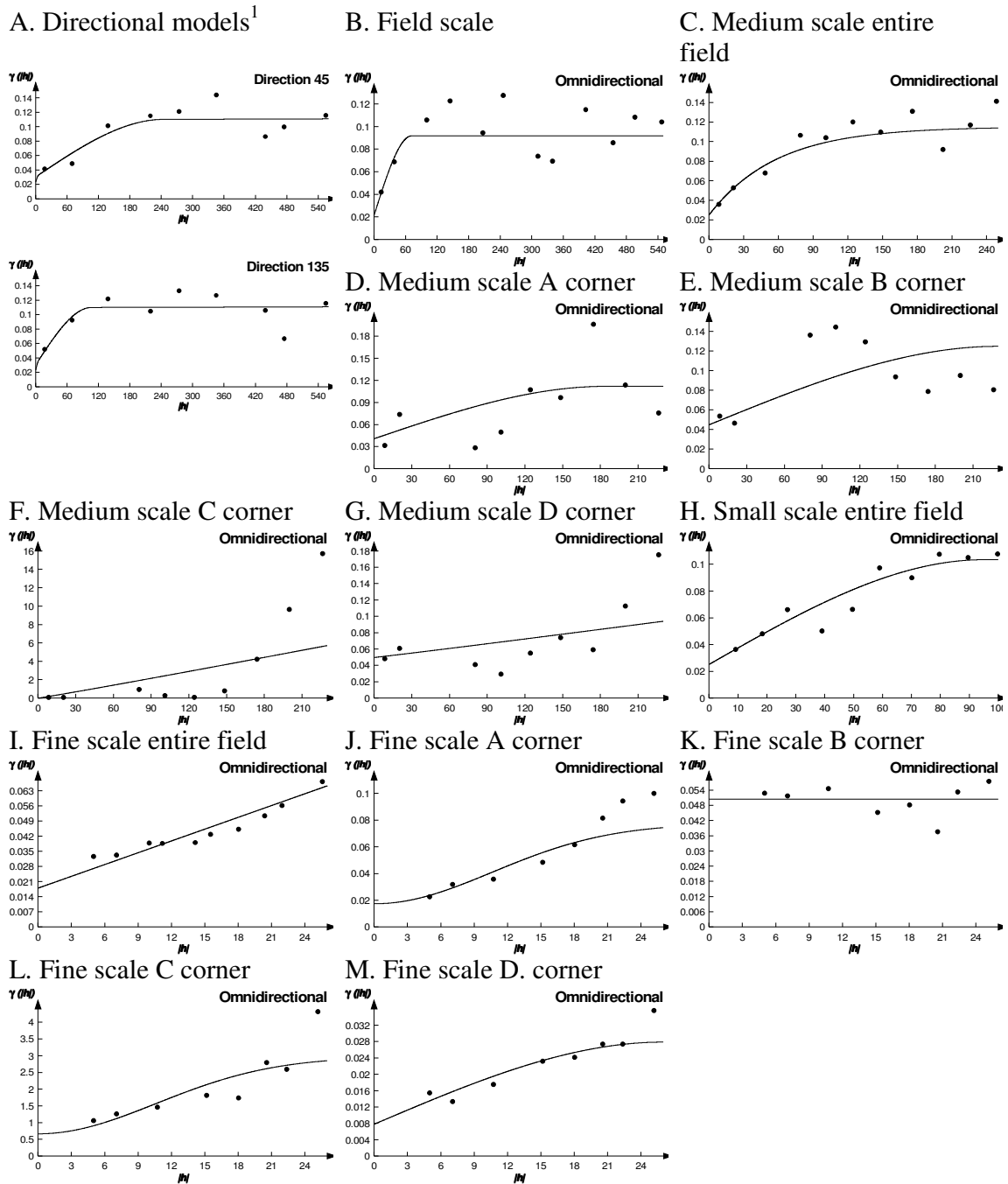


Figure 3.19. Semivariogram models of pH measurement. All models are omnidirectional unless specified.

1. Major and minor directional semivariogram models as simultaneously modeled for further analyses.

All region models fitted with different types of models with their corresponding field scale models. The ranges of the medium scale region model were longer than that of the medium scale field model, but all ranges of the fine scale models were close to or beyond the limit of the scale. The spatial dependence was also relatively constant and high (0.64 ~ 0.78) regardless of scales and regions of the field.

It is rather clear the spatial structures represented by the field models with various scales were divided into two groups: isotropic variables and anisotropic variables. The existence of directionality in the experimental field was the prominent feature separating them resulting in very different approaches in estimation procedures (search strategy in kriging and stochastic simulation). The identities of spatial structures were distinguishable between them in that two nested ranges of isotropic variables were relatively short (< 0.6 m) and all three anisotropic variables' largest range was 2.7 m (Table 4.2). The spatial dependence was not really separated between two groups; rather there was certain trend among different scales and regions within each variable: smaller scales and region models were more spatially structured compared to larger scales and field models. One notable but inexplicable result was that the all experimental semivariogram models of C corner at the medium scale were almost identical and were very poorly fitted using any of the 4 standard semivariogram models.

3.5.1.2. Kriging analysis

Using best fit semivariogram models, soil characteristics were estimated for the experimental field by using kriging analysis (Figure 3.20). Search strategies were determined based on the parameters of semivariogram models. Search areas, which were determined by the range values from the model, were circles for isotropic semivariogram

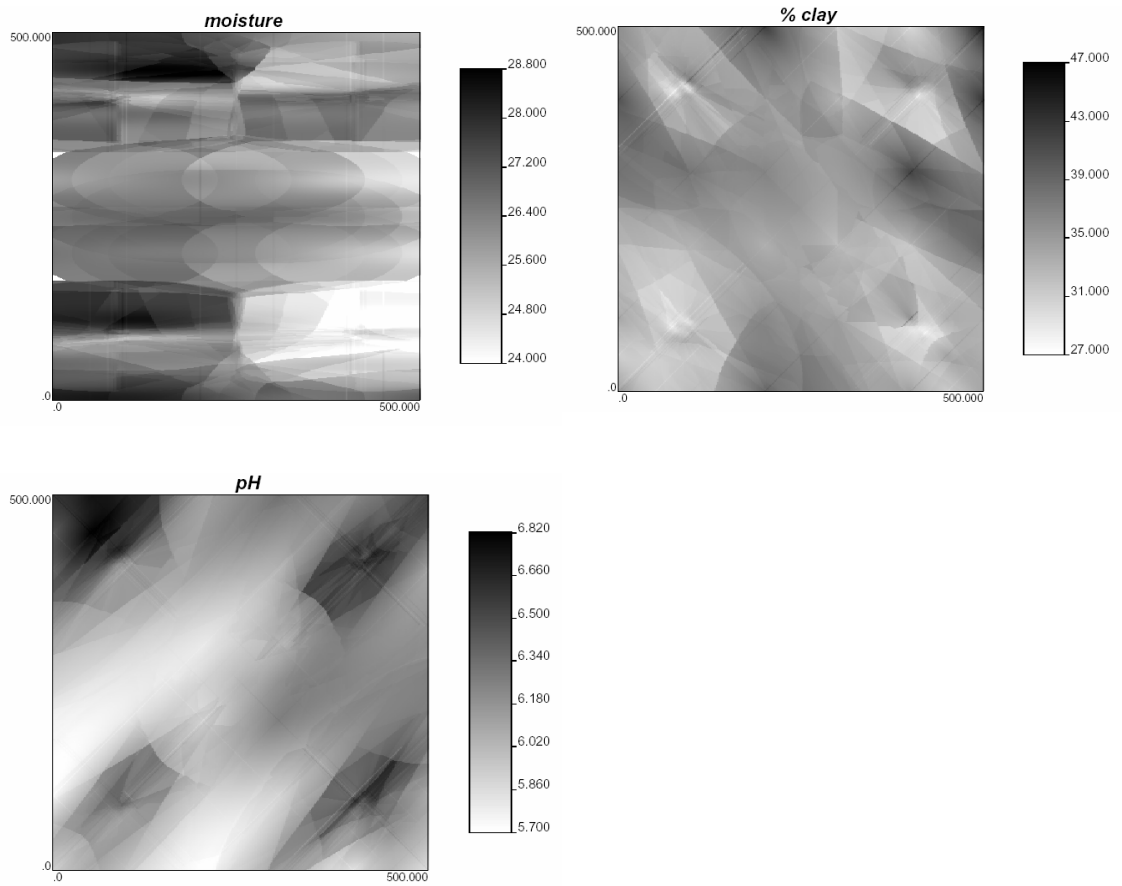


Figure 3.20. Kriged maps of soil characteristics. Maps for carbon, nitrogen, N/C ratio are not presented because of the extensive area of no-estimation due to short ranges.

models and ellipses for anisotropic semivariogram models. The maximum number of samples per estimation was set to 10 for all variables, and minimum number was chosen between 3 and 5 due to the different range values. The ranges of three isotropic models (carbon & nitrogen contents and C/N ratio) were relatively short (0.65, 0.84 and 0.5 m, respectively), so search areas were not large enough to have the minimum number of observations in large portion of the field. Therefore, kriged maps for those isotropic variables are not presented here. Not only the ranges of anisotropic semivariogram models, but also their anisotropy directions were important factor in the search strategy such that kriged maps of moisture content, % clay, and pH measurement had obvious directionality in the of estimation ellipses.

As shown in Figure 3.20, kriging estimates looked more variable in densely sampled areas (near four corners of the field) than in sparsely sampled areas. This is because of the inherent properties of kriging estimation and the search strategy. When samples were sparse, kriging estimation tends to estimate close to the mean of the field and that might create severe bias in some of those areas (Isobel Clark, personal communication). The rule of determining minimum number of samples in the search area indicate that 10 would be a reasonable number (Goovaerts, 1997) but it was not obtainable in large portion of the area at the experimental field, unless the search area covered almost the entire field. That is mainly because the sampling design did not contain fine scale sample distance (Level III) in the middle of the field. After consulting with Mr. Greg Okin, researcher decided to use stochastic simulation analysis, which is relatively free from the sample number constraint. That is probably because the

sequential simulation algorithm utilizes all the available observation around including previously simulated observation.

3.5.1.3. Stochastic simulation analysis

The sequential Gaussian algorithm (sgsim) was chosen for the stochastic simulation because it includes previously simulated values for subsequent simulation, thus minimizes constraints of limited number of sample in the middle of the experimental field. Required parameters were not much different from the kriging estimation (kt3d) since the most important search strategy was obtained from the ranges and directions (in the case of anisotropy) just like in the kriging estimation. When simulated maps were compared with available kriged maps, simulation maps were universally better in that there were not the apparent remnants of the search ellipse that was found in kriged maps (Figure 3.21). The most noticeable difference between two types of maps was the ranges of observation values between maps that the values from the simulation maps represent match the corresponding original value ranges but the ranges of values from the kriged maps are much narrower than the original value ranges. For example, minimum and maximum values of moisture content were 21.26% and 31.17%, and ranges of index bars were 24 – 28.8% (Kriging) and 21 – 31.5% (simulation).

The simulation map of total carbon content indicated existence of overall higher values throughout the entire field. Total nitrogen content map was little lighter overall than carbon content indicating more abundant existence of the area with value from the lower part of the index, but the general patterns of the locations of higher values are very similar. The simulation map of C/N ratio was dominated with midrange value, which is the commonly observed ratio 10:1. It is because of well-matching of simulation maps of

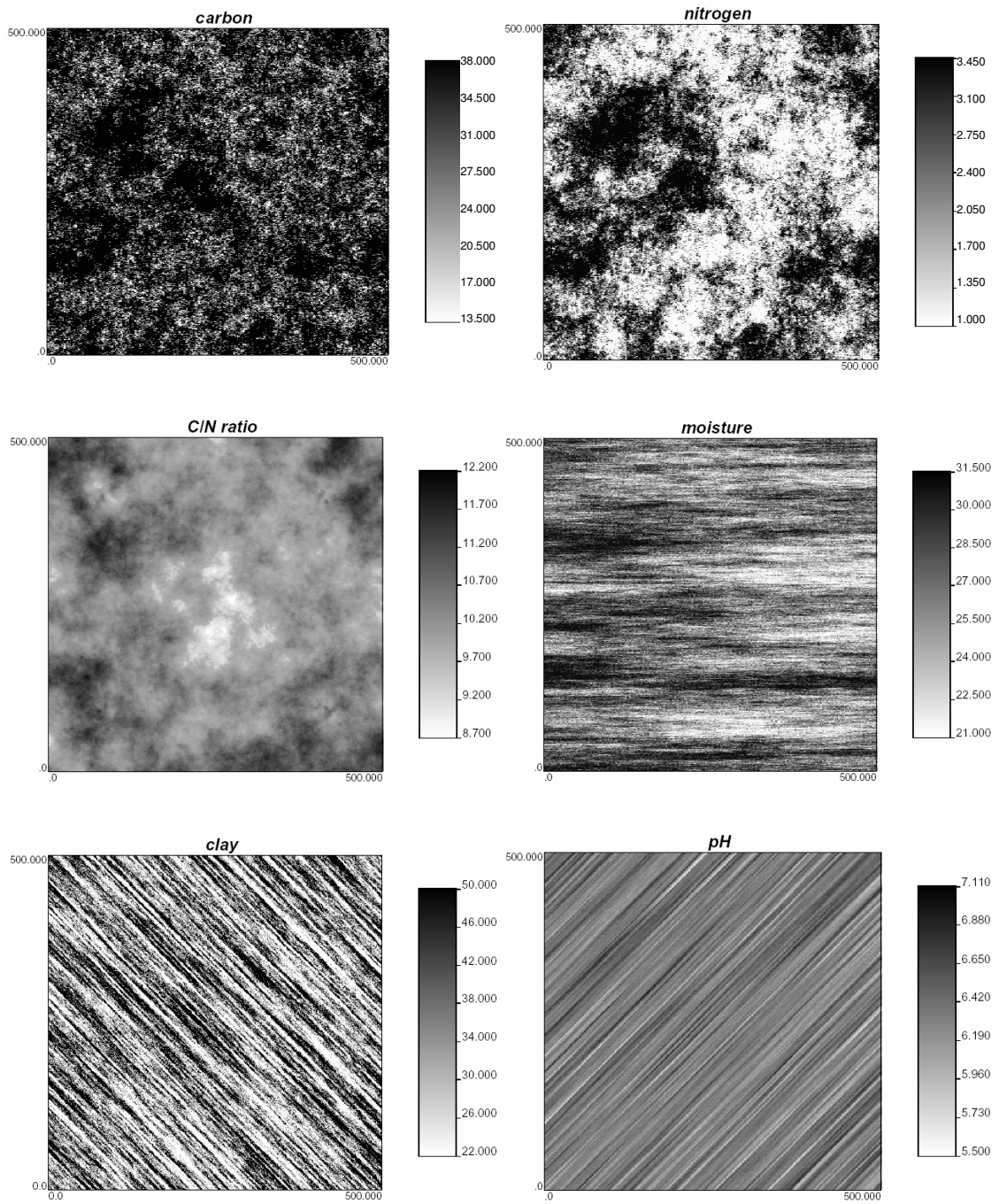


Figure 3.21. Simulated mps of soil characteristics.

total carbon and nitrogen content. The simulation maps of moisture content, % clay and pH possessed the evidence of strong anisotropy by clear linear shapes in the directions of the anisotropy. Moisture content map showed rather clear trend of the field that left section was, in general wetter than right section, and wetter bands were identified in lower section of the field as well. % clay map was simply very noisy, and pH map was relatively uniform in the field compared to other soil characteristics. Also both C/N ratio and pH maps looked very smooth compared to others, because the ranges of indices were significantly less than others (3.5 and 1.61 respectively) (Table 3.5).

As in the semivariogram modeling, it is obvious to group simulation maps into two groups of isotropic and anisotropic variables. The fact of existence of directionalities in the field made big difference in the process of simulation and the output maps. Total carbon and nitrogen content maps were almost identical in the patterns of high and low values, and as a result, the C/N ratio map was not very variable. Moisture maps indicated certain relatively wetter area of the field, but the maps of % clay and pH were showed relatively uniform distributions of values, unless the overall patterns were interfered by strong anisotropy.

3.5.2. Geostatistical analysis of soil microbial community

3.5.2.1. Semivariogram modeling analysis

Microbial abundance and microbial community structure information was used in semivariogram modeling analysis. Microbial abundance was first normalized with mean prior to geostatistical analysis. PCA was performed with microbial community structure data to produce estimation maps using kriging and stochastic simulation. However, the experimental semivariograms based on first three PCs were very noisy to be modeled

reasonably with any of four standard semivariogram models. Pseudo-semivariogram modeling based on relative dissimilarities between pairs of bacterial and fungal communities was the approach used for spatial structure study of microbial communities. Process of binning and issues related to that are basically same with those for soil characteristics (section 3.5.1.1) that lag distances were individually determined for each variable and 1% cut-off rule was also applied.

TOTAL ABUNDANCE

Microbial abundance data were first normalized by dividing with the mean in the beginning of analysis. Lag distances of experimental semivariogram field models were determined: 1 m (field scale), 0.25 m (medium scale), 0.2 m (small scale) and 0.025 m (fine scale). Strong zonal and geometric semivariogram anisotropy was identified: 90° (principal axis) and 180° (minor axis). The lag distances of all the region models were same with the corresponding field model with two exceptions: B (0.3 m) and D (0.2 m) corners in medium scale.

Semivariogram models were chosen by best fitting in both IGF value and graphical examinations (Table 3.7 and Figure 3.22). Like the factors of soil characteristics, region models in medium scale fit least well (A corner, IGF: 0.419) and field model in fine scale fit best (IGF: 0.00234). Fitting of the principal axis directional model was much better than the minor axis directional model and the omnidirectional model. The transformed reference model fitting was between two directional models but was less than the omnidirectional model. 12 out of the 14 models obtained were bounded: 7 were spherical models, 3 were exponential models and 2 were Gaussian models. All the field models were bounded: a spherical model was obtained for the field and fine scale,

Table 3.7. Summary of results from semivariogram analysis of total microbial abundance.

Factor	Scale	Corner	Model ¹	IGF ²	Nugget (C ₀)	Sill (C ₁)	Range (a) ³	NMSD ⁴	
AO	Field	Entire	S	0.0134	0.12	0.078	77	0.39	
		90° ³	S	0.00555	0.144	0.076	432	0.35	
		180°	E	0.0430	0.102	0.068	72	0.4	
	Medium	Entire	E	0.0254	0.118	0.088	142.5	0.43	
		A	P	0.491	0.102	0.002	0.75		
		B	S	0.315	0.142	0.072	> 250	0.34	
		C	P	0.317	0.153	0.02	0.8		
		D	S	0.0378	0.14	0.04	121.9	0.22	
		Entire	G	0.0111	0.13	0.05	56	0.28	
	Small	Fine	Entire	S	0.00234	0.078	0.064	11.2	0.45
			A	S	0.00368	0.048	0.11	> 25	0.70
			B	E	0.0304	0.078	0.062	7.8	0.44
			C	G	0.0120	0.141	0.108	> 25	0.43
			D	S	0.0130	0.066	0.07	9.1	0.51

1. E. Exponential model, G. Gaussian model, P. Power model, and S. Spherical model. For power model, slope and power are recorded under sill and range, respectively and NMSD was not calculated for power model either. By definition, the range of power model was beyond the limit of each scale.

2. Indicative Goodness of fit (2.5.1.2.).

3. Unit of range is cm.

4. Normalized measure of spatial dependence ($C_1 / (C_1 + C_0)$).

* Direction is measured from East in counter-clockwise following the convention of VARIOWIN. Directional models were identified separately, but presented as transformed model in Figure 3. 22.

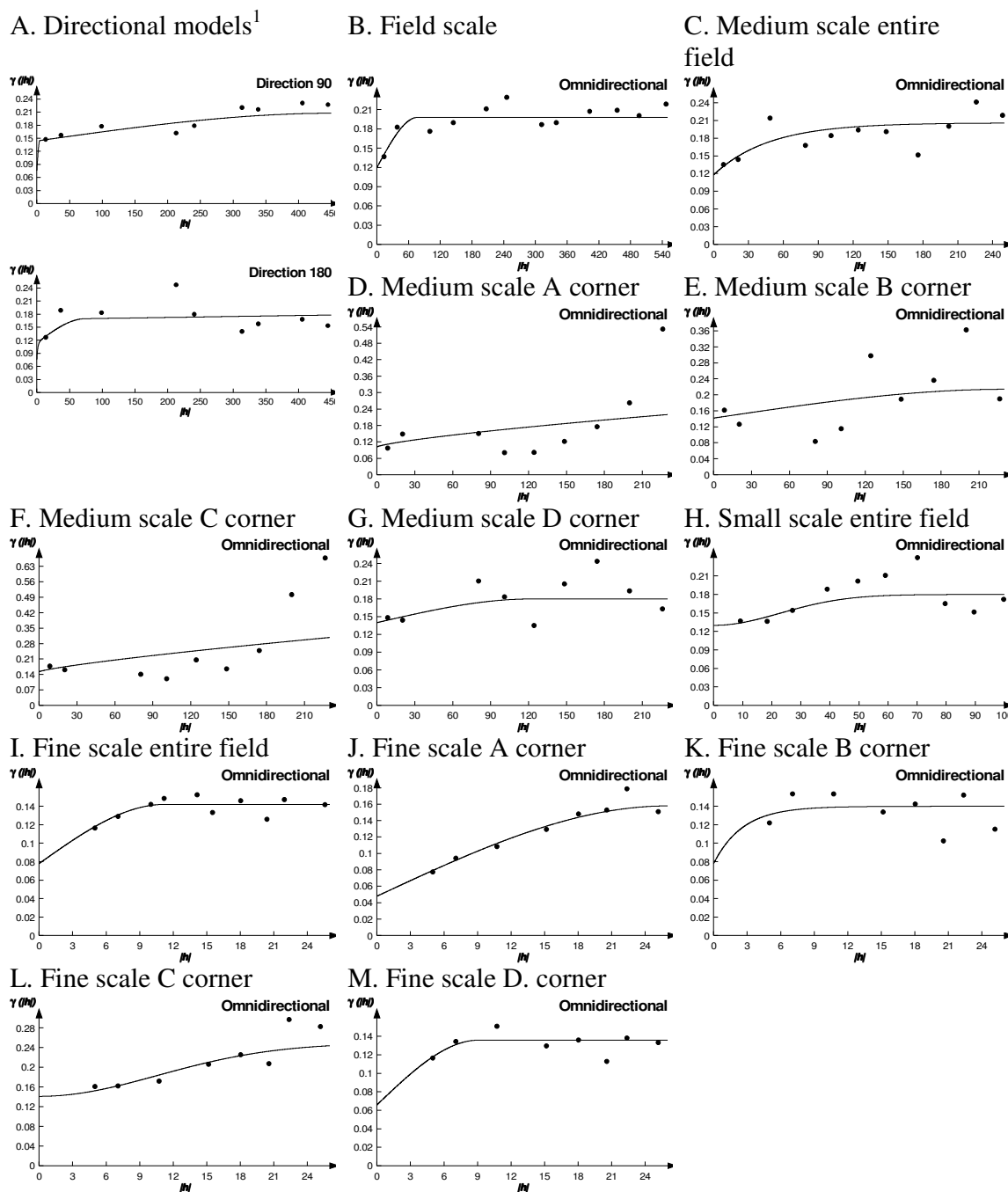


Figure 3.22. Semivariogram models of total microbial abundance measurement. All models are omnidirectional unless specified.

1. Major and minor directional semivariogram models as simultaneously modeled for further analyses

an exponential model for the medium scale and a Gaussian model at the small scale.

The first spatial structure was apparent from the fine scale and the second structure was identified at the small and medium scale at around 0.75 m. The last spatial structure was found at round 2.7 m where three anisotropic soil characteristics (moisture, % clay and pH) showed spatial structure. Spatial dependence was not very high, but it was relatively constant across different scales.

All the region models at the medium scale were modeled with different types of semivariogram models to the medium scale field model, but some of the fine scale region models, especially D corner, were very similar to the field model in both shape and parameters. The ranges of three medium scale region models and two fine scale region models were beyond the limits of the scales, and rest of the ranges were slightly smaller than the ranges of corresponding field models. Spatial dependence was higher in fine scale region models (0.43 – 0.70) compared to rest of models.

Like moisture content, % clay, and pH, total microbial abundance in the field showed strong anisotropy (6:1). The principal axis was Northing – 90° off from the direction of vegetation setting, which was the only obvious environmental gradient present in the experimental field (Table 3.7). In this case, the obvious conclusion is that microbial abundance is unrelated to plant identity. Strong anisotropy was apparent on the both kriged and simulation maps as remnants of search ellipses in the directions of principal axes, as was seen for the soil characteristics (Figure 3.27).

PRINCIPAL COMPONENTS OF BACTERIAL COMMUNITIES

Lag distances of experimental semivariograms field models were determined: 0.5 m (field scale), 0.25 m (medium scale), 0.1 m (small scale) and 0.025 m (fine scale). Lag

Table 3.8. Summary of results from semivariogram analysis of microbial community structure by both dissimilarity and PCs.

Factor	Scale	Corner	Model ¹	IGF ²	Nugget (C ₀)	Sill (C ₁)	Range (a)	NMSD ³		
Bac. PC1	Field	Entire	E	0.0431	0.71	0.27	82.5 cm	0.28		
		Entire	N	0.119	1.01					
	Medium	Left ⁴	P	0.0702	0.79	0.0008	0.94 cm			
		Right	N	0.0411	0.924					
		Entire	G	0.0607	0.83	0.25	17 cm	0.23		
	Small	Entire	S	0.0108	0.74	0.36	18.98 cm	0.33		
			A	N	0.0569	0.485				
		B	N	0.0229	0.749					
		C	P	0.0313	0	0.1068	1			
		D	N	0.0519	1.62					
G		0.0412	0.82	0.2	242 cm	0.20				
Bac. PC2	Field	Entire	G	0.0234	0.83	0.19	170.5 cm	0.19		
Bac. PC3	Field	Entire	G	0.0532	0.79	0.19	127.33 cm	0.19		
Fun. PC1	Field	Entire	G	0.0532	0.79	0.19	127.33 cm	0.19		
		Entire	N	0.0929	0.78					
	Medium	Left	G	0.186	0.141	0.156	110 cm	0.53		
		Right	N	0.0286	1.309					
		Entire	N	0.106	1.02					
	Small	Entire	N	0.0407	0.104					
			A	P	0.0488	0.035	0.00132	0.87		
		B	P	0.0192	0.702	0.082	0.91			
		C	P	0.00783	2.496	0.021	0.83			
		D	S	0.00541	0	0.3	18.2 cm	1.00		
S		0.00354	0.7	0.4	425.9 cm	0.36				
Fun. PC2	Field	Entire	S	0.00354	0.7	0.4	425.9 cm	0.36		
Fun. PC3	Field	Entire	N	0.00476	1					
Bacteria Dissimil.	Field	Entire	S	0.000238	0.8	0.13	49.5 cm	0.14		
		Entire	S	0.000249	0.75	0.18	32.5 cm	0.19		
	Medium	Left	S	0.000617	0.8	0.11	45 cm	0.12		
		Right	S	0.000384	0.82	0.11	45 cm	0.12		
		Entire	E	0.000151	0.88	0.04	70 cm	0.04		
	Small	Entire	S	0.000076	0.76	0.14	7.8 cm	0.16		
			A	S	0.000129	0.75	0.11	7.54 cm	0.13	
		B	S	0.00148	0.71	0.19	8.32 cm	0.21		
		C	S	0.000105	0.83	0.1	7.8 cm	0.11		
		D	S	0.000369	0.86	0.05	10.4 cm	0.05		
		Field	Entire	S	0.000165	0.75	0.12	60.5 cm	0.14	
		Fungi Dissimil.	Medium	Entire	S	0.000252	0.65	0.23	32.5 cm	0.26
				Left	S	0.000269	0.72	0.14	65 cm	0.16
	Right			S	0.000227	0.77	0.11	50 cm	0.13	
Small	Entire		E	0.000141	0.79	0.07	58 cm	0.08		
	Entire		S	0.000171	0.39	0.45	8.32 cm	0.54		
Fine	A		S	0.000357	0.38	0.46	8.06 cm	0.55		
	B		E	0.000082	0.6	0.25	12.22 cm	0.29		
	C		S	0.000537	0.49	0.41	8.58 cm	0.46		
	D		S	0.00101	0.29	0.5	8.58 cm	0.63		

1. E. Exponential model, G. Gaussian model, N. Pure nugget model, P. Power model, and S. Spherical model. For power model, slope and power are recorded under sill and range, respectively and NMSD was not calculated for power model either. By definition, the range of power model was beyond the limit of each scale.

2. Indicative Goodness of fit (2.5.1.2.). 3. Normalized measure of spatial dependence ($C_1 / (C_1 + C_0)$).

4. Left is the left portion of the field with *A. gerardii* is dominant and Right is the right portion of the field with diverse plants are co-dominant.

distances of the Left and Right section of the field models were 0.5 m, and all but one fine scale region models' lag distances were same as the field model (A corner: 0.02 m). Apparent anisotropy was not identified from any of three PCs.

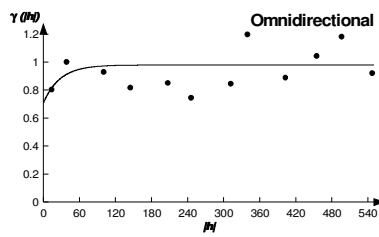
Among three PCs, PC1 was used with different scales, and only field scale semivariogram model was determined with PC2 and PC3 (Table 3.8 and Figure 3.23). Unlikely soil characteristics or microbial abundance data, model fitting was relatively similar throughout different scales and sets of data (PCs). However, region models fit less well than field model. Looking at those field models, even if model fits were better than pure nugget models in IGF value, visual examination did not really confirm it. Therefore, very large amount of unknown explainable variance with poorly fitted models resulted in discarding them for further estimation analyses.

The Left and Right sections of the field were very distinctive in above ground plant composition; the Left section was dominated by the monoculture of *A. gerardii* and the Right section was covered with mixture of 13 plant species. However, the spatial structures of bacterial communities represented by PC1 were not that much different between the sections: Both were unbounded models with relatively low IGF values. Since all region models including fine scale were unbounded models (power or pure nugget model), ranges and spatial dependence were not determined.

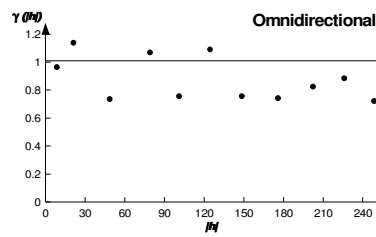
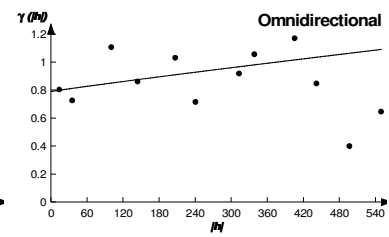
PRINCIPAL COMPONENTS OF FUNGAL COMMUNITIES

The process and results of binning was same as for the PCs of bacterial communities and the lag distances of all regional semivariogram models were same as field model. No apparent anisotropy was identified from any of fungal PCs either.

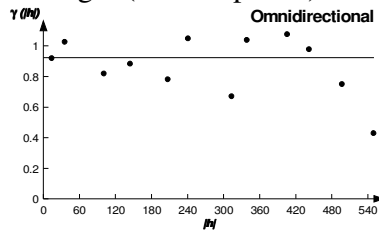
A. Field scale for PC1



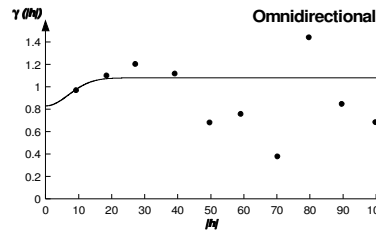
B. Medium scale entire field

C. Left (*A. gerardii*)

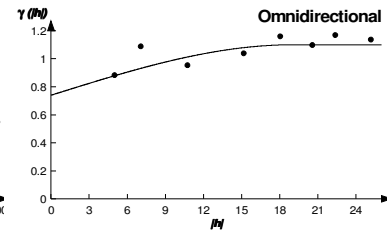
D. Right (diverse plants)



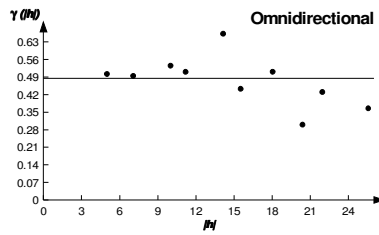
E. Small scale entire field



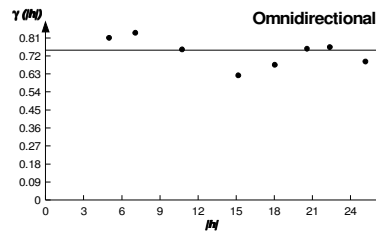
F. Fine scale entire field



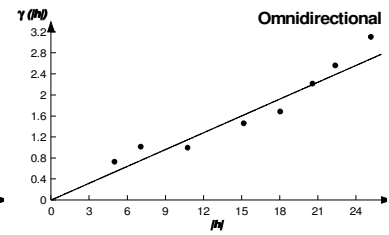
G. Fine scale A corner



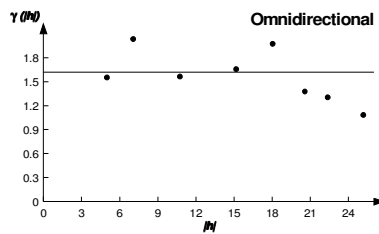
H. Fine scale B corner



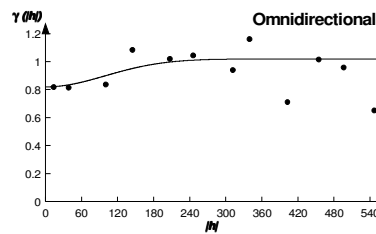
I. Fine scale C corner



J. Fine scale D corner



K. Field scale for PC2



L. Field scale for PC3

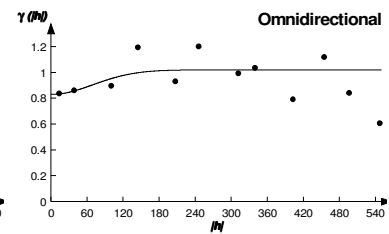
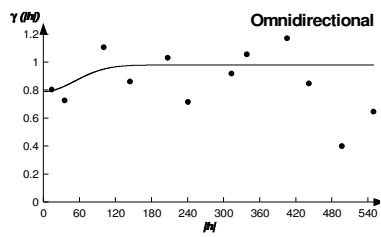
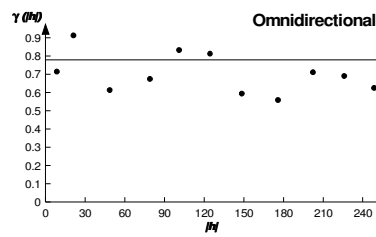
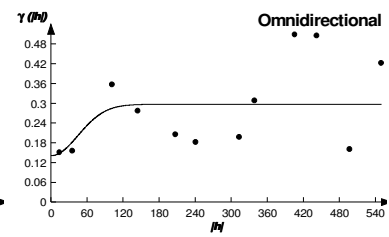


Figure 3.23. Semivariogram models of soil bacterial community structure calculated by first 3 PCs. PC1 was used for regional analysis.

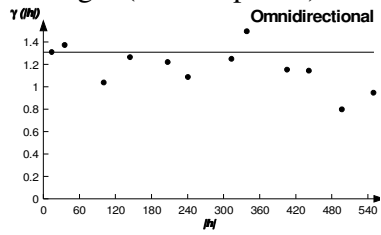
A. Field scale for PC1



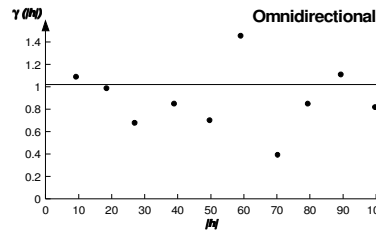
B. Medium scale entire field

C. Left (*A. gerardii*)

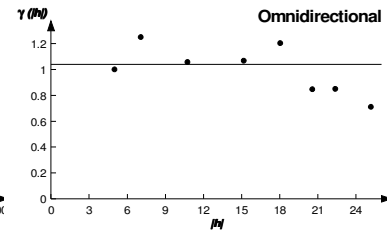
D. Right (diverse plants)



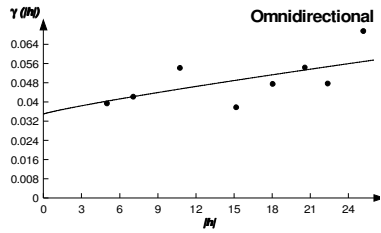
E. Small scale entire field



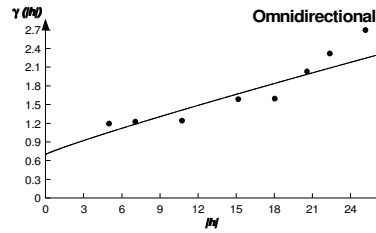
F. Fine scale entire field



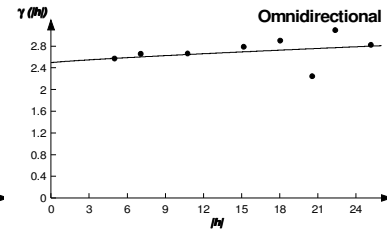
G. Fine scale A corner



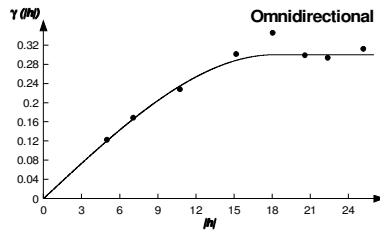
H. Fine scale B corner



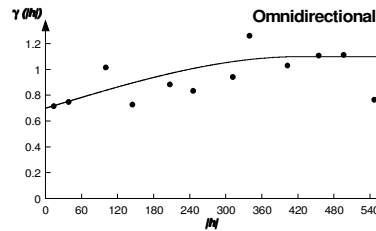
I. Fine scale C corner



J. Fine scale D corner



K. Field scale for PC2



L. Field scale for PC3

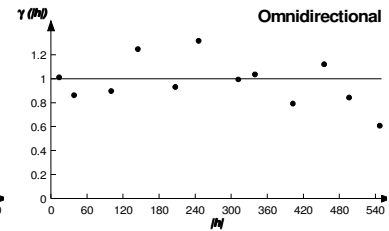


Figure 3.24. Semivariogram models of soil fungal community structure calculated by first 3 PCs. PC1 was used for regional analysis.

Like the bacterial case, only PC1 was analyzed at different scales, while field scale analysis was performed with PC2 and PC3 (Table 3.8 and Figure 3.24). Model fitting trend of fungal PCs across different scales was unique from all previously presented analyses such that some region models (C and D corner) were better fitted than the field model at the fine scale, while all other region models fitted better than their corresponding field models. Like bacterial PCs, the field models of fungal PCs were not very convincing except PC2. With the same reasons of bacterial PCs, it was decided that fungal PCs were not worth to be analyzed with geostatistics and the estimation analyses were not further pursued.

The Left and Right sections of the field were distinguishable in fungal communities, whereas the sections were not obvious from the bacterial communities. The Left section was modeled with a Gaussian with range of 1.1 m and spatial dependence of 0.53. On the contrary, the Right section fungal communities did not show any apparent spatial structure (a pure nugget). The field model at the fine scale was modeled as pure nugget, but all 4 regions showed a certain spatial structure: 3 power models and 1 spherical model (Figure 3.24.G, H, I and J) were obtained.

BACTERIA COMMUNITY ANALYSIS USING RELATIVE DISSIMILARITY

Determined lag distances of experimental semivariogram field models were 0.5 m (field scale), 0.25 m (medium scale), 0.1 m (small scale) and 0.025 m (fine scale). The lag distances of the Left and Right section models were determined to be 0.5 m, and the region models at the fine scale had same lag distance with the field model. There was no apparent anisotropy in bacterial community structures the field.

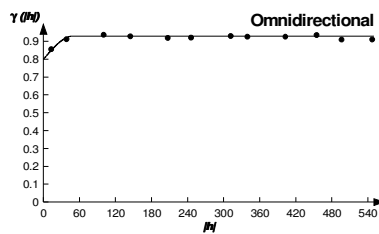
The pseudo-semivariogram models based on relative dissimilarity fit very well in that IGF values were at least one order of magnitude lower than all the previous analyses (Table 3.8 and Figure 3.25). Among the field models, the fine scale model fit best, and it fitted better than all of the fine scale region models. As shown in Figure 3.25, all the experimental semivariograms were very stable after lag 1 (lag 3 at the fine scale models), and they were all bounded model. In fact, all models except small scale field model, which was an exponential, were modeled as a spherical. Since all the models were same or very similar in their shape, the identification of nested structure was rather obvious in that two ranges were identified at 0.078 m (from fine scale) and at around 0.4 m (from field and medium scale). Although small scale was modeled as an exponential with range of 0.7 m, visual observation could not confirm it. Prevalent large nuggets resulted in very low spatial dependence (0.04 – 0.19).

Spatial structures of bacterial communities at the Left and Right sections of the experimental field were very similar to each other. Except nugget, which had difference of 0.02, all other parameters (sill, range and NMSD) were identical. The region models at the fine scale showed some variances among them but it was trivial compared to previously analyzed variables. The A-corner region model was most similar to the field model in both shape and model parameters. The ranges of region models at the fine scale were compatible to each other and the field model (0.075 – 0.104 m). Spatial dependences of the region models were still very low (0.05 – 0.21).

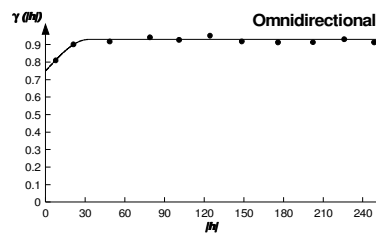
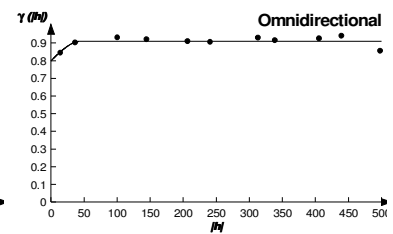
FUNGI COMMUNITY ANALYSIS USING RELATIVE DISSIMILARITY

The process and results of binning was same as the bacterial community analysis, and no apparent anisotropy was identified. Models fit of fungal communities was as good

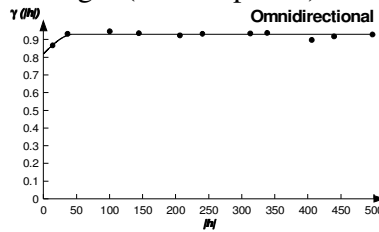
A. Field scale



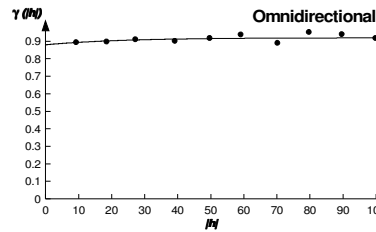
B. Medium scale entire field

C. Left (*A. gerardii*)

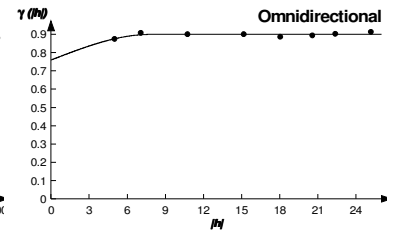
D. Right (diverse plants)



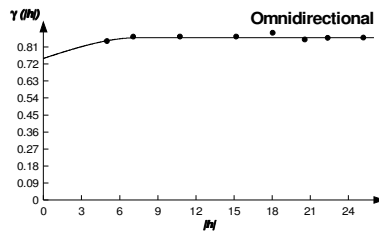
E. Small scale entire field



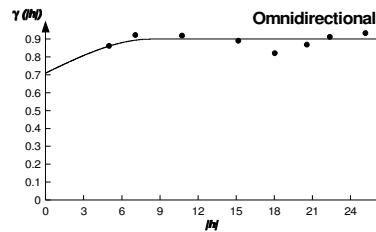
F. Fine scale entire field



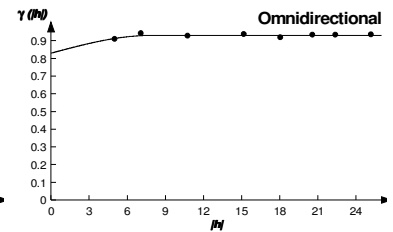
G. Fine scale A corner



H. Fine scale B corner



I. Fine scale C corner



J. Fine scale D corner

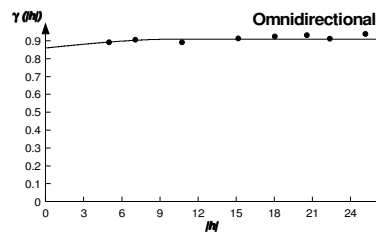
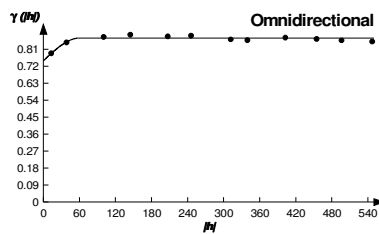
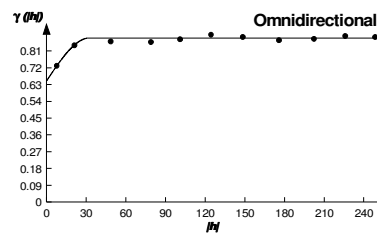
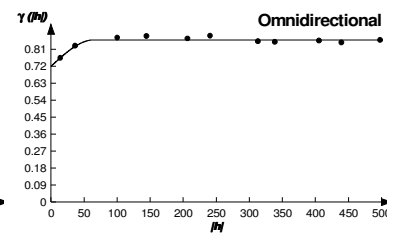


Figure 3.25. Pseudo-semivariogram models of soil bacterial community structure calculated by dissimilarity.

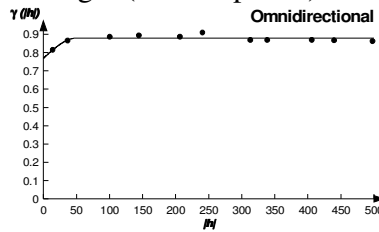
A. Field scale



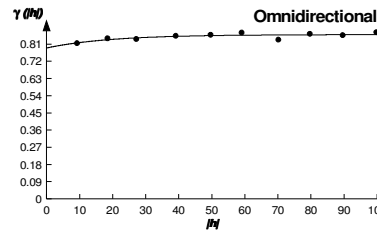
B. Medium scale entire field

C. Left (*A. gerardii*)

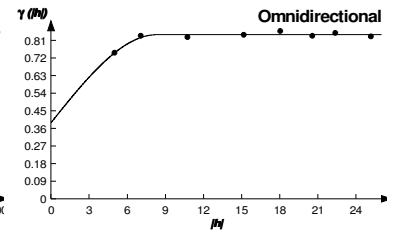
D. Right (diverse plants)



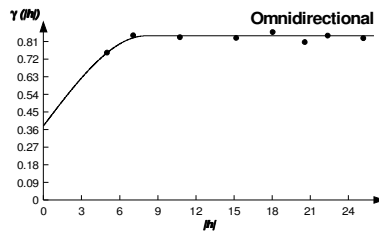
E. Small scale entire field



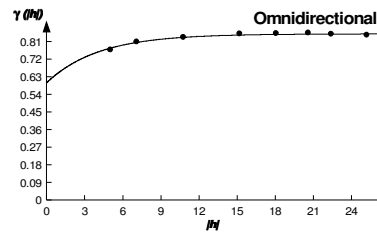
F. Fine scale entire field



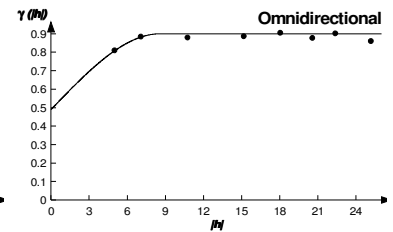
G. Fine scale A corner



H. Fine scale B corner



I. Fine scale C corner



J. Fine scale D corner

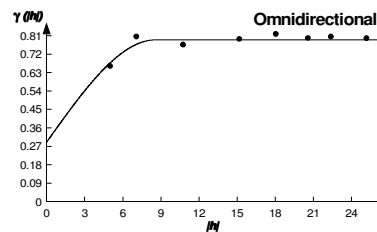


Figure 3.26. Pseudo-semivariogram models of soil fungal community structure calculated by dissimilarity.

as bacterial communities in that all but one IGF value was 10^{-4} or lower (Table 3.8 and Figure 3.26). All the field models fit almost equally well. From visual examination, models were very much like those based on relative dissimilarities of bacterial communities such that, for example, all models were bounded. 8 of the 10 models were a spherical: the small scale field model and the fine scale B corner model were an exponential. Due to the similarity of models to bacterial communities, two spatial structures were identified in fungal communities (0.083 m and 0.6 m). Spatial dependence was very low for the field, medium and small scale models, but the fine scale field model's spatial dependence was highest among all pseudo-semivariogram models (0.54).

Spatial structures of the Left and Right sections of the experimental field were less similar to each other compared to ones from bacterial communities, as they were in semivariogram models by the first PC of bacterial and fungal communities. Both were spherical models and had similar total sill ($C_0 + C_1$), but nugget, range and spatial dependence were distinctive. The region models at the fine scale were more similar to their field model compared to bacterial communities, and the A-corner region model was most similar to the field model, just like bacterial communities. The range of the Left section was 0.65 m and the Right section was 0.5 m. The ranges of region models at the fine scale were compatible when compared with same types of model, but the B-corner model, which was the only exponential model at the fine scale, showed much larger range (0.12 m). The spatial dependences were slightly higher than bacterial communities in comparison with corresponding models, but fine scale models' spatial dependence was higher than others (0.29 – 0.63).

The prevalent large nugget in the spatial structures of bacterial and fungal communities measured by relative dissimilarities of their structure might raise a question of usefulness of geostatistics. If what appeared from those pseudo-semivariograms is what they really were, bacterial and fungal communities might have not been spatially autocorrelated at the experimental field. This question is further discussed in the next chapter.

3.5.2.2. Kriging and stochastic simulation analysis

Normalized total microbial abundance data were used in kriging analysis with the same procedure of soil characteristics for estimation of the microbial abundance distribution of the experimental field (Figure 3.27). Large search area in elliptical shape left no area of the field unestimated with 5 as a minimum number of samples for estimation, but kriging generated a map with weird human face-like shape in the middle of the experimental field. This artificial structure was also thought to be generated due to the sparsely sampled large portion of the field. As a matter of fact, this particular kriged map actually brought the conclusion to conduct stochastic simulation rather than kriging for estimation purpose.

GSLIB's sgsim module was used for stochastic simulation of normalized total microbial abundance (Figure 3.27). Vertically long elliptical search area left vertical lines in simulation map. Soil microorganisms were determined to be most abundant at the vertical zone with about 0.3 m width from 20 to 25 m and the edge of the D corner region. The map was relatively abundant with mid-range value indicating relatively universal microbial abundance distribution throughout the field.

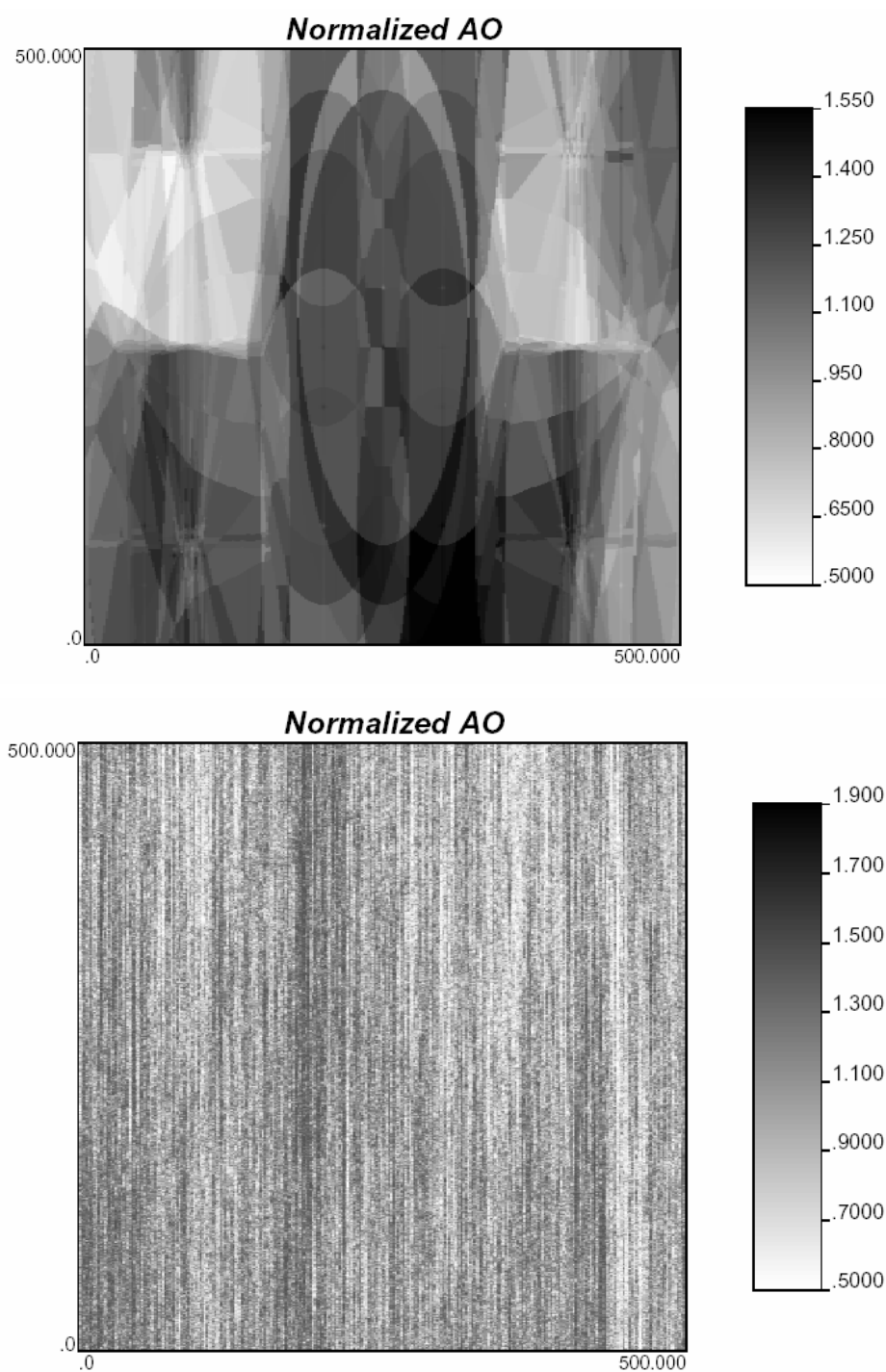


Figure 3.27. Kriged and simulated map of total microbial abundance measurement.

3.5.3. Correlation among variables

Measured soil characteristics were relatively well correlated to each other in that 2/3 pairs of them were significantly correlated to each other by Pearson's correlation analysis (SAS 8.2) (Table 3.9). However, correlation analysis based on dissimilarity matrices by Mantel test showed only 1/3 of them was significant (R Package 4.0) (Table 3.10). Among 10 significant pairs by Pearson's analysis, 3 pairs involved with % clay (vs. total carbon and nitrogen content, and pH) were negatively correlated. All 5 significant pairs by Mantel test were positively correlated even when % clay was involved (vs. pH). Total microbial abundance was significantly correlated only with the C/N ratio in Pearson's analysis, and negatively correlated with many of soil characteristics in both analyses, even if they were insignificant.

Pairwise Mantel test was conducted to determine the correlation rank of soil characteristics with soil microbial community structures. In later section (3.6.3), all soil characteristic measurements were combined into a single dissimilarity matrix to be compared with soil microbial community structures for causal modeling. All soil characteristics were significantly correlated with both bacterial and fungal community structures. All but total nitrogen content and pH were more significantly correlated with bacterial community structure than fungal community structure. Although negatively correlated, pH was most significantly correlated with bacterial community structure and % clay was most positively correlated with bacterial community structure. % clay was most significantly correlated with fungal community structure and the C/N ratio was most negatively correlated with fungal community structure. Total carbon and nitrogen contents were negatively correlated with both bacterial and fungal community structures,

Table 3.9. Results of pairwise correlation analysis of all the measured factors and total microbial abundance.

C	<i>r</i>	-0.04934				
	<i>p</i>	0.4889				
clay	<i>r</i>	-0.05114	-0.14926			
	<i>p</i>	0.4732	0.0354			
mois	<i>r</i>	-0.04509	0.28364	0.00177		
	<i>p</i>	0.5272	<.0001	0.9802		
N	<i>r</i>	-0.11452	0.95935	-0.14870	0.24080	
	<i>p</i>	0.1073	<.0001	0.0361	0.0006	
C/N	<i>r</i>	0.19749	0.36166	-0.02440	0.21137	0.08831
	<i>p</i>	0.0052	<.0001	0.7322	0.0027	0.2148
pH	<i>r</i>	0.02015	0.27417	-0.24073	-0.04663	0.30812
	<i>p</i>	0.7776	<.0001	0.0006	0.5131	<.0001
		AO	C	clay	mois	N
						C/N

Bold characters indicate significant correlations at the Bonferroni-corrected probability level of (0.05/21 =) 0.0023 for an overall significance test of 0.05 over 21 simultaneous tests.

Table 3.10. Results of pairwise Mantel test of soil characteristics and microbial community structures based on dissimilarity matrices. The significant test was based on Mantel's t approximation.

C	r	-0.0180							
	P	0.249							
clay	r	-0.0160	0.0520						
	P	0.306	0.090						
mois	r	-0.0223	0.0442	-0.0052					
	P	0.199	0.087	0.446					
N	r	0.0044	0.9010	0.0576	0.0363				
	P	0.433	<0.001	0.063	0.125				
C/N	r	0.0353	0.1892	-0.0312	0.0405	0.1005			
	P	0.110	<0.001	0.228	0.124	0.002			
pH	r	0.0104	0.0468	0.0938	0.0143	0.0909	-0.0336		
	P	0.350	0.079	0.008	0.331	0.002	0.174		
Bact	r	0.0760	-0.0812	0.2171	0.0879	-0.0953	0.0879	-0.3566	
	P	0.048	0.028	0.010	0.029	0.025	0.028	0.006	
Fungi	r	0.1872	-0.2062	0.7297	0.2327	-0.0940	-0.4438	0.2563	0.2829
	P	0.018	0.020	0.001	0.017	0.035	0.005	0.015	0.006
		AO	C	clay	mois	N	C/N	pH	Bact

Bold characters indicate significant correlations at the Bonferroni-corrected probability level of $(0.05/36 =) 0.0014$ for an overall significance test of 0.05 over 21 simultaneous tests.

but their correlations were relatively less significant. They were also negatively correlated with total microbial abundance in both Pearson's analysis and Mantel test with the exception of total nitrogen content in Mantel test. Community structures between bacteria and fungi were significantly correlated ($r = 0.2829$, $P = 0.006$).

Some selected experimental cross semivariograms were presented for identification of common microscale variability (Figure 3.28). Only three cross semivariograms had relatively low nuggets among all 21 cross semivariograms (A, B and C). Some almost reached sill within the limits of the experimental field (A and C), and the other was rather random in its experimental cross semivariance (B). There was one with almost flat cross semivariance with large nugget (D). Experimental cross semivariograms with large nugget and real fluctuations of cross semivariance throughout the lag distances were most abundant (E and F). Prevalent large nugget in cross semivariograms indicates that sizable nuggets found in semivariograms of individual variables are originated from the variability existing below the measurement unit (0.05 m).

3.6. EXPERIMENT 5: CONTROLLING FACTORS OF SOIL MICROBIAL COMMUNITY STRUCTURE

3.6.1. Spatial distribution of microbial community structures

Bacterial and fungal community structures were analyzed using DFA to compare the separate levels of sampling. Bacterial communities of the five regions from the level II sampling design were visualized in DF space using the first two discriminant functions (Figure 3.29.A). All regions contained five samples, and the regions were well separated from each other. The separation of the regions was obvious on the first function, but only

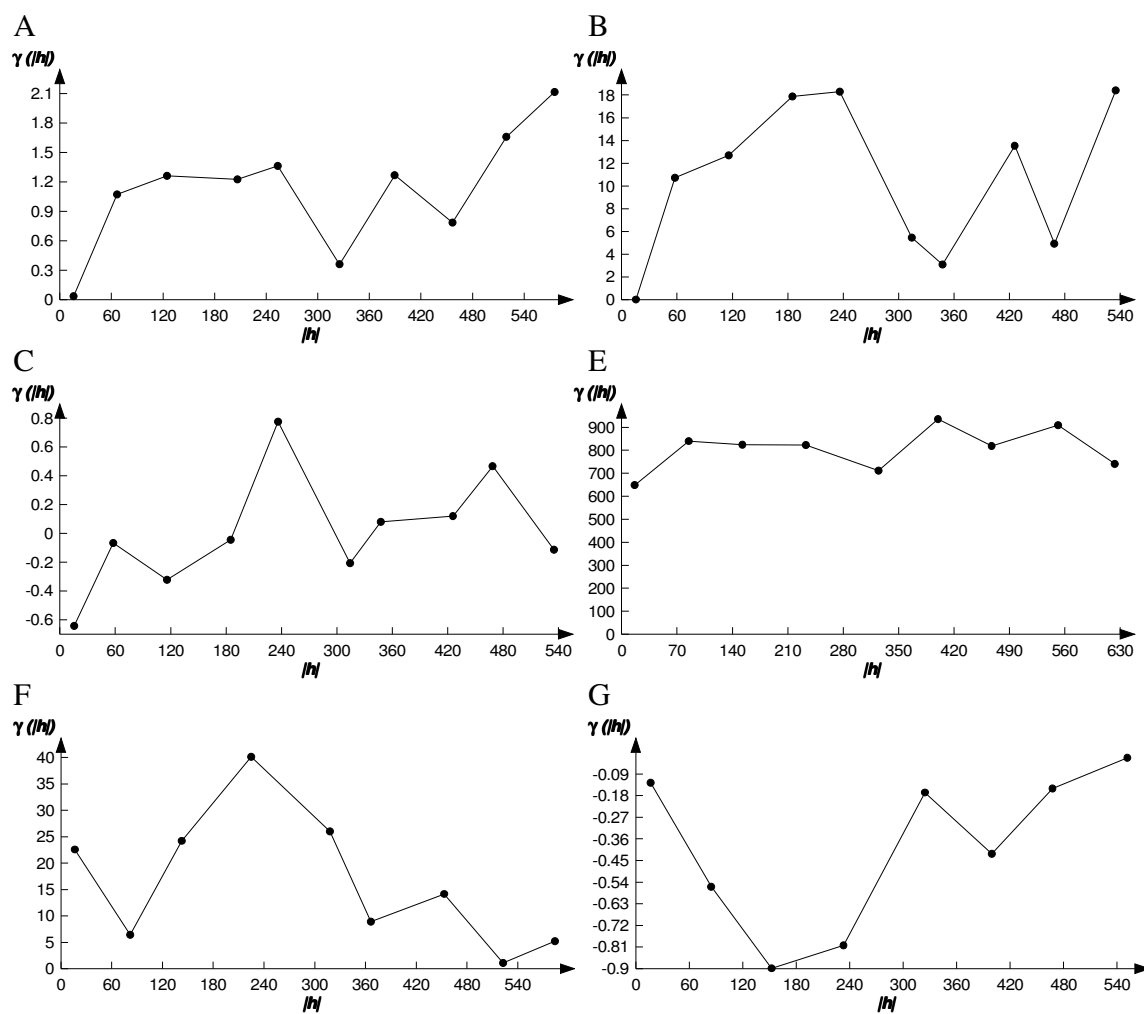


Figure 3.28. Cross semivariograms of selected pairs of variables. A. total N content and pH (Pearson's $r = 0.308$ & Mantel's $r = 0.091$), B. total C content and pH (0.274 & 0.047), C. % clay and moisture content (0.002 & -0.005), D. total C and total N content (0.959 & 0.901), E. total C content and C/N ratio (0.362 & 0.190) and F. % clay and pH (-0.241 & 0.094).

the C region separated from the other 4 on the second function axis. Overall matching of the groups was 96% (24 out of 25) of the original membership and the first two functions explained 89.8% of total variance. Bacterial communities of the four corners of the field 35 samples, except for C which comprised 33 samples (Figure 3.29.B). The analysis yielded a large overlap between the B and C corner groups, such that 5 samples from the C corner group were reassigned into the B corner group. Overall matching with the original group assignment was 87.7%, and explainable variance by the two functions was 86.2%.

Fungal communities analyzed by DFA yielded (Figure 3.30.A). Five groups that were well separated on the first function, but only the C region separated on the second function axis, as was seen for the bacterial communities. Overall matching was 100% of the original group membership and two functions explained 92.7% of total variance. Fungal communities of level III sampling design were visualized on DFA field in the same way (Figure 3.30.B). In this case there was close overlap between A and B region groups, but the rest of groups were well separated on both axes. Overall matching was 94.2% and explainable portion of total variance was 85.6%.

The distribution of both bacterial and fungal communities were very similar on level II samples DF plot in that the C region was clearly separated from rest of the regions on the second function axis. This might be generated by the fact highest plant abundance and diversity from the samples in the C region. Level III samples were well separated to each other on DF plot except the B corner, which was largely overlapped with different corner samples between bacterial and fungal communities. Level III

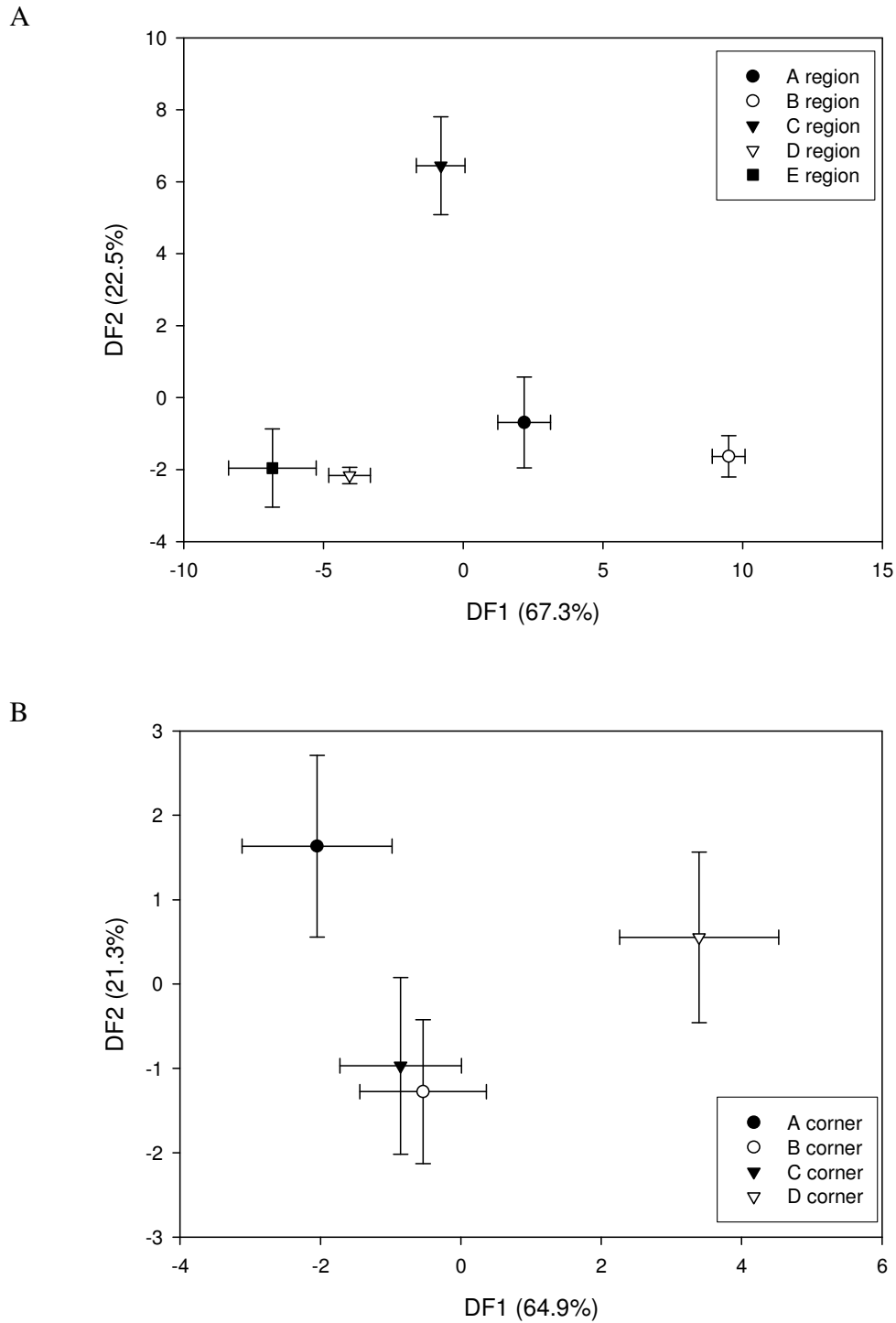
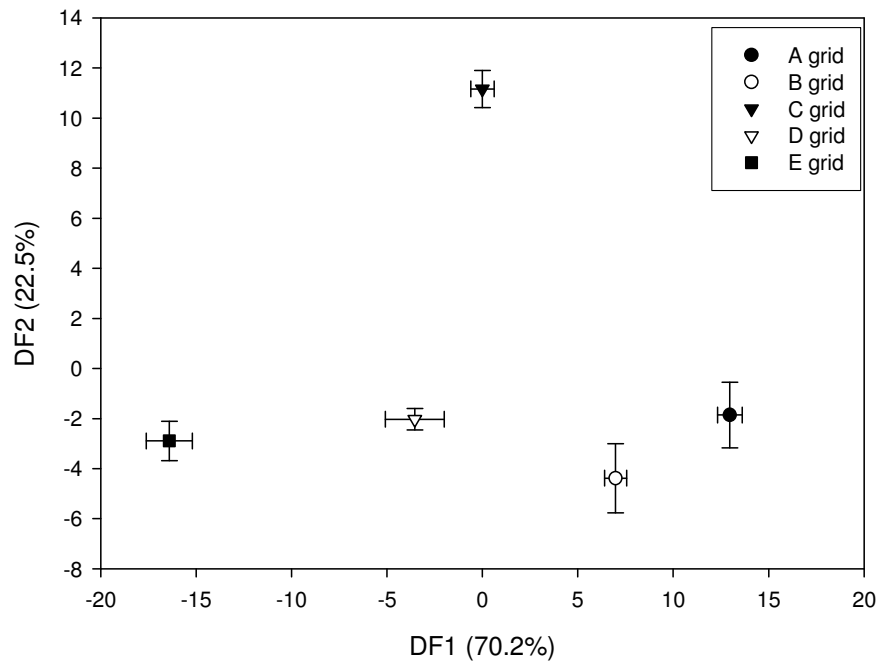


Figure 3.29. DF plots of bacterial community structure of different locations of the field at different level of sampling design. Error bars represent 1 standard deviation. A. 5 regions from level II, B. 4 corners from level III.

A



B

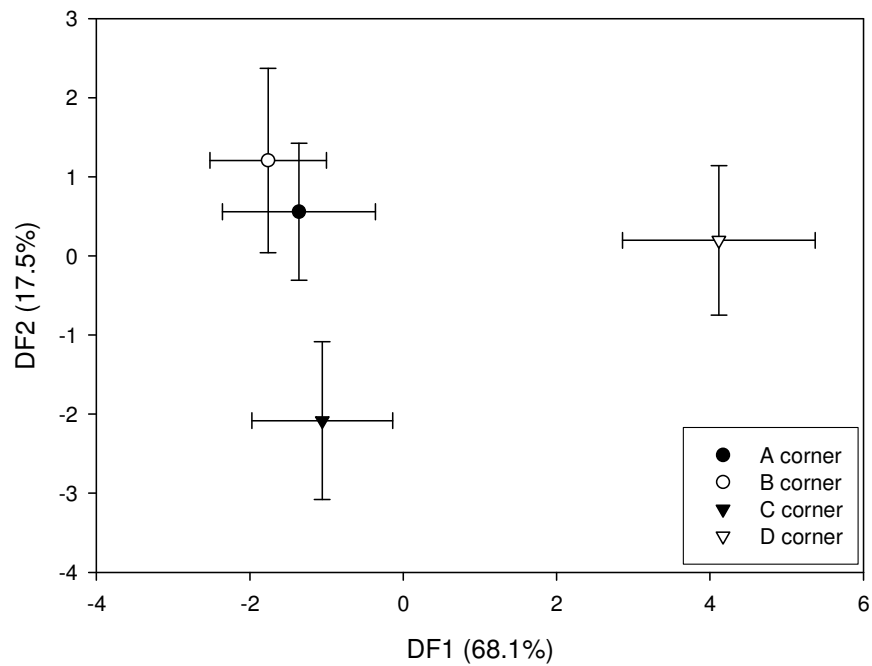


Figure 3.30. DF plots of fungal community structure of different locations of the field at different level of sampling design. Error bars represent 1 standard deviation.
 A. 5 regions from level II, B. 4 corners from level III.

sampling, which involved more samples than level II, may give more general phenomenon of distinction between bacterial and fungal communities (Zak *et al.*, 2003).

3.6.2. Mantel tests applied to the microbial community, soil characteristics, and distance

Dissimilarity matrices were prepared with multivariate binary data of bacterial and fungal community structures each using the Jaccard coefficient. A soil characteristics dissimilarity matrix was prepared from the 5 measured and one derived variables: moisture content, % clay, pH, carbon content, nitrogen content, and C/N ratio. Attempt to construct a dissimilarity matrix of plant communities was not successful, because the vegetation data, especially in the level III sampling points, was too sparse. Of the 199 sampling points in the field, only 97 points (48.7%) had even a single vegetation datum within the 3-cm diameter range made necessary by the limitation of sample-point distance in the level III sampling design.

A total of 13 plant species were identified from the field (using all the sampling points): *Andropogon gerardii*, *Andropogon virginicus*, *Anthozanthum odoratum*, *Apocynum cannabinum*, *Carduus acanthoides*, *Coreopsis lanceolata*, *Festuca elatior*, *Gelium verum*, *Phytolacca americana*, *Plantago lanciolata*, *Rudbeckia herta*, *Schizachyrium scoparium* and *Solidago* spp. Among them, 7 species were found in the X1 plot section and 12 species were found in the X4 plot section. *A. gerardii* was the grass species planted in the X1 section, and *A. virginicus* and *S. scoparium* were two of the three grass species planted in the X4 section. There were four forb species planted throughout the field that were also found: *A. cannabinum*, *C. lanceolata*, *R. herta* and *Solidago* spp. The most abundant species found in each corner of the level III sampling

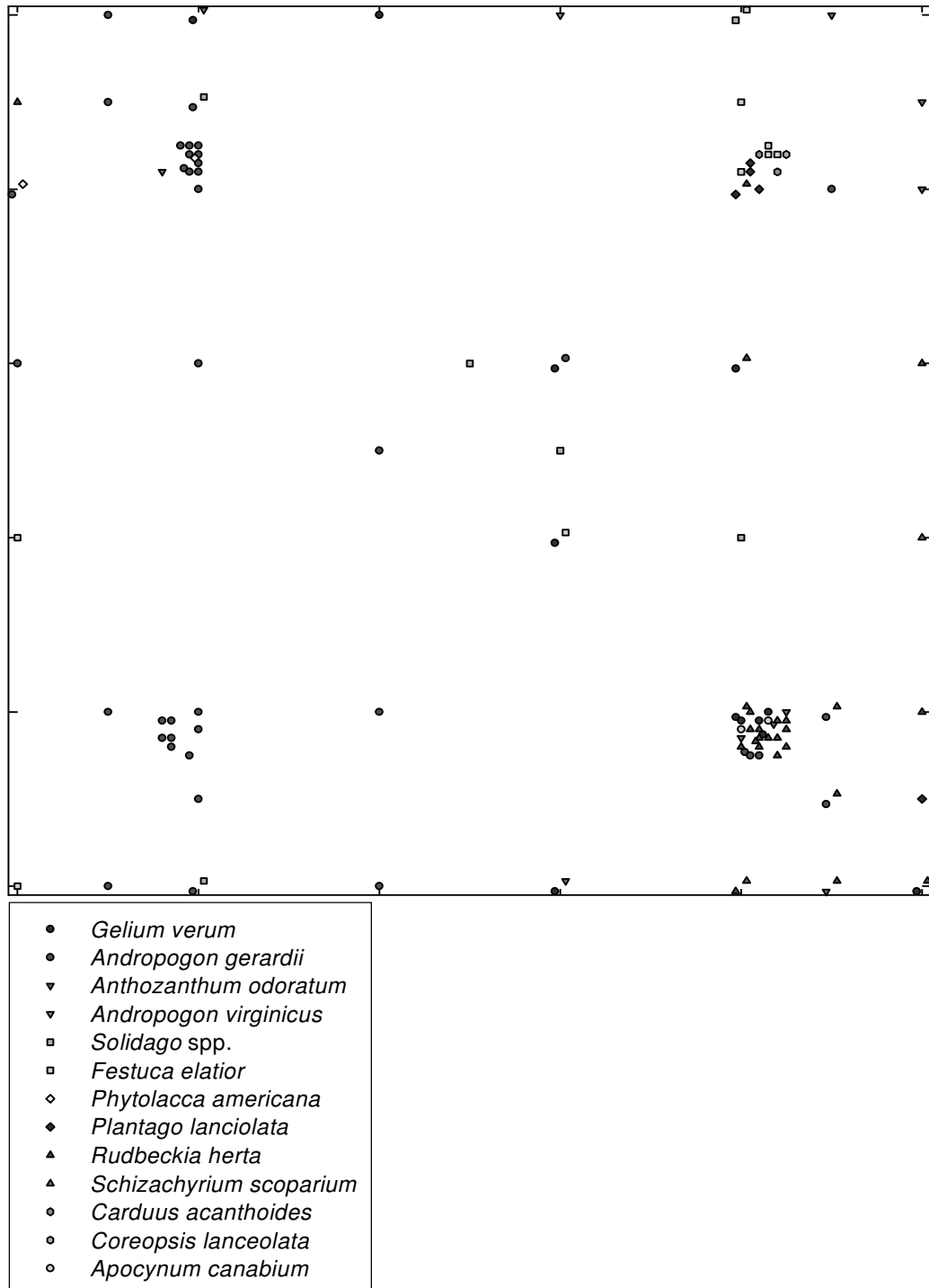


Figure 3.31. Location map of vegetation distribution on each sampling point.

areas were *A. gerardii* (A and D), *F. elatior* (B) and *R. herta* (C) (Figure 3.31). Of the 42 individual plants identified from X1, 30 (71.4%) were *A. gerardii*. There was higher density of vegetation in X4, which identified 72 species from the similar area at X1, and 33 of 72 (45.8%) individuals were among those grasses and forbs planted as part of the experiment.

Because vegetation could not be compared simultaneously with the other soil components and microbial community structures using the Mantel test, a stand-alone vegetation effect was measured instead. DFA was used, and group membership matching between X1 and X4 was measured for soil characteristics and microbial community structures to show the differences in vegetation (Table 3.11). The overall matching of the original membership between X1 and X4 of the field was fairly high in microbial community structure: 78.1% for bacterial communities and 79.6% for fungal communities. Soil characteristics matching was lower than that of microbial community structures, but still considered to be high enough (66.3%). However, significance tests between left and right section of the field on individual soil characteristics measurements revealed inconsistency of vegetation effect among soil characteristics measurements (Table 3.12). Only moisture content and pH are significantly different between left and right sections of the field with distinct vegetation development. Total microbial abundance did not show vegetational effect either ($P = 0.5553$).

A Mantel test was used to analyze bacterial and fungal community structure individually with respect to the soil characteristics and distance. Due to large datasets (199×199 matrices) significance was determined based on the Mantel's t approximation probability, which was suggested to be used for larger matrix (50 X 50) to save computer

Table 3.11. Summary of results of discriminant function analysis of soil characteristics and microbial community structure for vegetation effect. Percentage of overall matching of original memberships were presented.

	Left	Right	Total
Bacterial community	71.7% (71/99)	84.5% (82/97)	78.1% (153/196)
Fungal community	82.8% (82/99)	76.3% (74/97)	79.6% (156/196)
Soil characteristics	60.6% (60/99)	72.2% (70/97)	66.3% (130/196)

Table 3.12. Summary of the results of description statistics on soil characteristics and total microbial abundance between distinct vegetation patterns.

	Left (<i>n</i> = 99)		Right (<i>n</i> = 97)		<i>t</i>	<i>P</i>
	Mean	Variance	Mean	Variance		
Total microbial abundance	1.04E+9	2.10E+17	1.08E+9	2.11E+17	-0.59	0.5553
Total carbon content	535.13	10047.82	521.33	8433.70	1.00	0.3162
Total nitrogen content	51.16	72.27	50.30	71.91	0.71	0.4794
C / N ratio	10.44	0.34	10.36	0.21	1.06	0.2912
% clay	32.43	16.90	32.54	19.50	-0.18	0.8588
Moisture content	26.76	2.58	25.53	2.95	5.17	<.0001
pH	6.25	0.079	6.39	0.080	-3.49	0.0006

time (Sokal, and Rohlf, 1995; Casgrain, and Legendre, 2001). Prepared matrices were fed into the R package and multiple Mantel and partial Mantel tests for all the possible combinations were performed (Table 3.13). All tests were significant and Mantel coefficient (r) were larger for the fungal community structure than for the bacterial community structure, except partial Mantel test for soil characteristics and distance with fungal community structure was partialled out. These results were sort of corresponding what appeared from Table 3.10 in that all individual soil characteristics were significant with both bacterial and fungal communities and the associations were more significant with fungal communities in general. The high association between microbial communities and distance indicates the existence of spatial autocorrelation and suggests the usefulness of geostatistics in analyzing a distribution of soil microbial communities.

3.6.3. Causal modeling

The results of the simple Mantel and partial Mantel tests were used for causal modeling (Table 2.5). Since all of possible tests were significant for both bacterial and fungal community structures, model 4 was determined to be the one that best represented the relationships among microbial community structure, soil characteristics and distance (Table 3.13 and Figure 3.32). A problem here is that every components can be anywhere in the model. However, as it is stated in the legend of table 2.5, either one of the simple Mantel tests of AC or BC can be insignificant for model 4. This might indicate that the simple Mantel test between A and B is probably most significant of all the simple Mantel tests. In bacterial community structure, correlation between distance and soil characteristics was most significant ($r = 0.4039$, $P = 0.000001$). Between distance and soil characteristics, a distance control on soil properties made more sense than the reverse

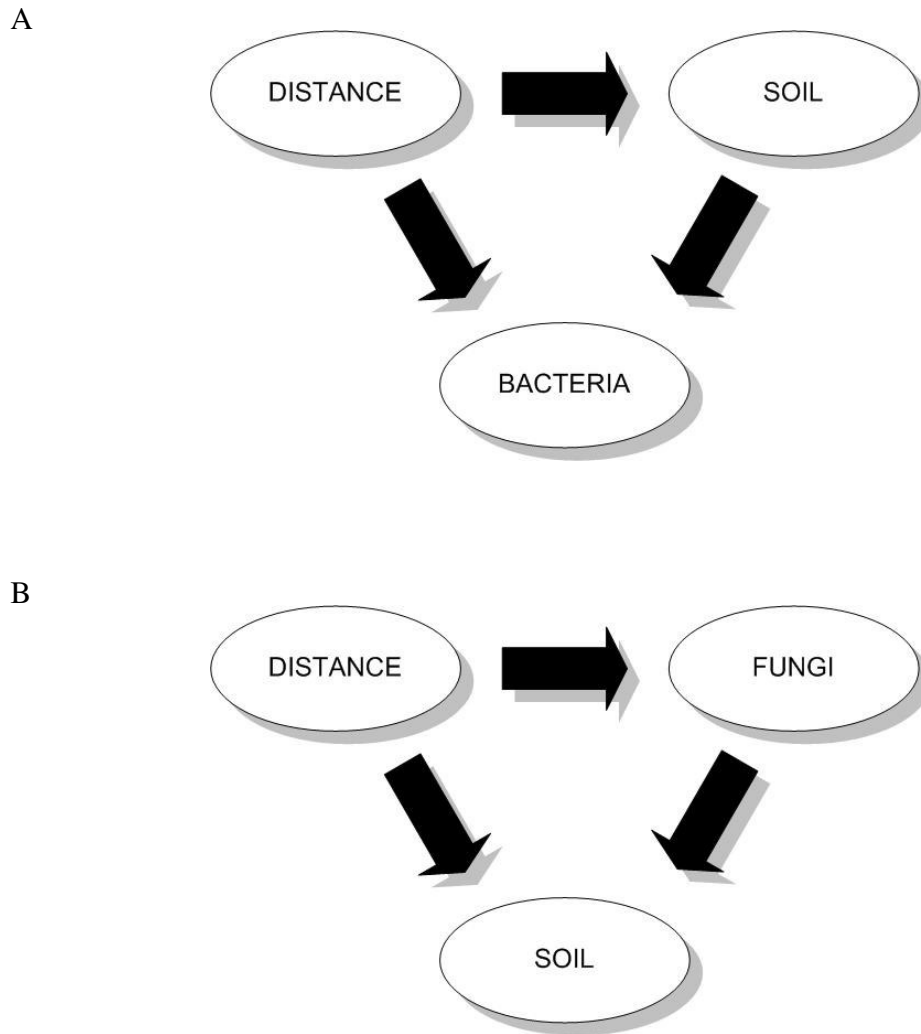


Figure 3.32. Causal models for bacterial community (A) and fungal community (B) with environmental factors based on Table 3.8. Plant was not included in the modeling because there was not enough data to make plant factor statistically significant.

such that in the final model distance influences both soil characteristics and bacterial community structure, and bacterial community structure is also controlled by soil characteristics (Figure 3.32.A). Following similar logic, distance was determined to be the major controlling factor, however in this case the fungal community structure was placed in a controlling position due to its high coefficient ($r = 0.4613$, $P = 0.000001$) (Figure 3.32.B).

Table 3.13. Summary of results from the Mantel tests for microbial community with environmental factors. Upper right section is the results of the Mantel tests and lower left section is the results of the partial Mantel tests.

	Bacteria	Soil	Distance
Bacteria	—	$r = 0.3004$ $P = 0.000010^*$	$r = 0.3466$ $P = 0.000010^*$
Soil	$r = 0.2879$ $P = 0.000305†$	—	$r = 0.4039$ $P = 0.000001^*$
Distance	$r = 0.2991$ $P = 0.000021†$	$r = 0.3815$ $P = 0.000012†$	—

	Fungi	Soil	Distance
Fungi	—	$r = 0.4322$ $P = 0.000001^*$	$r = 0.4613$ $P = 0.000001^*$
Soil	$r = 0.3614$ $P = 0.000031†$	—	$r = 0.4039$ $P = 0.000001^*$
Distance	$r = 0.3908$ $P = 0.000026†$	$r = 0.3061$ $P = 0.000067†$	—

* and † indicate significant association by the Mantel test and the partial Mantel test.

4. DISCUSSION

4.1. EFFECT OF SAMPLE SIZE IN THE STUDY OF SOIL MICROBIAL COMMUNITY

Among many issues in a sampling strategy of microbial ecology, sample size is often ignored (Ranjard, and Richaume, 2001). However, both sample size and distance between sampling locations are critical for proper interpretation of the results from highly heterogeneous soil systems (Grundmann, and Gourbiere, 1999). Microbial measurements for the present study were analyzed systematically and formal statistical analyses were employed to determine amount of samples for the best representing of the microbial communities.

4.1.1. Enumeration

Viable abundance on 1/10 diluted R2A media separated 0.25-g samples from rest of sample sizes (Figure 3.1.A). Both 0.1-g and 0.25-g samples had very large error bars due to large among-replicate variations and the source of variation was identified as an experimental error. Colonies were found on several plates of 0.1-g and 0.25-g samples with high dilution (10^{-3} and 10^{-4}), and they had a higher impact on final counts than counts from lower dilutions plates, so the final counts of 0.1-g and 0.25-g samples had much larger standard deviation. Plate countings with serially diluted soil samples were off from what they were supposed to be by the calculations using dilution factors (Polyanskaya *et al.*, 2000). It is generally expected that smaller sample sizes would be more variable among replicates with respect to viable abundance, but it turned out that 0.01-g samples were the least variable of all sizes examined (Figure 3.1.B). This could be explained at least partially by the existence of experimental errors for other sample sizes (0.1 and 0.25 g).

The limitations of viable counts have been discussed (e.g., Polyanskaya *et al.*, 2000); inaccuracy by inflation due to higher dilution factors and lack of real non-selective media such that only 1-10% of the total community is represented on currently used general media. The difference in count trends of different sample sizes between viable and direct counts is consistent with the perceived weakness in cultural counts. Abundance is measured as the average of cell counts from the highly heterogeneous microbial habitats in the soil. Therefore, smaller sample sizes are expected to have very variable abundance leading higher variance. Larger sample sizes tend to have more counts with lower variance (Figure 4.1), because larger sample sizes would have averaging effect by homogenization of smaller sample sizes. In the present study these criteria were better demonstrated with direct count measurements than with viable counts.

4.1.2. Microbial community structure

The DNA-extraction yield can be displayed in two different ways. One is per unit volume of final solution (ml) and the other is per unit amount of sample soil (g). Due to the limited soil sample capacity of the UltraClean™ soil DNA isolation kit (MoBio Laboratories) for these soil samples, there were some variations in the extraction procedures; two tubes were used for the 1.0-g sample and UltraClean™ Mega Soil DNA isolation kit was used for the 10.0-g sample. The identification of the effect of different sample sizes was the purpose of the analysis. Therefore, data were presented as the amount of DNA obtained per unit of soil. The DNA yields ranged between 0.5 – 32.7 µg DNA / g of soil, a value that meets the DNA yield claimed by the manufacturer (Braid *et al.*, 1999). Yields from the UltraClean™ kit (0.5 – 14.1 µg / g soil) were lower than yields from UltraClean™ Mega kit (15.3 – 32.7 µg / g soil). These yields were about the

same or a little bit lower than most of the reported yields for several different soil DNA-extraction protocols (Cho *et al.*, 1996; Zhou *et al.*, 1996; Miller *et al.*, 1999; Blagodatskaya *et al.*, 2003).

Centroids and associated among-replicate variances from DFA were used to determine the sample size for the best representation of the microbial community structure (Figure 3.4). As was done for means and variances from the enumeration and DNA-yield data, centroids and variances were tested using ANOVA and Hartley's F_{\max} test. Clustering on 2-D DF plot and statistical tests on each axis was used to identify ranges of sample sizes for reliable analysis of microbial community structure. The optimal ranges were different between the two microbial components of the soil community; the bacterial community structures were clustered in the larger sample size (0.25 – 10.0 g), and the fungal community structures were clustered in the intermediate sample sizes (0.1 – 1.0 g). It is known that larger samples with their averaging effect are often more suitable to get general description of the overall communities because available genetic fingerprinting technique with ssu rDNA gene can only detect several most abundant ribotypes in the communities, whereas smaller samples are more heterogeneous and are therefore better for use for a diversity inventory (Figure 4.1). The overall patterns of tighter clustering in larger sample sizes; therefore, makes sense, but the unique location of the 10.0-g sample for the fungal community is difficult to interpret. The overall patterns of distributions of bacterial and fungal community structures on DF plots were roughly similar, even if their identities of sample sizes were different. Except for the 0.01-g sample, the relative location of every sample size was changed between bacterial and fungal community structures on DF plot. This was probably caused by the

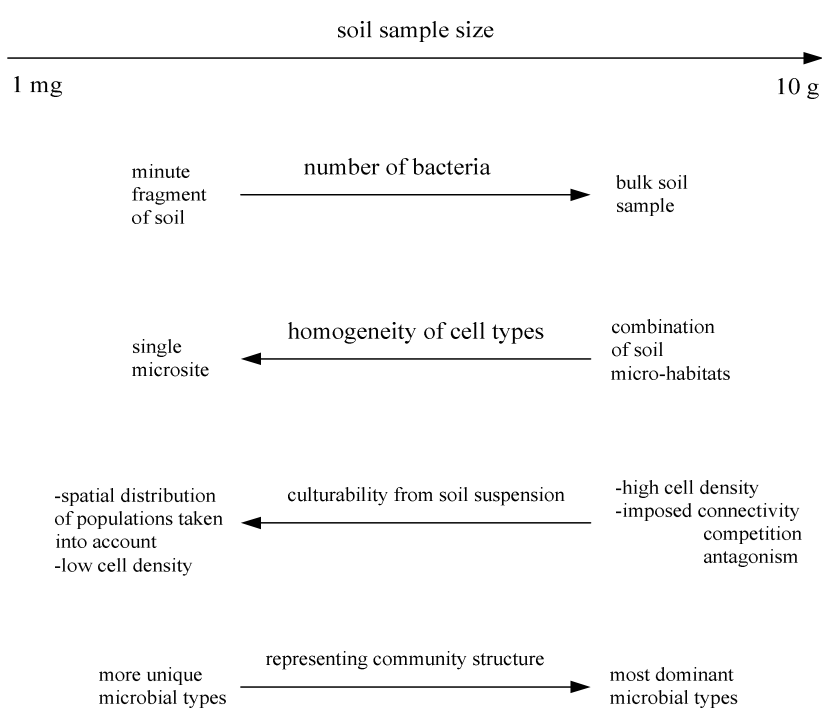


Figure 4.1. Conceptual diagram of the effect of sample sizes on different aspects of soil microbial community ecology study. Redrawn with modification from Grundmann & Gourbiere (1999).

difference between bacteria and fungi in both physiological and ecological aspects, which will be discussed in detail later (Section 4.4.2).

Surprisingly, there have not been long list of extensive studies of sample size effects on microbial community analysis, but two recent papers approached the topic with some rigorous methodology. Ranjard *et al.* (2003) evaluated the effect of sample size on microbial community study using ARISA (automated ribosomal intergenic spacer analysis) from three agricultural field in France. Their sample sizes were 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 g. The DNA was extracted using the “bead beating” method; results were not consistent among fields. Their PC plot was drawn to include all the individual samples (no centroids), and they concluded that the bacterial community structures represented were not influenced by sample sizes. Fungal communities, on the other hand, were very variable in sample sizes smaller than 1.0 g. Ellingsøe and Johnsen (2002) published the first paper dealing with the effect of sample size on the recovered microbial community structure. They subsampled 0.01, 0.1, 1.0 and 10.0 g from soil collected from a virgin forest soil in Denmark. Although they did not perform any formal statistical analysis on the polyphasic DGGE patterns of bacterial community they obtained, they concluded bacterial community structures of larger sample sizes (1.0 and 10.0 g) were virtually consistent among replicates. Visual determination of differences in patterns on gels without any quantitative analysis is a common, but dangerous, practice among microbial ecologists. As the present study and previous two studies were different in various aspects (experimental setting and analytical approach), the results of constant samples sizes were inconsistent among them.

Different procedures for extraction of soil DNA can influence both DNA yields and the qualitative results of subsequent analysis (DGGE) (Niemi *et al.*, 2001; de Liphay *et al.*, 2004). de Liphay *et al.* (2004) compared three different methods of physical cell disruption for DNA extraction; sonic disruption, grinding-freezing-thawing, and bead beating (FastDNA[®] Spin Kit for Soil (Bio101, Vista, CA)) for six different Danish soils. Niemi *et al.* (2001) compared five different soil DNA extraction procedures, including the commercial kit from Mo Bio Laboratories, on rhizosphere soil. DNA yields were not significantly different among different extraction methods in either study, except for one soil from Denmark in de Liphay study. However, both studies concluded that the results of DGGE, including DNA band patterns, number, and intensity of bands were substantially different among different extraction methods, although again, both studies lacked formal statistical evaluations. In the study of Niemi *et al.* (2001), DNA extracted with the MoBio kit yielded the broadest and the most intense band pattern among the five extraction methods. Therefore, we now know that both sample size and method of DNA extraction influence the analysis of soil microbial community structure, and it is important, therefore, to be very consistent in all the preparation steps for such studies. In addition, it is also necessary to note the preparation steps along with the setting of the studies when comparing results with those from other studies. Otherwise, the results are basically incomparable because differences from sample sizes or extraction methods could hide the real differences or create artificial ones.

4.1.3. Suggestions

The purpose of evaluating the effect of sample size for the present study was to find the sample size that best represents the microbial community, so clustered centroids

was the target. If the purpose of a particular study is to inventory microbial diversity, one should use a large number of small samples to generate a large number of polymorphisms, thus capturing a greater portion of the community. The sample sizes determined in the present study were for internal comparisons of the general structure of the soil microbial community. Sample sizes excluded from the selected ranges were more diverse in the measurements and inconsistent among replicates, and that suggests higher chances of finding rare microorganisms. Molecular approaches to Microbial Ecology are very popular; however, they are limited to visualizing the most dominant types of microorganism in the community (Chandler *et al.*, 1997). To capture larger numbers of microbial types, it seems more appropriate to use larger numbers of very small samples.

As Table 3.1 shows, the optimal ranges among different microbiological methods for the enumeration of microorganisms are not very consistent among the methods; no single sample size was determined to be universally good for all the analytical techniques used in present study. Some approaches were more amenable to smaller sample sizes and others were more favorable to larger sample sizes. Thus, different amount of samples were chosen for different analytical techniques. Along with the fact that optimal sample size ranges should be chosen based on the technique to be used, different environmental settings (Ranjard *et al.*, 2003) and different DNA extraction methods also generate inconsistent results (Table 4.1). Therefore, one should perform a preliminary reconnaissance study to determine the optimal range of sample sizes at the beginning of each project for each proposed analytical technique, especially when researchers are examining unfamiliar locations.

Table 4.1. Range of acceptable sample size on microbial community structure study.

	Range of sample size	Molecular method	Reference
Fungi	1.0 – 4.0 g	ARISA	Ranjard <i>et al.</i> , 2003
Fungi	0.1 – 1.0 g	DGGE	Current study
Bacteria	0.125 – 4.0 g	ARISA	Ranjard <i>et al.</i> , 2003
Bacteria	1.0 – 10.0 g	DGGE	Ellingsøe & Johnsen, 2002
Bacteria	10.0 g	DGGE	Nicol <i>et al.</i> , 2003
Bacteria	0.25 – 10.0 g	DGGE	Current study

4.2. VERTICAL PROFILES OF SOIL MICROBIAL COMMUNITY STRUCTURE

4.2.1. Vertical profiles of soil characteristics

Since the experimental field was located in grassland vegetation, a strong O horizon was not expected to be found. O horizons require large accumulations of organic matter and most often are associated with soils developing in forests (Brady, and Weil, 1999). The 25- cm trenches included the A and upper portion of the B horizons. Although the root zone was 5 cm deeper in the D trenches, the dark, organic-rich A horizons were identified at about same depth (10 cm) in all the trenches. Note that in the vertical profile of organic matter (Figure 3.6.A), organic matter content became almost identical below 10 cm in both trenches after a monotonic decrease in concentration from the surface to 10 cm. The 10-cm criterion was well matched with all other observations of soil characteristics and total microbial abundance; all were relatively constant below the 10-cm depth. Vertical profiles were very similar among all measurements except pH. Organic matter content profile could be the key for explanation for all others in that moisture content, total carbon and nitrogen content, and total microbial abundance have close relationships with organic matter content. The opposite pH trend is also explainable by the acidification effect of organic matter decomposition (Brady, and Weil, 1999).

Moisture content was one of the properties showing a decrease with depth. In some studies, vertical profiles of moisture content were constant with depth (Kostov, 1993; Sait *et al.*, 2002), whereas others have reported mixed profiles (i.e., increasing or decreasing moisture with depth) dependent on the types of field cover (Schulze *et al.*, 1996). Schulze *et al.* (1996) measured volumetric water content (%) from five sites with

distinctly different soil covers (from desert to forest) on a 140-km transect in Argentina. Except for the *Stipa* covered grassland site, which showed some downward increase in moisture over a 50-cm distance or constant overall profile, all of the sites examined showed downwardly decreasing moisture content, similar to that in the present study. In particular, sites with deciduous scrub and *Festuca* grass had profiles with a clear decrease in moisture content down to where the moisture increased again at about 2 m below the surface. This decreasing trend of soil moisture content is probably caused mainly by the existence of root zone, which extended 13 – 18 cm. The decreasing trend portion of the profile could be the result of the balance between precipitation and evapotranspiration. Almost constant moisture content profile below root zone inversely verifies. Another causal factor would be the higher relative concentration of clay and organic matter (i.e., higher field capacity) near surface and excessive precipitation during the growing season of 2003 that larger amount of clay and organic matter could hold ample amount of moisture in the upper horizon minimizing downward infiltration and evaporation of the moisture. The average soil moisture content at the 5-cm depth in the same meadow in July 2002 (the last dry year), was about 8% and it increased to about 22% in 2003.

Unique vertical profile of pH measurement is explainable by the fact of higher moisture content near surface and the organic matter content profile (Figure 3.6.A). Organic matter (C_2H_4ONS , generalized form) decomposition can produce carbonic acid, carboxylic acid and inorganic acids (Brady, and Weil, 1999):



The ionization of those acids would have very strong acidification effect, especially from strong sulfuric acid ($pK_a \approx 0$) and moderately strong carboxylic acid ($pK_a = 4.75$). The carbon dioxide availability profile could be another contribution. Soil carbon dioxide is generated mostly by soil respiration (microbial and plant root). The vertical profile of soil carbon dioxide must be similar to the general soil characteristics pattern, because of existence of root zone and soil microbial abundance profile. Water and carbon dioxide form carbonic acid (H_2CO_3), and dissociation of carbonic acid produces bicarbonate (HCO_3^-) lowers pH.

Soil depth profiles from subtropical and semi-arid regions of Mexico reported by Rodriguez *et al.* (2004) were relatively well matched with those of the present study. The soil depth profile reached 50 cm from the surface, and the depth profiles of pH, organic matter content and moisture content were of the same general trend as those of the present study. The only difference is in clay relative content that there was virtually no difference in 50-cm gradient. The trends of vertical profiles of soil characteristics might be extended even deeper. Blume *et al.* (2002) measured the same soil properties as measured in the present study, along with the microbial community properties (biomass, diversity and activity) in a vertical profile in an agricultural field in Indiana. The soil pits were 1.7 m deep but the general trends of pH, moisture content, and total organic carbon were similar to those found in the present study in the 25 cm. For example, the pH of upper 20 cm was lowest regardless of the location of pits and seasons.

4.2.2. Vertical profiles of soil microbial communities

The vertical profile of total microbial abundance was well matched with the expectation from the vertical profiles of soil nutrients (organic matter content, total

carbon and nitrogen content), i.e., microbial numbers were highest at the soil surface where organics and nutrients (including oxygen diffusing into the soil from above ground) were highest. Although overall patterns between trenches were not significantly different ($r = 0.822$, $P = 0.012$ in correlation analysis), large between-trench difference in the upper 5 cm was identified (B trench was less abundant than D trench). Two trenches were located in two distinctive vegetation settings. Two species were identified at the B trench (*P. lanciolata* and *R. herta*), whereas only *A. gerardii* was identified at the D trench. Different plants species are known to produce and release different types of organic carbon compounds (Rovira, 1959; Hale *et al.*, 1978; Lynch, and Whipps, 1990), and those root exudates are the main carbon and energy source, and they are, therefore, a major controlling factor for the composition of soil microbial communities in the vicinity of plant roots (Grayston, 2000; Kozdroj, and van Elsas, 2000; Baudoin *et al.*, 2003; Bertin *et al.*, 2003). It can be hypothesized that a more diverse suite of organic compounds from a more diverse set of plant species would, in turn, support a more diverse microbial community. Such a diverse community might, under some circumstances, be highly internally competitive, resulting in lower overall abundance, for example through the antibiotic activities of genera such as *Streptomyces* (Lancini, and Parenti, 1982).

The decreasing trend of microbial abundance in the downward direction is commonly seen and is intuitively obvious given the fact the majority of soil microorganisms are heterotrophs and that the source of organic compounds is at or near the surface. A 10-cm deep profile from a pasture in Australia showed a very similar decreasing trend in total microbial abundance along with a nearly constant moisture

profile (Sait *et al.*, 2002). A similar trend was also found in a 15-cm deep profile of freshwater sediment in Illinois; here the cell number reached a nearly constant value at about 4 – 5 cm below the surface (Gough, and Stahl, 2003). Almost constant profile in abundance was also seen in a 10-cm deep profile also reported in the Arctic Ocean (Svalbard) sediment. The abundance of the anaerobic sulfate-reducing bacteria, however, increased down to 3 cm (Ravenschlag *et al.*, 2000).

The abundance of negative bands in the DGGE profiles (i.e., bands absent in most samples and only found in a few, assumed to be rare ribotypes) caused major problem in DFA such that DFA including each sample depth separately failed in SPSS. There were insufficient data to compensate for the instability caused by the prevalence of negatives (actually 0s) in the data set. Instead, DFA with grouping of the samples based on soil characteristics (in several depth ranges) was tried. The trends of microbial community structures trajectory (change with depth) in DF space were different between bacteria and fungi; they had the same counter-clockwise direction but there was a different location for each group's centroid (Figure 3.9). The vertical profile of microbial community structures in a 200-cm soil column measured by PLFA followed by PCA in a California soil also showed a continuous trajectory in PC space from the surface to the bottom of the profile (Fierer *et al.*, 2003). The 1.3-m vertical profiles of PLFA data obtained by Blume *et al.* (2002) also showed smooth trajectories in both summer and winter measurements – their directions were same but the centroid locations were different in PC space, as was observed in the present study. Griffiths *et al.* (2003) described bacterial communities in 20-cm vertical profile from a grassland in the UK. Community structures by DGGE and physiological trends by community-level physiological profiling (CLPP) both showed

relatively smooth trajectories of centroids from top to bottom in the profile on the PC field. The trajectories of the profiles obtained by Griffiths *et al.* (2003) showed a convergence at the bottom of the profile (i.e., 15-20 cm) compared with all the other groups.

The change in microbial community structures with depth is less intuitive than are decreasing vertical profiles of microbial abundance. However, the simplest explanation might come from the close relationship with same soil variables that control overall abundance. Organic matter, including total carbon, content generally decreases from the surface to the bottom of the soil profile (Figure 3.6.A and 3.7.A). In addition to the quantity of carbon, the carbon quality is also known to be lesser at greater soil depths (Ajwa *et al.*, 1998; Trumbore, 2000). Therefore, the composition of microbial communities might be expected to differ as total quantity and quality of major nutrients are changed. The microbial community near the soil surface probably is dominated by those heterotrophs that readily utilize labile carbon sources, and as profile goes deeper, microorganisms adapted to more recalcitrant carbon sources would dominate in the community.

4.3. EFFECT OF THE OVERLYING PLANT COMMUNITY ON SOIL BACTERIAL COMMUNITY STRUCTURE

4.3.1. Sampling and devegetation treatment effect

The Difference in the resident plant species has long been thought to be a driving force in structure of bacterial community within the rhizosphere (rhizosphere effect) (Starkey, 1929; Katznelson *et al.*, 1948; Clark, 1949; Krasil'nikov, 1954). Even though grasses have diffuse root systems that permeate the soil, some investigators showed the

effects of plants on soil bacterial community to be different in the rhizosphere and in the bulk soil (Kowalchuk *et al.*, 2002; Soderberg *et al.*, 2004). Because, in the present study, a shovel was used for sampling, and no effort was made to separate the root-attached soil from the bulk soil, differentiation between the rhizosphere and bulk soil was unobtainable. Given the original definition of rhizosphere – the zone where microorganisms are influenced by plant roots (Hiltner, 1904), and given the density of the root mat, we expected all of the soil collected to be influenced by the plants in this case. However, some reduction in differentiation might have resulted from mixing the root-associated soil with the bulk soil in the samples. This is also shown in the abundance data which showed lower counts than what is found (10^9 / g soil) in other studies measured rhizosphere bacterial abundance (Tate, 2000). It may be, therefore, that the inability to detect differences in the microbial communities under different plant species is partially related to the sampling method used.

Several earlier studies of the effects of fire on soil microbes clearly indicated a significant decrease in bacterial biomass and a drastic change in bacterial community structure (Baath *et al.*, 1995) after the burn event. Thus, it was surprising that there was no drastic decrease in bacterial abundance during the earliest part of the study. Indeed, the bacterial abundance increased between the first and second sampling, which was collected right after the burning (Figure 3.10). A partial explanation for the observation might be that the increase occurred because of the combined warming of the soil and the input of a large amount of organic matter because of plant death due to the herbicide treatment. Because the enumerations were done by direct counts for the present study, the immediate effect of the fire may have been different than expected based on cultural

counts, which was usual methods employed by earlier fire effect studies. Cells may have been killed by the heat, but not destroyed so as to be uncountable with Acridine Orange staining. Furthermore, the depth at which the samples were collected (5-10 cm) could have provided protection of the cells from the heat of the fire, which was not very intense due lack of enough fuel. Another possibility is that the increase might be a part of a general spring warming of the soil.

The bacterial community structure, unlikely the abundance, changed significantly in association with the fire (Figure 3.11), i.e., in the period between samplings 1 and 2, and sampling 3. The reason there was no significant structural change between sampling 1 and 2 was probably because there was little immediate effect of fire on bacterial community structure. The change of abundance is rather instant result of burning, while structural change is not necessarily to be same case, since the latter is more likely influenced by the selective enhancement or suppression by the excessive input of organic matter, and burning has limited selective pressure. The inconsistency between DFA and the Mantel test for the immediate of fire might be explained by the difference of the ways of dealing with raw data between them. DFA works on directly the binary data of band patterns, while the Mantel test is based on the similarity index calculated from the binary data. A discrepancy might arise because of this; however, two statistical analyses were relatively well matched to each other overall.

Bacterial community recovery can be very rapid (Ahlgren, and Ahlgren, 1965); in that study, plating data indicated a sharp increase in abundance after burning a grassy field in each of two successive years. The first rainfall was about a month after the burning and a drastic increase in abundance (by plating) was observed a week after that

rainfall. In the second year, it rained within 48 hours after the burning and a highly significant increase in bacterial abundance was observed in that period. In neither case was any time course sampling carried out, so there was no determination of the actual time to take for bacterial community recovery. In a study of Choromanska and DeLuca (2002), a drastically reduced bacterial biomass C was observed immediately after the fire, but the numbers exceeded the original state in samples collected 14 days after the fire; no community structure data were obtained, and no comment about rainfall or moisture was provided. In the present study, the first three samples were collected roughly 20 days apart, and this may be too sparse to detect rapid changes in bacterial abundance. Other types of communities are also known to respond quickly to disturbance. For example, in a study of impoundment sediments, Bell *et al.* (1990) noted that the anaerobic community returned to a normal level of abundance and function within about 14 days after the bed was scoured and covered with 15 cm of fresh, aerobic sediment. The commonality of the two-week time frame in the present and all of the aforementioned studies suggests this may be a reasonable recovery time for microorganisms in many productive habitats.

4.3.2. Genetic fingerprinting techniques

By combining two different genetic fingerprinting techniques for the analysis, it was hoped that a more complete understanding of the bacterial community could be obtained than if only a single measure was used. Several other studies applied a similar strategy, analyzing samples with two different genetic fingerprinting techniques, and those results were relatively consistent with respect to matching results of the methods to each other within the same samples. For example, studies with individual genome samples have shown that RAPD provides results that are very consistent with amplified

fragment length polymorphism (AFLP) (Powell *et al.*, 1996; Renganayaki *et al.*, 2001). In the present study, the general trend in the results of DFA was roughly consistent between RAPD and DGGE. 100% of the RAPD and 98.1% of the DGGE profiles matched up with their sample membership for sampling times 3, 6, and 8. However, the acceptance of null hypothesis in the Mantel test between two genetic fingerprinting techniques indicates that the two methods perceived the bacterial community structure differently. This is a sensible result since DGGE analysis discriminates among communities based on differences in the phylogenetic makeup (i.e., ribotypes based on 16S rDNA sequences), whereas RAPD differentiates the community based on frequency of sequences distributed throughout the total genome. Both techniques are useful tools for bacterial community structure analysis (Franklin *et al.*, 1999). Indeed, Franklin *et al.* (2001) reached identical conclusions about community structure after applying AFLP, TRFLP and CLPP (community level physiological profiling) (Garland, and Mills, 1991) to the same sample set, although the methods do not always generate the same patterns because of the different community structure properties examined by each.

4.3.3. Temporal and spatial trends in the soil bacterial community

The bacterial community structure within the soil changed substantially during the two years after the disturbance and revegetation (Figure 3.11). Changes associated with the initial disturbance events were evident followed by successional stages as the plant community developed after seeding. The successional pattern displayed in DF field was, with the single exception of the last sampling time (time 10), monotonic as the vegetation developed. Given the trajectory over the two years, and given the observations associated

with the slow rise to dominance of the native grasses planted, we conclude that the bacterial communities and the plant communities were developing in parallel.

Although a study by Blume *et al.* (2002) reported that soil bacterial biomass was relatively constant despite seasonal change, most reports indicate that bacterial abundance undergoes a seasonal trend (Smalla *et al.*, 2001), particularly between summer and winter (Grayston *et al.*, 2001; Corre *et al.*, 2002). The seasonal pattern in the present study was clear, although only a single winter sampling was taken. The annual temperature cycle is very closely related to bacterial abundance and activity (Biederbeck, and Campbell., 1971; Dormaar *et al.*, 1984; Kaiser, and Heinemeyer, 1993) due to direct effects on bacterial growth and indirectly by factors such as increased root growth and turnover in the summer months (Lynch, and Panting, 1982).

The revegetation of native grass species did not appear to be very successful during the first two years. In the first year, the field was almost completely overrun with weeds, although the sown grasses were present in clumps throughout the plots in which they were planted. Overall, 127 plant species were identified in the first growing season including the 6 grasses sown (Howard Epstein, personal communication). There was a clear increase in the degree of dominance of the sown grasses between the first and second year; the frequency of patches of the seeded grasses was significantly greater in each plot in the second year. Nevertheless, the native vegetation still did not dominate the field. This fact can certainly account for the failure to distinguish among the different plant treatments over the experimental period. The differences in the visually observable dominance of the native grasses between the first and second years of the experiment is consistent with the differentiation of the bacterial communities from the first and second

year into two distinct groups when analyzed by DFA. The vegetation recovery has been improved every year that the native grass began to dominate the majority of the field in fifth year (2004).

The shifts in bacterial community structure with time were evident prior to establishment of the sown grasses as the dominant vegetation, a result that is consistent with observations elsewhere. In salt marsh sediments, for example, changes in bacterial community activity (dissolved organic-carbon mineralization rates) were correlated with changes in the relative contribution of *Spartina patens* and *Juncus roemerianus* roots to the total belowground biomass (Aiosa, 1996). These belowground responses preceded changes that were documented in above-ground plant community structure in experimental plots that were exposed to altered hydroperiod (Tolley, and Christian, 1999). Aiosa (1996) speculated that the altered bacterial community activity measured reflected changes in bacterial community composition and that the bacterial community could provide an early indication of plant community structure changes. The obvious seasonality between growing and non-growing season also has been reported in study with bacterial PLFA (Bardgett *et al.*, 1999) and DGGE (Smit *et al.*, 2001).

The established blocks used in the experimental design were based on the observed topography of the field. Topography can have a major influence on soil moisture, microclimate (Sobieraj *et al.*, 2002), soil type (Pennock *et al.*, 1987) and differences in vegetation (Burke *et al.*, 1989) along elevational gradients. Given the obvious wet area at the bottom of the hill slope and the fact that soil moisture has a strong influence on many soil factors (Merz, and Plate, 1997), researcher considered a gradient in soil moisture content to be a potentially important controlling factor for bacterial

abundance and community structure (van Gestel *et al.*, 1992; Schimel *et al.*, 1999; Corre *et al.*, 2002). The total bacterial abundance from this field was virtually identical among blocks representing the elevation gradient, and, by inference, the soil moisture gradient (data not shown); but genetic fingerprinting techniques were able to reveal substantial differences in the bacterial community structure. *In situ* soil moisture was measured only once during the experimental period, and no gradient was observed at that time. However, Kim and Barros (2002) showed that strong connection between topography and soil moisture content was observed only during and immediately after rainfall, and that the duration of the effect was heavily dependent on soil texture. A database search of precipitation was done through www.weatherunderground.com at Winchester for two days prior and the sampling day for the 10 sampling times (total of 30 days). As 2000 and 2001 were among the recent very dry years in Virginia, only one day (July 18, 2001, two days before sampling 6) had a noticeable amount of precipitation (2.29 cm). Thus, one might conclude that there was something other than moisture gradient influencing the topographic blocks, since 9 out of 10 samplings would not be expected to show strong differences in moisture regimes.

4.4. SPATIAL STRUCTURE OF SOIL MICROBIAL COMMUNITIES

4.4.1. Spatial structure of soil characteristics

Measured soil characteristics were selected due to their known relationships with the general distribution of soil microorganisms with respect to chemical (pH and C/N ratio), nutritional (total carbon and nitrogen content), and physical attributes (moisture content and % clay). If more specialized groups of microorganisms were to be examined,

different sets of measurements might have been chosen that would specifically target those organisms. For example, species of organic or inorganic nitrogen, along with total nitrogen, could be measured if ammonium oxidizing bacteria (AOB) were of most interest. This, in fact, could be pursued in the future by using specific probes sets for 16S rDNA of those groups of microorganisms (Mobarry *et al.*, 1996; Kowalchuk *et al.*, 1998; Horsch *et al.*, 2004) or specific primer sets for genes responsible for the ammonium oxidizing activity such as *amoA* (Nicolaisen *et al.*, 2004{Aoi, 2004 #359}).

Since spatially distributed ecological data tend to be autocorrelated (Legendre, and Legendre, 1998; Webster, and Oliver, 2001), an appropriate analysis that takes into consideration spatial autocorrelation was required for the current project. The most formal geostatistical analysis for spatial structure is semivariogram analysis (Enriquez *et al.*, 1993; Goovaerts, 1998; Bengtsson *et al.*, 2003; Ritz *et al.*, 2004). Series of semivariogram analyses on different scales and regions of the experimental field for each soil characteristics measurements revealed spatial structures of the soil characteristics in detail.

Total carbon and nitrogen content, and the ratio of carbon and nitrogen content (C/N ratio) were identified to be isotropic at the field scale. Semivariogram models of different scales indicated that there was more than one structure; two spatial structures were identified from all three isotropic soil characteristic measurements (Table 4.2). Overall trends of semivariogram models from different scales and regions between total carbon and nitrogen content were very similar (Figure 3.14 and 3.15), and as a result, the simulation maps of them were very similar in their overall patterns (Figure 3.21) as well. The C/N ratio in mature soil is approximately 10:1 (Atlas, and Bartha, 1998). The C/N

Table. 4.3. Spatial structures of soil characteristics and microbial community.

	1st structure	2nd structure	3rd structure
C	0.16 m	0.4 m	
N	0.16 m	0.5 m	
C/N	0.13 m	0.6 m	
Moisture	0.5 m	2.7 m	
% clay	0.38 m	0.8 m	2.7 m
pH	0.95 m	2.7 m	
Abundance	0.11 m	0.75 m	2.7 m
Bacterial comm..	0.078 m	0.5 m	
Fungal comm..	0.083 m	0.6 m	

ratio is a function of mineralization and immobilization of nitrogen in the soil (Bengtsson *et al.*, 2003) and is used as an index of the quality of substrate (Enríquez *et al.*, 1993; Bossuyt *et al.*, 2001) and decomposition rate (Hobbie, and Vitousek, 2000). The C/N ratio is also reported to be an important factor in the determination of bacterial and fungal community structure (Eiland *et al.*, 2001; Marschner *et al.*, 2003). Even if the C/N ratio of entire experimental field were relatively uniform (8.71 – 12.26:1) in the present study, a spatial structure was identified (Figure 3.16 and 3.20). This would indicate relatively uniform existence of soil microbial community structures with certain degree of spatial autocorrelation. The spatial dependence of three isotropic variables was high enough to be analyzed with geostatistics. In general, very low spatial dependence as indicated by a high nugget value (C_0) indicates either large variation below the measurement scale or large experimental error. Most importantly, high nugget suggests that geostatistics does not provide much benefit over the conventional statistics due to the lack of, or very weak, spatial autocorrelation.

Spatial dependence of total nitrogen content was similar to that in an unimproved grazed upland grassland in Scotland (Ritz *et al.*, 2004), a newly abandoned agricultural field, and a second-growth forest in southwestern Michigan (Gross *et al.*, 1995). Spatial dependence identified in a mid- to late-successional field was higher than that observed in the present study (Gross *et al.*, 1995). However, ranges of those studies were greater by an order of magnitude, probably due to the different sizes of the experimental fields use in each study: 12 m × 12 m (Scotland) and 25 m × 15 m (Michigan) vs. 5 m × 5 m (present study).

The rest of the soil characteristics measured showed strong anisotropy in the experimental field: moisture content (14.3:1), % clay (1.6:1) and pH (2.2:1). The principal axes of the variables; however, differed from one another, and the principal and minor axes of % clay and pH were opposite to one another. Two spatial structures were identified for moisture content and pH, and % clay showed three spatial structures (Table 4.2). In different magnitudes, but all soil characteristics, whether isotropic or anisotropic, were patched in multiple scales inferring that there were several governing factors on the distributions of them. The moisture content distribution showed very strong anisotropy, and directional models were combined into a Gaussian semivariogram model, whereas the omnidirectional field model was an exponential model. The % clay and pH were much less anisotropic, and were modeled with the same type of semivariogram model as the corresponding omnidirectional variograms. Anisotropy is frequently seen in the ecological measurements along environmental gradients: velvetleaf and sunflower population (Johnson *et al.*, 1996), radon concentration in groundwater (Zhu *et al.*, 1996), potassium concentration in savanna (Couto *et al.*, 1997), soil zinc concentration in US (White *et al.*, 1997), soil arsenic concentration in Taiwan (Chang *et al.*, 1999), soil nematode population (Gavassoni *et al.*, 2001). For example, nitrate-nitrogen (NO_3^- -N) content in shallow wells in Wisconsin-age till in Iowa showed anisotropy in the general direction of groundwater flow (Ella *et al.*, 2001).

Although the meadow containing the experimental field in the present study sloped at about 3.7 % from north to south, the experimental field was not large enough to have apparent elevation difference. Additionally, the experimental field was within a single soil map unit, the Poplimento silt loam. The only environmental gradient apparent

from the vegetation differences that might be expected to represent anisotropy is reflected in an east-west direction, especially for soil characteristics have strong relationships with plants composition such as total carbon and nitrogen content. Moisture content met that expectation, but the weaker anisotropy shown by the % clay and pH was in a different direction than that of the vegetation gradient. This might have been caused by factors controlling the clay and pH having a different directional distribution than the vegetation gradient. For those variables with an isotropic distribution, there might have been almost equally effective factor governing mainly in north-south eliminating the vegetation effect or simply that the vegetation had no effect, although that explanation is difficult to understand for total carbon and nitrogen content. A relationship between moisture content and % clay is obvious from the similarities in the semivariogram models and / or estimation maps (overall trend). That relationship is often seen as a result of location in the topographic setting (Birkeland, 1999).

Moisture content was analyzed in a successional field in Michigan by Gross *et al.* (1995), and the spatial dependence from newly abandoned field was compatible with but spatial dependence of mid-late successional field and ranges were higher than ones from present study; this trend was rather consistent in comparison with the results of nitrogen content from their study as well. Ritz (2004) presented a semivariogram model of pH and obtained model parameters that were very similar to the ones from the present study.

All measured soil characteristics showed an agreement in model fitting between the field semivariogram models and regional semivariogram models at the corresponding scales. By both visual inspection and IGF values, all of the field models fit the data as well or better than the corresponding regional models. This trend was more obvious in

the fine-scale models than medium-scale models in that the IGF value of the field models was an order of magnitude lower than the regional models and model fits shown in the graphs were also clearly better than the regional models. This might be explained by the difference in calculating the experimental models between field and regional models. Field experimental models were calculated based on all possible pairs of values within the limit of each scale in the entire field, whereas the regional models used only pairs of values located in each corner. It is more obvious in the fine scale models that the field model was a direct average of its four corner regional models, since scale size and lag distance were limited in the level III sampling design. Throughout the analyses on all soil characteristics measurements, certain amounts of nugget effects were prevalent. Even if there are two possibilities for the origin of a nugget effect, this consistency might indicate that fine scale measurement (0.05 m interval) is still too coarse to detect micro-heterogeneity of soil characteristics. Existence of multiple spatial structures of soil characteristics are reflects the fact of existing multiple factors operating in different scales (Robertson, and Gross, 1994).

Kriged maps and simulation maps showed the evidence of anisotropy along with the principal axes (total nitrogen and carbon, and C/N ratio). That is because the search strategy used a search area defined by ranges of directional models in both kriging and stochastic simulation, such that the search area for anisotropic variables was an ellipse. For the isotropic variables, no apparent directionality was found because the search area was defined as a circle (Figure 3.21). Some responses from the members on AI-GEOSTATS mailing list (www.ai-geostats.org) verified that the outcomes of the stochastic simulation analysis did not seem to be artificial (Pierre Goovaerts, personal

communication). However, anisotropy from semivariogram analysis is not always obvious on the estimation maps (White *et al.*, 1997; Chang *et al.*, 1999). In study by Johnson *et al.* (1996), both velvetleaf (*Abutilon theophrasti*) and common sunflower (*Helianthus annuus*) populations in an agricultural field in Nebraska were identified to be anisotropically distributed with Easting as the principal axis. The anisotropy was apparent on the kriged maps as elliptical shapes along the direction of the principal axis. White and his colleagues (1997) analyzed soil zinc concentration data from USGS and USDA for an estimation map of the entire USA, and they found weak zonal anisotropy had no major effect on the kriged map. Crawford and Hergert (1997) used soil organic matter content data from a corn field in Nebraska to produce estimation maps with five different kriging methods: ordinary kriging with an isotropic semivariogram, ordinary kriging with an anisotropic semivariogram, ordinary kriging within local neighborhoods, universal kriging and median-polish kriging. Even if two directional semivariogram models were very different with a combination of geometric and zonal anisotropy, all estimation maps, including kriged maps between isotropic and anisotropic semivariogram models were virtually identical. However, an experiment with isotropy and anisotropy in semivariogram modeling generated clearly different estimation maps. Therefore, the degree of criticality of appropriate semivariogram modeling for more accurate estimation seems to be dependent upon both observations and environment.

Kriged maps of total carbon and nitrogen content and the C/N ratio were not provided here, because the patterns in the three kriged maps that were presented did not look very natural. The fundamental problem here may be in the sampling design (Figure 2.3), in that the large area in the middle of the field was very sparsely sampled. The

ranges of the three isotropic variables were relatively short (0.5 – 0.84 m), so the large central area of the field did not have enough values to allow meaningful interpolation. Because they had longer ranges in their anisotropic models, the three anisotropic variables had fairly large elliptical search areas to allow coverage of the entire field with a minimum number of values for interpolation. However, generated maps showed apparent artificial structures that were likely due to incompatible number of values for interpolation in different regions of the field (Greg Okin, personal communication). Therefore, kriged maps were not extensively discussed here.

4.4.2. Spatial structure of the soil microbial community

Total microbial abundance was measured as a continuous variable, so the procedure of semivariogram modeling and estimation analysis was exactly same as for the various soil properties. Unlike, however, the soil characteristics measurements which were measured as continuous variables, microbial community structure was measured using a binary format. Such data have not been analyzed using geostatistical approaches until recently. Two approaches for entry of binary data into a geostatistical format are proposed so far: relative dissimilarity and transformation using scores from PCA. The definition of semivariance is half the squared difference between two samples separated by a given lag distance. Franklin and Mills (2002) used the general concept to describe dissimilarity over a lag distance. The approach has been used and accepted in the microbial ecology community, especially by the European journals (FEMS Microbiology Ecology and Soil Biology & Biochemistry) in recent years (Franklin *et al.*, 2002; Mummey, and Stahl, 2003; Lilleskov *et al.*, 2004; Ritz *et al.*, 2004). However, relative dissimilarity has a disadvantage in that it cannot be used for estimation analysis.

Estimation analysis estimates values at unsampled locations by interpolation of sampled values at specific point, but dissimilarities are calculated between *pairs* of samples by the patterns of existence of microbial types. There is nothing to be interpolated in this case.

Another approach used for microbial community structure is using PCA to transform the multivariate binary data into multiple continuous variables. Some investigators have transformed multivariate binary data (genetic fingerprinting, PLFA, FAME and so on) into continuous variables with PCA (Cavigelli *et al.*, 1995; Saetre, and Baath, 2000; Sampson *et al.*, 2001). By definition, all PCs are orthogonal to each other and subsequent PCs are determined from the variance left unexplained by all the previous PCs. The orthogonality of PCs make it useless for employing geostatistical analysis for combining multiple variables (cross semivariogram and cokriging), because for those procedures, variables should be somewhat correlated to provide better results using cokriging than kriging with a single variable. High numbers of polymorphisms from microbial community analysis makes a large number of original variables so that the variance explained by the first PCs very low. The sum of first three PCs of soil microbial community structure data in agricultural soil from AFLP was merely 26.4% (Franklin, and Mills, 2003). In the present study using DGGE to define the polymorphisms, it was about 15% (14.18% for the bacterial community, 15.77% for the fungal community). The property of decreasing explainable variance for successive PCs suggests that the pattern for the results of semivariogram and estimation analysis based on PCs should yield variograms in which that PC1 should be the most structured (longer ranges in semivariograms and simpler kriged maps) and subsequent PCs should be less structured

(shorter ranges in semivariograms and noisy kriged maps). Results from the present study, however, did not conform to this idea and most of the experimental semivariogram models were too erratic to be used to generate meaningful semivariogram models.

Thus the two geostatistical approaches currently available for analyzing the spatial distribution of microbial community structure are not close to perfect. Modeling of multivariate binary data into a single continuous variable was attempted during summer of 2004. Extensive literature search was not very successful in terms of finding solution, although a couple of papers with promising questions and approaches (Billheime *et al.*, 1998; Gelfand *et al.*, 2000) became available. Thus it is proposed as future project to model multivariate binary data into a single continuous variable without loss of large portion of variations in the original data set. It is also desirable to evaluate current approach of using relative dissimilarity is, in fact, appropriate method of analyzing spatial structure of microbial community structures (Appendix A). As discussed, no estimation maps could be developed for the distribution of microbial community structures.

Spatial structure in microbial abundance has not been widely reported yet. Nunan *et al.* (2002) measured soil bacterial density from a 3 m × 3 m × 0.9 m intact soil core. They prepared fixed thin sections from samples in the core and composite digital images were taken using an epifluorescent microscope for counting and locating bacterial cells. Nunan *et al.* examined multiple scales (μm to mm scale, mm to cm scale and cm to m scale), and spatial structure of bacterial density was identified only at the microscale (μm to mm) in the upper 30 cm. Spatial dependence was 0.65 and range was 558 μm in 3.5-cm scale limit. Semivariogram models showed pure nugget in intermediate-scale and

large-scale views of the topsoil. A similar result was found from the viable (culturable) abundance (soil-extract agar) in a large area at the Kellogg Biological Station - Long Term Ecological Research (KBS-LTER) site. Viable abundance was modeled as pure nugget with the maximum lag distance of 216 m (Robertson *et al.*, 1997).

In the present study, overall patterns in 'pseudo-semivariogram' models based on relative dissimilarity were almost identical within certain scales ranges, i.e., field and medium scale, and small scale and fine scale (Figure 3.25 and 3.26). In particular, 4 larger scale models (field and medium scale) were almost identical when scales in y axis (semivariance, γ) are ignored. Although total microbial abundance was anisotropically structured in the experimental field, neither bacterial nor fungal community structure showed evidence of apparent anisotropy. Certainly, the abundance and structure of microbial community are not necessary highly correlated to each other. Figures 3.10 and 3.11 show temporal changes of the microbial community abundance and structure, respectively, following a major fire disturbance; the responses in abundance and structure were different between them. For example, sampling 2 represented a fire effect; abundance changed significantly, but the change in community structure was insignificant. The degree of anisotropy in bacterial and fungal community structures displayed by the field models were supported by comparing the semivariogram models obtained for the experimental field's two sections. The field comprised two sections: the left section was close to a monoculture of *A. gerardii*, whereas the right section contained a diverse collection of vegetation. The shapes and parameters of the variogram models for the two zones were virtually identical for the bacterial community structures. However, as compared with the bacterial community, the spatial structure of the fungal

communities showed more distinction between the two zones (Table 3.8). The summarizing effect, which found in the field scale models over region models in terms of better fit of the model, was also apparent with both total abundance and structures of microbial community. Two very similar spatial structures were identified for the bacterial and fungal community structures, and they were among the smallest in patch sizes (~0.08 m) along with total carbon and nitrogen contents (Table 4.2) of all the variables examined.

Bacterial and fungal community structures differed in various analyses throughout the study. The comparisons made were effect of sample size effect (Figure 3.4), the relationship among summarized soil characteristics and distance (Figure 3.31), spatial structures compared between two distinct vegetation settings (Table 3.8, Figure 3.25 and 3.26), the association with pH and C/N ratio (Table 3.10), spatial distributions (Figure 3.29 and 3.30) and vegetation diversity effect (Table 3.11). The direct association between bacterial and fungal community structures in vertical gradient turned out not to be significantly correlated (Table 3.3). However, the spatial distribution of some soil characteristics (total carbon and nitrogen contents, % clay, and moisture content) and the general soil characteristics, which was summarized in a single variable by Euclidean-distance calculation (section 2.4.4), were similar between the bacterial and fungal community (Table 3.10 and 3.13). Both bacterial and fungal community structures (measured as genetic distance) were determined to be positively associated to geographical distance (Table 3.13), and their analysis in spatially distributed samples by Mantel tests showed significant association (Table 3.10).

Several studies using PLFA (Phospholipid fatty acid) profiles were able to distinguish between bacterial and fungal community structure responses to different factors (Burke *et al.*, 2003; Clegg *et al.*, 2003; Grayston *et al.*, 2004). Clegg and his colleagues (2003) used PLFA to measure the difference of soil fungal and bacterial community structures under different management treatments on grassland in United Kingdom. The most abundant PLFA signals for fungal and bacterial communities were unique under different management regimes. Pennanen *et al.* (2004) used DGGE with separate primer sets for soil bacterial and fungal community to see the effect of amendment with high quality substrate (L-asparagine), and found that change in community structure was more drastic in bacteria than in fungi, suggesting the bacteria were more responsive to the asparagine. Ranjard *et al.* (2003) showed a sample size effect on community structure analysis for bacterial and fungal communities using ARISA (automated ribosomal intergenic spacer analysis), and the overall patterns of different sample sizes on PC plot were very different between bacteria and fungi, just like one found from the present study using DF plot with DGGE (Figure 3.4). As was found in the present study, others also have shown similar overall responses for the two microbial components (Sanyal, and Kulshrestha, 1999; Girvan *et al.*, 2004). Sanyal and Kulshrestha (1999) studied herbicide degradation capability by the bacterial and fungal community in soil, and showed that repeated treatment induced adaptation of microbial communities equally for bacteria and fungi; both bacterial and fungal communities were ultimately able to degrade over 80% of applied herbicide. Girvan *et al.* (2004) evaluated different fertilizer treatments effect and found very similar seasonal pattern between soil bacterial and fungal community structures using DGGE.

The differences between the two cell types lead to fairly complex differences in their ecology. As discussed in Chapter 1, their habitats are generally spatially segregated due to cell-size differences and that translates into major differences in living conditions. Since bacteria often reside within aggregates in spaces too small for fungi or other organisms (Foster, 1988), bacteria are generally protected from predation (Postma *et al.*, 1990; Heijnen *et al.*, 1993; Young *et al.*, 1994) and catastrophic events such as drought (van Gestel *et al.*, 1996); as a result, bacterial biomass is relatively stable over time. Part of the stability is due to the fact that in many situations, bacteria live at near starvation conditions much of the time (Morita, 1990) due to limited mobility (Bass *et al.*, 1998). Although total abundance of bacteria is several orders of magnitude higher than that of fungi in most soil ecosystems (Foster, 1988; Frey *et al.*, 1999; Polyanskaya *et al.*, 2000; Tiquia *et al.*, 2002), their relationship in terms of biomass is more variable (Frostegard, and Baath, 1996; Frey *et al.*, 1999). The ratios of fungal to bacterial biomass are reported as 1% (Bloem *et al.*, 1994) to 42% (Yang, and Insam, 1991). In some soils; however, fungal biomass dominates soil biomass such that it exceeds biomass of all other constituents in soil, including bacteria, plants, and animals (Anderson, and Domsch, 1978; Kjoller, and Struwe, 1982; Schmidt, and Bolter, 2002) (Franklin *et al.*, 2002; Franklin, and Mills, 2003; Mummey, and Stahl, 2003; Lilleskov *et al.*, 2004).

These inconsistent results of the relationship between bacterial and fungal communities lead to the conclusion that while there are always differences between bacteria and fungi, whether or not differences are observable in any study is dependent on the analytical methods used and the systems in which the studies were done.

Nugget effects were apparent in variograms describing microbial community data, and the effect was especially prevalent in community structure analyses (86% of the sill in field scale models). Similar results based on relative dissimilarity have been reported by others (Franklin *et al.*, 2002; Franklin, and Mills, 2003; Mummey, and Stahl, 2003; Lilleskov *et al.*, 2004). However, it is not known for sure whether the high nuggets are properties of the distribution of microbial community structure, or whether they are artificially generated in the currently used approach that uses relative dissimilarity. The Mantel test results show that the association between microbial community structures and distances among communities was significant, thus demonstrates the benefit of employing geostatistics on microbial community structure analysis, but the results from ‘pseudo-semivariogram’ analysis kind of suggests the opposite. As discussed earlier, high nugget indicates low spatial dependence and less advantage of using geostatistics over conventional statistics extracting inference out of spatially distributed data. That is one additional reason of the proposal of future research on mathematical modeling of multivariate binary data into a single continuous variable.

4.4.3. Linkage between soil microbial communities and soil characteristics

The identification of structure in the spatial distribution of soil characteristics and soil microbial communities begs the question of spatial correlation between them. This question is unanswered at this point because the studies that have asked questions about the relationship between microbial community structure and environmental factors have not explicitly dealt with the spatial settings of the collected samples (Johnson, and Wedin, 1997; Hrselova *et al.*, 1999; Bardgett *et al.*, 2001; Joergensen, and Castillo, 2001; Grayston *et al.*, 2004). The present study is one of the very first to attempt correlate

spatial structure in environmental factors with the structure of microbial communities. Franklin and Mills (2003) began to address the question, however the set of variables they selected was limited to a few nutritional items (organic matter, C, N, clay content) than included in the present study, and their vegetational setting was a wheat field (monoculture) with little plant diversity. Additionally, Lowit (Michael Lowit, personal communication) has examined similar questions in the estuarine water column, but that study is at a much larger scale, and the number of samples and their distribution precludes any mapping analysis.

The present statistical analysis began with the Pearson's product-moment correlation analysis among soil characteristics and total microbial abundance (Table 3.9). Soil characteristics themselves were well correlated; 10 out of 15 analyzed pairs were significantly correlated. Total microbial abundance was not, however, significantly correlated with any of the factors. Since correlation between the soil characteristics and the microbial community structure could not be tested by the Pearson's analysis, the Mantel test was used to determine association between them (Table 3.10). The results of the Mantel tests were differed from those of the Pearson's correlation analysis in that only 5 out of the 15 pairwise comparisons of soil characteristics were significant. That difference is due to the differences in the way each analysis handles the data sets. The Pearson's analysis uses the data themselves to calculate a correlation coefficient based on covariance (sum of products divided by degree of freedom) and standard deviation product of the two variables (Equation 4.1).

$$r_{jk} = \frac{\sum y_j y_k}{(n-1)} = \frac{\sum y_j y_k}{\sqrt{\sum y_j^2 \sum y_k^2}} = \frac{s_{jk}}{s_j s_k} \quad (4.1)$$

The Mantel test, however, is based on the matrices constructed with differences among pairs of data. The Mantel statistic (equation 4.2) is calculated as the sum of cross-products of two matrices (Z statistics) and is standardized to eliminate units, as is the Pearson's coefficient. The standardized Mantel coefficient, r , behaves just like Pearson's r (2004).

$$r_{jk} = \frac{\sum_{j=1}^{n-1} \sum_{k=j+1}^n X_{jk} Y_{jk}}{\frac{n(n-1)}{2} - 1} \quad (4.2)$$

This relationship between soil microbial communities and soil characteristics has been studied and reported using various approaches, and the results agree well with the results of the present study. For example, Grayston *et al.* (2004) analyzed soil microbial communities using physiological and genetic markers from several grasslands in Scotland, England, and Wales. By comparing soil properties with signature lipid biomarker of all bacteria (15:0 and 17:0), the authors showed that bacterial components were positively correlated with extractable calcium and total phosphorous contents, and negatively correlated with total nitrogen and organic matter contents. Total nitrogen content was also measured in the present study and it was negatively associated with bacterial community structure. In a study by Taylor *et al.* (2002) from two agricultural fields, one each in Iowa and Michigan, a series of soil characteristics, along with microbial abundance and enzymatic activities, were measured. Among various comparisons made

by Taylor *et al.*, % clay and total microbial abundance coincided with the present study. The correlations differed in the two fields in that the correlation was negative in the Iowa field (silty clay loam) and positive in the Michigan field (loamy sand), but neither correlation was statistically significant. The experimental field in the present study was a silty clay loam and the correlation between % clay at various points within that field and total microbial abundance at each of those points was weakly negative in both the Pearson's and Mantel tests. Soil microbial communities measured by CLPP (community level physiological profiling) and DGGE were clustered according to soil types (10-15% clay vs. 15-20% clay) in an English agricultural field (Girvan *et al.*, 2003). Mycorrhizal spore counts and soil nutrients from a field in Costa Rica showed significant correlations between spore counts and total nitrogen content, total carbon content, and C/N ratio (Johnson, and Wedin, 1997). The results of correlation analysis (Pearson's and Mantel) from the present study and other studies clearly suggest that there is strong association between soil microbial community structures and soil characteristics, although some constituents of soil characteristics have negative relationships with microbial communities.

As previously discussed, anisotropy in the spatial structure might indicate the existence of governing factors acting in different directions within the field. Thus, figures 3.20 and 3.27 indicate that there were different governing factors for moisture content, % clay, pH, and total microbial abundance and all the isotropic variables. However, the results of correlation analyses were rather confusing. High correlation coefficients and a similar direction of anisotropy were obtained for some pairs or variables, viz., total carbon content and total nitrogen content, total carbon content and C/N ratio, and total

nitrogen content and C/N ratio (Mantel test only). In the mean time, the majority of pairs of observations did not match as well. For example, pH and % clay were anisotropic in perpendicular directions, but they were significantly correlated in both analyses (Pearson and Mantel). Although geostatistical procedures consider the spatial distribution of samples as important in the calculation, neither Pearson's correlation analyses nor Mantel tests do so directly. The discrepancy, therefore, could be due to the differences between the geostatistics and conventional statistics, as far as the validity of 'pseudo-semivariogram' is concerned that the results of geostatistical analysis were true. The tendency of discrepancy is also shown from the comparisons among ranges and degree of spatial dependence that both fungal and bacterial community structures were rather similar with total microbial abundance, and all isotropic soil characteristics (Table 3.6, 3.7 and 4.1); because all of those pairs were relatively weakly associated from the Mantel test. Among measured soil characteristics, isotropic soil characteristics were nutritional constituents of the soil. Since there are no formal statistical procedures for comparing semivariogram (George Cutter, personal communication), qualitative comparisons using ranges of nested spatial structures was used. Kriged maps could be nice another good tool for comparison (Ritz *et al.* 2004), but they were not available for the present study due to the problem with PCA of microbial community structure data. It turned out both bacterial and fungal communities were best matched with soil nutrients (total carbon and nitrogen content), and the total abundance was best matched with % clay, which are reasonable because soil nutrients probably possess a selective microbial pressure and clay particles have larger surface area.

As several of the cross semivariograms suggested (Figure 3.28), joint variability of pairs of soil characteristics were diverse. The nugget shown by the cross semivariogram indicates the identity of the nugget between experimental error and microscale variance (Goovaerts, and Chiang, 1993). The nugget effect in the cross semivariograms between total carbon content and pH, and total nitrogen content and pH shows that there was a very small amount of common variance (Figure 3.28.A and B). Semivariograms of those individual variables imply; therefore, there was more experimental error in the measurement of total carbon and nitrogen content than in pH measurement, because nuggets of carbon and nitrogen content were larger than that of pH (55 – 66% of sill vs. 24% of sill respectively). There was also a very small amount of microscale variation in common between % clay and moisture content (Figure 3.28.C), a fact which suggests that the moisture content measurement probably contained some sizable experimental error. Total carbon and nitrogen content and moisture content were determined to have sizable experimental errors from cross semivariogram analysis. All three have larger nuggets (49 – 66% of the sill) in field scale models, but their nuggets became significantly reduced in smaller scale models (28 – 45% of the sill in medium scale), and their relationship with other factors did not support the larger experimental error. Automated elemental analyzer was used for total carbon and nitrogen content measurement, and manual weighing and drying oven were used in moisture content measurement. Both procedures were followed standard protocols and under careful supervision during entire experiment, so the determination of the identity of nuggets from cross semivariogram analysis seems to be more related to the scale (or number of samples), at least in this study.

The rest of the pairs of soil characteristic measurements had large nugget in their cross semivariograms, indicating the prevalence of common microscale variation. This suggests the association of microscale variations between most pairs of soil characteristics, even if four of those pairs (% clay and C/N ratio, pH and C/N ratio, total nitrogen content and C/N ratio, and moisture content and pH) were not significantly correlated by Pearson's correlation analysis. Among the three pairs of variables without common microscale variations, two of them were significantly correlated by both analyses (total carbon content and pH, and total nitrogen content and pH). Thus, it can be concluded that the overall correlations and microscale spatial correlations are not always consistent in that relatedness of variables could be scale-dependent. Same conclusion can be drawn by the fact that cross semivariogram between % clay and pH, which are supposed to be spatially uncorrelated due to their perpendicular direction of anisotropy, showed large common microscale variations. As a matter of fact, different factors or mechanisms at different scales are not surprising to see in such a diverse and heterogeneous soil ecosystem.

Overall the uncertainty of common correlation analysis to infer relationships among spatially distributed variables, because the results between conventional statistics and geostatistics were inconsistent. In spatial sense, soil bacterial and fungal community structure were well correlated with total carbon and nitrogen content, and C/N ratio in the experimental field of the present study.

4.5. FACTORS CONTROLLING MICROBIAL COMMUNITY STRUCTURE

4.5.1. Spatial distribution of microbial community structures

Visualization of the bacterial and fungal community structures from different areas of the experimental field in DF space revealed interesting patterns (Figure 3.29 and 3.30). The level II regions represent the 4 corners and the center of the experimental field, and consisted of 5 samples each. The actual factor loadings on the DF axes differed between bacteria and fungi, but the overall patterns that resulted were almost identical in that the C region was well separated from rest of the regions on the DF2 axis. While there is some risk in drawing inference from these plots due to small number of samples involved, the patterns displayed by the two components of the microbial community are too clear to be ignored. The obvious separation of the C region from the rest is likely related to vegetation abundance and diversity. The relationships between plants and soil microorganisms should be fairly strong here, due to higher percentage of rhizosphere soil in the samples. In studies comparing rhizosphere communities among plants, the microbial communities were closely related to the species of plants (Zak *et al.*, 2003). For example, Westover *et al.* (1997) reported a clear separation of soil bacterial and fungal community structures within the rhizosphere of different plant species (between *Poa secunda* and *Festuca idahoensis*, and between *Anthoxanthum odoratum* and *Plantago lanceolata*) in canonical discriminant function space based on culturing on tryptic soy agar and potato dextrose agar. *A. odoratum* and *P. lanceolata* were also fairly abundant in certain regions of the experimental field (C corner region and B corner region, respectively), and clear separation found in fungal community structures must have been influenced by them in some degree. Malmivaara-Lamsa and Fritze (2003) determined

that the most important factor influencing soil microbial community structure in an urban forest in Finland was the dominant tree types and understory vegetation composition. Marschner *et al.* (2001) examined soil bacterial community structures with three plant species (chickpea, rape and Sudan grass) in three types of Californian soils (sandy, sandy loam and clay), and determined both plant species and soil types are equally effective measured by PCR-DGGE. In a study by Ravit *et al.* (2003), sediment microbial communities of Eastern shore were significantly different between two marsh grass species (*Phragmites australis* and *Spartina alterniflora*) measured by PLFA. From the five samples locations in region C, 7 individual plants of 4 different species identified, the most among all the regions in level II sampling. There were total of 6 plant species identified from all level II sampling locations and two of them, *P. lanciolata* and *Rudbeckia herta*, were found only in region C. Two other species (*Andropogon gerardii* and *A. odoratum*) in region C were rather prevalent in all regions. The uniqueness of the plants present in region C might accommodate distinct microbial communities, and account for the separation of those communities from the rest of the regions.

The only plant species in the D region was *A. gerardii*, and it was also found in the other 4 regions as part of a more diverse community. Given the ubiquity of *A. gerardii* in all the sampling regions, the microbial communities might have a lot of commonality. Another point can be made from the DF plots about the identities of axes. All 5 regions were relatively well separated on the DF1 axis, but all of the regions except for C were clustered closely on the DF2 axis. The DF1 axis, therefore, probably represents heavy weighting of microorganisms associated with the most dominant plant species within each region; A – mixture, B – *A. odoratum*, C – *R. herta*, D – *A. gerardii*

and E – *Solidago* spp. The DF2 axis, on the other hand, might reflect those microorganisms associated with the *A. gerardii* that was found in all the regions. The relationships could be confirmed by a greenhouse experiment with recovered microorganisms from gels and suggested plant species

DF plots for the level III samples also showed very interesting patterns between bacterial and fungal community structures. The overall locations of centroids were relatively constant between bacteria and fungi except corner B, which was closer to corner C in bacterial community structures and was closer to corner A in fungal community structures on DF space. Since there were more samples involved in level III sampling than in the level II effort, the pattern of distinction between bacterial and fungal community structures in the former are probably more meaningful than the lack of distinction seen in the latter (Zak *et al.*, 2003; Innes *et al.*, 2004). Again, based on the close relationship between plant and soil microorganisms, unique plant species found in corner B could be responsible for the differentiation between bacteria and fungi. Among 10 species found in the level III sampling locations, *Festuca elatior* and *P. lanciolata* were found only in corner B. In the level II sampling, *P. lanciolata* was one of two unique species found in region C to separate it from rest of the sampling locations for both bacterial and fungal communities. Thus, it might be assumed that *P. lanciolata* would be less effective than *F. elatior* in differentiation on microbial community structures, and in fact, *P. lanciolata* was less abundant than other co-existing species in both level II and III samplings. Observations on overlapped pairs of corners might provide more insights on the controlling factors of soil bacterial and fungal community structures. In both pairs of corners B-C and A-B, there was no single commonly

occurring plant species, even if B and C corners are in more diverse X4 plot (Right) section of the experimental field. ANOVA on all soil characteristics indicated that bacterial community structures were probably influenced more than other soil characteristics by C/N ratio and pH, as Tukey test failed to reject null hypothesis between B and C corners. In the same way, total carbon and nitrogen content, C/N ratio, % clay, and moisture content were determined to be the differentiating factors among all soil characteristics for fungal community structure.

Since the plant dissimilarity matrix was not constructed to feed into the Mantel test for simultaneous comparisons with matrices of the other variables, indirect inferences were made from DFA and t tests (Table 3.11 and 3.12). Both bacterial and fungal community structures seemed to be relatively well separated by different vegetation settings (mainly diversity) in DFA. Different plant diversity can have a selective influence on soil characteristics that only moisture content and pH were significantly different between two sections of the field. Moisture content was significantly higher in the left section. Precipitation is the input source of soil moisture, and evapotranspiration and runoff are the two soil moisture outputs (equation 3). In equation 3,

$$\frac{dC}{dt} = p - r - e \quad (4.3)$$

C is the soil water content, p is the precipitation rate, r is the runoff rate, and e is the evapotranspiration rate. Among these three fluxes, precipitation can be eliminated from consideration, because there must have been little or no difference in precipitation within the 5 m × 5 m field. Runoff is basically the difference between precipitation, and the sum of evapotranspiration and storage (Chapin III *et al.*, 2002). Important factors of runoff are

topography and water holding capacity (organic matter content and % clay) (Brady, and Weil, 1999). Since all three important factors were virtually same between sections (Table 3.12 and Figure 3.6.A), runoff is probably also similar. Thus, evapotranspiration, which is strongly influenced by plant species and amount of canopy, may be the major cause of the difference in soil moisture content. The right section was more completely covered (74.2% of all sample locations had plants) than the left section (42.4% coverage). The factor or combination of factors responsible for the clear difference in microbial community structures between the left and right sections of the field cannot be definitively identified from the results available here. However, because only two of all six soil characteristics were significantly different between sections and membership matching of soil characteristics by DFA was fairly lower than that of both microbial community structures, clear distinction of vegetation diversity of two sections is probably more comprehensively controlling factor than soil characteristics.

4.5.2. Interrelationships of soil microbial communities and relating components

Soil microbial communities interact with biotic and abiotic components of their environment in close proximity to their cells (England *et al.*, 1993; Baath *et al.*, 1995; Schimel *et al.*, 1999; Broughton, and Gross, 2000; Ranjard *et al.*, 2000; Malmivaara-Lamsa, and Fritze, 2003). Although variables that were investigated in the present study were directed at those that might influence microbial communities, the possible effects of the microbes on the environment should not be ignored. The list of such factors can be extensive as discussed in Chapter 1: plant growth promoting activity, phytopathogenesis, activities on soil structure improvement, mediating soil nutrient cycles, etc. Those effects

condition the environment and the variables that influence community structure such that the interrelationships in both directions are important for study.

In the current work, the plant effect was measured separately from the other variables due to the availability of plant data on the experimental field, and soil characteristics were collectively used to construct dissimilarity matrices along with a geographic distance matrix for comparison with bacterial and fungal community structure matrices. Conceptual models of the interrelationships were developed with the results of the Mantel tests and causal modeling (Figure 3.32). Distance was the dominant controlling factor for both bacterial and fungal communities. In the geostatistical analysis of microbial community structure, the use of relative dissimilarity did not generate convincing evidence of the existence of spatial structure within the experimental field; in other words, spatial distance was not an important factor in the differentiation of microbial community structures. However, the soil properties that were shown to be correlated with soil microbial communities were highly spatially structured. In addition, distance controls the spatial distribution of soil characteristics in the causal models.

The causal model showed differences between the relationship with soil characteristics and the bacterial or fungal communities. The arrows point in opposite directions; bacterial community structures are controlled by soil characteristics and fungal community structures are controlling soil characteristics. As the magnitude of Mantel's coefficients indicate, the directions of the arrow is not absolute. Rather they represent overall trends aggregated for all the interactions between the two components. It means that there are may be several arrows pointing each way and adding all those

arrows is combined into a single arrow representing overall trend of influence between two components.

The soil characteristics measured in present study can be categorized into physical components (% clay and moisture content) and chemical components (total carbon and nitrogen content, C/N ratio and pH). Soil nutrients are, in general, equally influencing on both bacteria and fungi in soil (Hrselova *et al.*, 1999). Carbon sources are made available to soil organisms from the large unavailable pool through decomposition by heterotrophic bacteria and fungi. Soil structure affects bacteria and fungi differently, mainly through habitat separation based on size differences in the organisms (Foster, 1988). Bacterial communities are generally stable because they reside within the micropores inside aggregates (Ranjard, and Richaume, 2001), where they are buffered from even small scale environmental fluctuations and predation from organisms like nematodes (England *et al.*, 1993). Fungal hyphae are important in the formation and stability of aggregates (Chenu, and Stotzky, 2002), and fungal communities could trap particulates and therefore influence the relative clay content. Although there are some extreme acidophilic bacteria, in general, bacteria are more susceptible to lower pH than fungi due to the increased toxicity of organic acids to the prokaryotes under acid conditions (Tate, 2000). These discussions confirm the overall conceptual models in Figure 3.32.

Some investigators have suggested that pairwise comparisons with Mantel tests should be performed with the Bonferroni-correction (Nantel, and Neumann, 1992). Bonferroni-correction is suggested in ANOVA multiple comparisons, when a global test of significance is performed before local tests of significance. In case of the Mantel test (and Pearson's correlation analysis), a global test of significance is normally not the

intent. For example, Table 3.14 summarized the pairwise Mantel tests for microbial community structure, soil characteristics, and distances. The question dealt with the significance of association among pairs of variables, not the global significance among all variables. As suggested by Cabin and Mitchell (2000), therefore, the Bonferroni-correction was not employed for the pairwise analyses in present study. The Bonferroni-correction is also criticized by its super-conservativeness resulting in over-inflated type II error (Legendre, and Legendre, 1998).

5. CONCLUSIONS

The soil microbial community was studied in a meadow along with associated environmental factors in order to determine the relationships between the communities and the measured factors. The work was initiated by testing the usefulness of a genetic fingerprinting technique and multivariate statistical analyses to examine the microbial community structure. The importance of sample size was evaluated for several analytical approaches to find the size that best represented the community. Then the main question of relationships between microbial community structure and the environmental factors was addressed in three different parts; vertical profiles, temporal trends, and spatial distribution at the field scale.

Upon determining that different sample sizes, along with different environmental settings and DNA extraction methods, affect the results of the microbial community analysis, optimal combinations of acceptable ranges of sample sizes, DNA extraction methods and community fingerprinting techniques were sought for the current study. Total microbial abundance was significantly different among the sample sizes (Figure 3.2.). Both bacterial and fungal community structures also were significantly different for different sample sizes, and the trends were inconsistent for bacteria as compared with fungi (Figure 3.4.). Sample sizes that generated variable results were excluded from the current work, but they might be useful for different purposes such as microbial (taxon) inventories or study of communities with more diverse types of microorganisms, in other words, for studies that do not rely on a need for rigorous quantitative analysis. The results of the current study, and also some previous studies, suggest that the range of acceptable sample sizes should be individually determined, at the beginning of each study, for different environmental settings and analytical methods.

Throughout this project, PCR-DGGE was used to assess soil microbial community structures. The possible existence of multiple gene copies in a single organism and the limitation of the approach to detection of only the most abundant ribotypes could present serious problems for some applications, especially those that rely on identifying individual bands from gels. Although there are well known weaknesses in using ssu rDNA gene based techniques for many purposes, PCR-DGGE has been reliable tool for quantitative assessment of differences or changes in microbial community structures, especially when those changes are reflected in the suite fo most abundant organisms. Because the research questions addressed in this study required measurement of relative changes in microbial community structures, those problems had no significant influence on the overall results of the study.

The soil characteristics were well structured spatially, but those components which showed anisotropic distributions (moisture content, % clay and pH) were more spatially dependent than components which were distributed isotropically (total carbon and nitrogen content and C/N ratio) (Table 3.7). The analysis from cross-semivariogram determined the prevalence of microscale (< 0.05 m) variance among all the soil characteristics measurements. Total microbial abundance was relatively well structured spatially. However, the distribution of both bacterial and fungal community structures was much less spatially autocorrelated (spatial dependence 0.1 – 0.3) than the continuous variables (soil characteristics and abundance) that were measured. The analysis of microbial community structures was conducted by using relative dissimilarity, which is a conceptual proxy of semivariance. The prevalence of very large nuggets and exceptional

fit in the 'pseudo-semivariogram' models raised a question of the validity of the approach as a formal geostatistical analysis.

As summarized in Table 5.1, major factors influencing the distribution of soil microbial community structures were identified in different experimental systems. Plant community composition was the dominant controlling factor on soil microbial community structure independent of experimental systems. Plant cover type apparently exerts a strong influence on the vertical profiles of soil microbial community structures, because almost all the vertical profiles of soil characteristics were virtually identical between the experimental trenches. Changes in the plant community were associated with changes in the structure of the soil bacterial community, and succession in bacterial community proceeded congruently with visually observable changes in the identity of the dominant plants. The distinctive plant setting of the Left and Right sections of the experimental field were readily apparent in the distribution of both bacterial and fungal community structure because group membership matching of both bacterial and fungal community structures was way higher than that of soil characteristics (Table 3.11). Because, among soil characteristics, only moisture content and pH were significantly different between the two sections (Table 3.13.), the strong separation of microbial community structures between the sections was determined to be more effectively caused by plants than soil characteristics. When plant effects were discounted, horizontal spatial structures of soil microbial communities were most highly correlated with soil nutrients. Distance was determined to be also significant controlling factor on the distribution on both bacterial and fungal community structures, while soil characteristics influenced the bacterial community but not the fungal community.

Literature reports credit different factors influencing the soil microbial community structures in the rhizosphere as compared with the bulk soil. Some studies identified plant type as a dominant factor for rhizosphere soil microbial communities (Dunfield, and Germida, 2001; Smalla *et al.*, 2001; Kowalchuk *et al.*, 2002). When bulk soil microbial communities were studied, soil characteristics were determined to be controlling factors (Duineveld *et al.*, 1998; Felske, and Akkermans, 1998; Kowalchuk *et al.*, 2000). In the present study, samples were not discriminated between rhizosphere and bulk soil, but the idea of relative amount is available. Spatial setting samples contain more rhizosphere soil than vertical profile soil which was, in some part, collected from below the root zone. However the main controlling factor, plant composition, was identified consistent from the experimental field.

Table 5.1. Summary of interrelationships between soil microbial community structures and candidate components in different settings.

Settings	Controlling factors
Vertical gradient	Plants (and/or distance between trenches)
Temporal trend	Plants (soil characteristics did not measured)
Spatial setting	Plants > Distance > Soil Characteristics (Bacteria)
	Plants (and/or distance among samples) (Fungi)

BIBLIOGRAPHY

- Ahlgren, I. F., and C. E. Ahlgren (1960). "Ecological effects of forest fires." *Botanical Review* 26: 483-533.
- Ahlgren, I. F., and C. E. Ahlgren (1965). "Effects of prescribed burning on soil microorganisms in a Minnesota Jack pine forest." *Ecology* 46: 304-310.
- Aiosa, J. D. (1996). The effects of inundation and vegetation on microbial metabolism of dissolved organic carbon. Environmental Sciences. Charlottesville, VA, University of Virginia: 143.
- Ajwa, H. A., C. W. Rice, and D. Sotomayor (1998). "Carbon and nitrogen mineralization in tallgrass prairie and agricultural soil profiles." *Soil Science Society of America Journal* 62: 942-951.
- Alexander, M. (1977). *Introduction to Soil Microbiology*. Malabar, FL, Krieger.
- Anderson, J. P. E., and K. H. Domsch (1978). "Mineralization of bacteria and fungi in chloroform-fumigated soils." *Soil Biology & Biochemistry* 10: 207-213.
- Atlas, R. M., and R. Bartha (1998). *Microbial Ecology: fundamentals and applications*. Menlo Park, CA, Benjamin/Cummings.
- Baath, E., A. Frostegard, T. Pennanen, and H. Fritze (1995). "Microbial community structure and pH response in relation to soil organic matter quality in wood-ash fertilized, clear-cut or burned coniferous forest soils." *Soil Biology & Biochemistry* 27: 229-240.
- Barber, D. A., and J. K. Martin (1976). "The release of organic substances by cereal roots into soil." *New Phytologist* 76: 69-80.
- Bardgett, R. D., A. C. Jones, D. L. Jones, S. J. Kemmitt, R. Cook, and P. J. Hobbs (2001). "Soil microbial community patterns related to the history and intensity of grazing in sub-montane ecosystems." *Soil Biology & Biochemistry* 33: 1653-1664.
- Bardgett, R. D., R. D. Lovell, P. J. Hobbs, and S. C. Jarvis (1999). "Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands." *Soil Biology & Biochemistry* 31: 1021-1030.
- Barrett, J. P., and L. Goldsmith (1976). "When is n sufficiently large?" *The American Statistician* 30: 67-70.
- Bass, C. J., R. A. Davey, and H. M. Lappin-Scott (1998). "Long-term starvation survival of a thermophilic sulfidogen consortium." *Geomicrobiology Journal* 15: 29-36.
- Baudoin, E., E. Benizri, and A. Guckert (2003). "Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere." *Soil Biology & Biochemistry* 35: 1183-1192.

- Becker, S., P. Boger, R. Oehlmann, and A. Ernst (2000). "PCR bias in ecological analysis: a case study for quantitative *Taq* nuclease assays in analysis of microbial communities." *Applied and Environmental Microbiology* 66: 4945-4953.
- Bell, P. E., A. T. Herlihy, and A. L. Mills (1990). "Establishment of anaerobic, reducing conditions in lake sediment after deposition of acidic, aerobic sediment after a major storm." *Biogeochemistry* 9: 99-116.
- Bending, G. D., M. K. Turner, and J. E. Jones (2002). "Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities." *Soil Biology & Biochemistry* 34: 1073-1082.
- Bengtsson, G., P. Bengtson, and K. F. Mansson (2003). "Gross nitrogen mineralization-, immobilization-, and nitrification rates as a function of soil C/N ratio and microbial activity." *Soil Biology & Biochemistry* 35: 143-154.
- Bertin, C., X. Yang, and L. A. Weston (2003). "The role of root exudates and allelochemicals in the rhizosphere." *Plant and Soil* 256: 67-83.
- Biederbeck, V. O., and C. A. Campbell. (1971). "Influence of simulated fall and spring conditions on the soil system. I. Effect on soil microflora." *Soil Science Society of America Journal* 35: 474-479.
- Billheime, D., P. Guttorp, and W. F. Fagan (1998). *Statistical analysis and interpretation of discrete compositional data*. NRCSE technical report series, The National Research Center for Statistics and the Environment: 1-29.
- Bird, S. B., J. E. Herrick, M. M. Wander, and S. F. Wright (2002). "Spatial heterogeneity of aggregate stability and soil carbon in semi-arid rangeland." *Environmental Pollution* 116: 445-455.
- Birkeland, P. W. (1999). *Soils and Geomorphology*. New York, NY, Oxford University Press.
- Blagodatskaya, E. V., O. S. Khokhlova, T. H. Anderson, and S. A. Blagodatskii (2003). "Extractable microbial DNA pool and microbial activity in paleosols of Southern Urals." *Microbiology* 72: 750-755.
- Bloem, J., G. Lebbink, K. B. Zwart, L. A. Bouwman, S. L. G. E. Burgers, J. A. Devos, and P. C. Deruiter (1994). "Dynamics of microorganisms, microbivores and nitrogen mineralization in winter wheat fields under conventional and integrated management." *Agriculture Ecosystems & Environment* 51: 129-143.
- Blume, E., M. Bischoff, J. M. Reichert, T. Moorman, A. Konopka, and R. F. Turco (2002). "Surface and subsurface microbial biomass, community structure and metabolic activity as a function of soil depth and season." *Applied Soil Ecology* 20: 171-181.
- Boer, P. H., and J. Ramamoorthy (1997). "How to correct for errors in mRNA quantification by competitive PCR due to heteroduplex formation of amplification products." *Cellular and Molecular Biology* 43: 841-850.

- Borchers, J. G., and D. A. Perry (1990). Effects of prescribed fire on soil organisms. *Natural and Prescribed Fire in Pacific Northwest Forests*. J. D. Walstad, S. R. Radosevich and D. V. Sandberg. Corvallis, OR., Oregon State University Press. 143-157.
- Bosma, T. N. P., P. J. M. Middelorp, G. Schraa, and A. J. B. Zehnder (1997). "Mass transfer limitation of biotransformation: Quantifying bioavailability." *Environmental Science & Technology* 31: 248-252.
- Bossuyt, H., K. Deneff, J. Six, S. D. Frey, R. Merckx, and K. Paustian (2001). "Influence of microbial populations and residue quality on aggregate stability." *Applied Soil Ecology* 16: 195-208.
- Bottomley, P. J. (1994). Light microscopic methods for studying soil microorganisms. *Methods of Soil Analysis, Part 2: Microbiological and Biochemical Properties*. R. W. Weaver, S. Angle, P. J. Bottomley et al. Madison, WI, Soil Science Society of America. 81-105.
- Brady, N. C., and R. R. Weil (1999). *The Nature and Properties of Soils*. Upper Saddle River, NJ, Prentice-Hall.
- Braid, M. D., L. M. Nicholas, B. G. clement, J. B. Kitner, C. L. Kitts, M. N. Brolaski, and R. J. Venugopal (1999). Testing the UltraClean™ Soil DNA Purification Kit on a diverse range of soils by PCR amplification of 16S rDNA. American Society of Microbiology General Meeting, Chicago, IL.
- Brock, T. D. (1987). *The study of microorganisms in situ: progress and problems*. Ecology of Microbial Ecommunities., The University of St. Andrews, Cambridge University Press.
- Brock, T. D., and M. T. Madigan (1991). *Biology of Microorganisms*. Elglewood Cliffs, NJ, Prentice-Hall.
- Brooks, S. C., A. L. Mills, J. S. Herman, and G. M. Hornberger (1997). "Kinetic evaluation of the effects of bioavailability of organic ligands on biodegradation in the presence of common sesquioxide grain coatings." *Environmental Toxicology and Chemistry* 16: 862-870.
- Broughton, L. C., and K. L. Gross (2000). "Patterns of diversity inplant and soil microbial communities along a productivity gradient in a Michigan old-field." *Oecologia* 125: 420-427.
- Bruggermann, J., J. R. Stephen, Y.-J. Chang, S. J. Macnaughton, G. A. Kowalchuk, E. Kline, and D. C. White (2000). "Competitive PCR-DGGE analysis of bacterial mixtures an internal standard and an appraisal of template enumeration accuracy." *Journal of Microbiological Method* 40: 111-123.
- Buckley, D. H., and T. M. Schmidt (2001). "The structure of microbial communities in soil and the lasting impact of cultivation." *Microbial Ecology* 42: 11-21.

- Burke, D. J., E. P. Hamerlynck, and D. Hahn (2002). "Interactions among plant species and microorganisms in salt marsh sediments." *Applied and Environmental Microbiology* 68: 1157-1164.
- Burke, I. C., W. A. Rainers, and R. O. Olson (1989). "Topographic control of vegetation in a mountain big sagebrush steppe." *Vegetatio* 84: 77-86.
- Burke, R. A., M. Molina, J. E. Cox, L. J. Osher, and M. C. Piccolo (2003). "Stable carbon isotope ratio and composition of microbial fatty acids in tropical soils." *Journal of Environmental Quality* 32: 198-206.
- Buyer, J. S., D. P. Roberts, and E. Russek-Cohen (2002). "Soil and plant effects on microbial community structure." *Canadian Journal of Microbiology* 48: 955-964.
- Cabin, R. J., and R. J. Mitchell (2000). "To Bonferroni or not to Bonferroni: when and how are the questions." *Bulletin of the Ecological Society of America* 81: 246-248.
- Cariello, N. F., J. A. Swenberg, and T. R. Skopek (1991). "Fidelity of *Thermococcus-litoralis* DNA-Polymerase (Vent) in Pcr Determined by Denaturing Gradient Gel-Electrophoresis." *Nucleic Acids Research* 19: 4193-4198.
- Casgrain, P., and P. Legendre (2001). *The R package for multivariate and spatial analysis version 4.0 (development release 6) user's manual*.
- Cavigelli, M. A., G. P. Robertson, and M. J. Klug (1995). "Fatty-acid methyl-ester (FAME) profiles as measures of soil microbial community structure." *Plant & Soil* 170: 99-113.
- Chandler, D. P., S.-M. Li, C. M. Spadoni, G. R. Drake, D. L. Balkwill, J. K. Fredrickson, and F. J. Brockman (1997). "A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment." *FEMS Microbiology Ecology* 23: 131-144.
- Chang, T. K., G. S. Shyu, Y. P. Lin, and N. C. Chang (1999). "Geostatistical, analysis of soil arsenic content in Taiwan." *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering* 34: 1485-1501.
- Chapin III, F. S., P. A. Matson, and H. A. Mooney (2002). *Principles of Terrestrial Ecosystem Ecology*. New York, NY, Springer.
- Chenu, C., and G. Stotzky (2002). *Interactions between Microorganisms and Soil Particles: An Overview*. *Interactions between Soil Particles and Microorganisms*. P. M. Huang, J.-M. Bollag and N. Senesi. Chichester, England, John Wiley & Sons. 3-40.
- Cho, J.-C., D.-H. Lee, Y.-C. Cho, J.-C. Cho, and S.-J. Kim (1996). "Direct extraction of DNA from soil for amplification of 16S s-RNA gene sequences by polymerase chain reaction." *Journal of microbiology* 34: 229.
- Choi, B. K., B. J. Paster, F. E. Dewhirst, and U. B. Gobel (1994). "Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis." *Infection and Immunity* 62: 1889-1895.

- Choromanska, U., and T. H. DeLuca (2002). "Microbial activity and nitrogen mineralization in forest mineral soils following heating: evaluation of post-fire effects." *Soil Biology and Biochemistry* 34: 263-271.
- Christensen, B. T. (1992). "Physical fractionation of soil and organic matter in primary particle size and density separates." *Advances in Soil Science* 20: 2-76.
- Clark, F. F. (1949). "Soil microorganisms and plant growth." *Advances in Agronomy* 1: 241-288.
- Clegg, C. D., R. D. L. Lovell, and P. J. Hobbs (2003). "The impact of grassland management regime on the community structure of selected bacterial groups in soils." *FEMS Microbiology Ecology* 43: 263-270.
- Cliff, A. D., and J. K. Ord (1981). *Spatial Process - Models and applications*. London, UK., Pion.
- Colwell, R. R., and B. Austin (1981). *Numerical taxonomy. Manual of Methods for General Bacteriology*. P. Gerhardt, R. G. E. Murray, R. N. Costilow et al. Washington, DC., American Society for Microbiology.
- Corre, M. D., R. R. Schnabel, and W. L. Stout (2002). "Spatial and seasonal variation of gross nitrogen transformations and microbial biomass in a Northeastern US grassland." *Soil Biology and Biochemistry* 34: 445-457.
- Couto, E. G., A. Stein, and E. Klamt (1997). "Large area spatial variability of soil chemical properties in central Brazil." *Agriculture Ecosystems & Environment* 66: 139-152.
- Crawford, C. A. G., and G. W. Hergert (1997). "Incorporating spatial trends and anisotropy in geostatistical mapping of soil properties." *Soil Science Society of America Journal* 61: 298-309.
- Curtis, T. P., W. T. Sloan, and J. W. Scannell (2002). "Estimating prokaryotic diversity and its limits." *Proceedings of the National Academy of Sciences of the United States of America* 99: 10494-10499.
- Dahllof, I., H. Baillie, and S. Kjelleberg (2000). "rpoB-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity." *Applied and Environmental Microbiology* 66: 3376-3380.
- Dashman, T., and G. Stotzky (1982). "Adsorption and binding of amino acids on homoionic montmorillonite and kaolinite." *Soil Biology & Biochemistry* 14: 447-456.
- Dashman, T., and G. Stotzky (1984). "Adsorption and binding of peptides on homoionic montmorillonite and kaolinite." *Soil Biology & Biochemistry* 16: 51-55.
- de Liphay, J. R., C. Enzinger, K. Johnsen, J. Aamand, and S. J. Sorensen (2004). "Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis." *Soil Biology & Biochemistry* 36: 1607-1614.

- Demeke, T., and R. P. Adams (1994). The use of PCR-RAPD analysis in plant taxonomy and evolution. PCR Technology: Current Innovations. H. G. Griffin and A. M. Griffin. Boca Raton, FL, CRC Press. 179-191.
- Deniel, F., P. Rey, M. Cherif, A. Guillou, and Y. Tirilly (2004). "Indigenous bacteria with antagonistic and plant-growth-promoting activities improve slow-filtration efficiency in soilless cultivation." *Canadian Journal of Microbiology* 50: 499-508.
- Deutsch, C. V., and A. G. Journel (1998). *GSLIB: Geostatistical Software Library and User's Guide*. New York, NY, Oxford University Press.
- Dighton, J., H. E. Jones, C. H. Robinson, and J. Beckett (1997). "The role of abiotic factors, cultivation practices and soil fauna in the dispersal of genetically modified microorganisms in soils." *Applied Soil Ecology* 5: 109-131.
- Dollhopf, S. L., S. A. Hashsham, and J. M. Tiedje (2001). "Interpreting 16S rDNA T-RFLP data: Application of self-organizing maps and principal component analysis to describe community dynamics and convergence." *Microbial Ecology* 42: 495-505.
- Dormaar, J. F., A. Johnston, and S. Smoliak (1984). "Seasonal changes in carbon content, and dehydrogenase, phosphatase and urease activities in mixed prairie and fescue grassland Ah horizons." *Journal of Range Management* 37: 31-35.
- Duineveld, B. M., A. S. Rosado, J. D. v. Elsas, and J. A. v. Veen (1998). "Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum *via* denaturing gradient gel electrophoresis and substrate utilization patterns." *Applied and Environmental Microbiology* 64: 4950-4957.
- Dunfield, K. E., and J. J. Germida (2001). "Diversity of bacterial communities in the rhizosphere and root interior of field-grown genetically modified *Brassica napus*." *FEMS Microbiology Ecology* 38: 1-9.
- Eiland, F., M. Klamer, A.-M. Lind, M. Leth, and E. B. 櫛 th (2001). "Influence of initial C/N ratio on chemical and microbial composition during long term composting of straw." *Microbial Ecology* 41: 272-280.
- El Fantroussi, S., L. Verschuere, W. Verstraete, and E. M. Top (1999). "Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles." *Applied and Environmental Microbiology* 65: 982-988.
- Ella, V. B., S. W. Melvin, and R. S. Kanwar (2001). "Spatial analysis of NO₃-N concentration in glacial till." *Transactions of the ASAE* 44: 317-327.
- Ellingsoe, P., and K. Johnsen (2002). "Influence of soil sample sizes on the assessment of bacterial community structure." *Soil Biology & Biochemistry* 34: 1701-1707.
- England, L. S., H. Lee, and J. T. Trevors (1993). "Bacterial survival in soil: effect of clays and protozoa." *Soil Biology & Biochemistry* 25: 525-531.

- Enríquez, S., C. M. Duarte, and K. Sand-Jensen (1993). "Patterns in decomposition rates among photosynthetic organisms: The importance of detritus C:N:P content." *Oecologia* 94.
- Fanning, S., and R. A. Gibbs (1997). PCR in genome analysis. *Genome Analysis: A laboratory manual*, vol. 1: Analyzing DNA. B. Birren, E. D. Green, S. Klapholz, R. M. Myers and J. Roskams. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press. 249-299.
- Felske, A., and A. D. L. Akkermans (1998). "Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soils." *Microbial Ecology* 36: 31-36.
- Fenwick, L. (1973). "Studies on the rhizosphere microflora of onion plants in relation to temperature changes." *Soil Biology & Biochemistry* 5: 315-320.
- Ferris, M. J., and D. M. Ward (1997). "Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis." *Applied and Environmental Microbiology* 63: 1375-1381.
- Fierer, N., J. P. Schimel, and P. A. Holden (2003). "Variations in microbial community composition through two soil depth profiles." *Soil Biology & Biochemistry* 35: 167-176.
- Fisher, S. G., and L. S. Lerman (1979). "Length-independent separation of DNA restriction fragments in two dimensional gel electrophoresis." *Cell* 16: 191-200.
- Fogel, G. B., C. R. Collins, J. Li, and C. F. Brunk (1999). "Prokaryotic genome size and SSU rDNA copy number: estimation of microbial relative abundance from a mixed population." *Microbial Ecology* 38: 93-113.
- Foster, R. C. (1988). "Microenvironments of soil microorganisms." *Biology and Fertility of Soils* 6: 189-203.
- Franklin, R. B. (2004). *Spatial patterns in microbial communities*. Department of Environmental Sciences. Charlottesville, University of Virginia: 342.
- Franklin, R. B., L. K. Blum, A. C. McComb, and A. L. Mills (2002). "A geostatistical analysis of small-scale spatial variability in bacterial abundance and community structure in salt marsh creek bank sediments." *FEMS Microbiology Ecology* 42: 71-80.
- Franklin, R. B., J. L. Garland, C. H. Bolster, and A. L. Mills (2001). "The impact of dilution on microbial community structure and functional potential: a comparison of numerical simulations and batch culture experiments." *Applied and Environmental Microbiology* 67: 702-712.
- Franklin, R. B., and A. L. Mills (2003). "Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field." *FEMS Microbiology Ecology* 44: 335-346.

- Franklin, R. B., D. R. Taylor, and A. L. Mills (1999). "Characterization of microbial communities using randomly amplified polymorphic DNA (RAPD)." *Journal of Microbiological Methods* 35: 225-235.
- Frey, S. D., E. T. Elliott, and K. Paustian (1999). "Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients." *Soil Biology & Biochemistry* 31: 573-585.
- Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi (2002). "Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns." *Environmental Microbiology* 4: 634-643.
- Frostegard, A., and E. Baath (1996). "The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil." *Biology and Fertility of Soil* 22: 59-65.
- Gallardo, A., J. J. Rodriguez-Saucedo, F. Covelo, and R. Fernandez-Ales (2000). "Soil nitrogen heterogeneity in a Dehesa ecosystem." *Plant and Soil* 222: 71-82.
- Garland, J. L., and A. L. Mills (1991). "Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level-sole-carbon-source utilization." *Applied and Environmental Microbiology* 57: 2351-2359.
- Gauch Jr., H. G. (1982). *Multivariate analysis in community ecology*. Cambridge, UK, Cambridge University press.
- Gavassoni, W. L., G. L. Tylka, and G. P. Munkvold (2001). "Relationships between tillage and spatial patterns of *Heterodera glycines*." *Phytopathology* 91: 534-545.
- Gee, G. W., and J. W. Bauder (1986). 15. Particle-size analysis. *Methods of Soil Analysis, Part 1: Physical and Mineralogical Methods*. A. Klute. Madison, WI, Soil Science Society of America. 383-411.
- Gelfand, A. E., N. Ravishanker, and M. D. Ecker (2000). Modeling and inference for point-referenced binary spatial data. *Generalized Linear Models: A Bayesian Perspective*. D. K. Dey, S. K. Ghosh and B. K. Mallick. New York, NY, Marcel Dekker. 373-386.
- Girvan, M. S., J. Bullimore, A. S. Ball, J. N. Pretty, and A. M. Osborn (2004). "Responses of active bacterial and fungal communities in soils under winter wheat to different fertilizer and pesticide regimens." *Applied and Environmental Microbiology* 70: 2692-2701.
- Girvan, M. S., J. Bullimore, J. N. Pretty, A. M. Osborn, and A. S. Ball (2003). "Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils." *Applied and Environmental Microbiology* 69: 1800-1809.
- Gomes, N. C. M., O. Fagbola, R. Costa, N. G. Rumjanek, A. Buchner, L. Mendona-Hagler, and K. Smalla (2003). "Dynamics of fungal communities in bulk and

- maize rhizosphere soil in the tropics." *Applied and Environmental Microbiology* 69: 3758-3766.
- Goovaerts, P. (1997). *Geostatistics for Natural Resources Evaluation*. New York, NY, Oxford University Press.
- Goovaerts, P. (1998). "Geostatistical tools for characterizing the spatial variability of microbiological and physico-chemical soil properties." *Biology and Fertility of Soil* 27: 315-334.
- Goovaerts, P., and C. N. Chiang (1993). "Temporal persistence of spatial patterns for mineralizable nitrogen and selected soil properties." *Soil Science Society of America Journal* 57: 372-381.
- Gough, H. L., and D. A. Stahl (2003). "Optimization of direct cell counting in sediment." *Journal of Microbiological Method* 52: 39-46.
- Grayston, S. J. (2000). "Rhizodeposition and its impact on microbial community structure and function in trees." *Phyton-Annales Rei Botanicae* 40: 27-36.
- Grayston, S. J., C. D. Campbell, R. D. Bardgett, J. L. Mawdsley, C. D. Clegg, K. Ritz, B. S. Griffiths, J. S. Rodwell, S. J. Edwards, W. J. Davies, D. J. Elston, and P. Millard (2004). "Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques." *Applied Soil Ecology* 25: 63-84.
- Grayston, S. J., G. S. Griffith, J. L. Mawdsley, C. D. Campbell, and R. D. Bardgett (2001). "Accounting for variability in soil microbial communities of temperate upland grassland ecosystems." *Soil Biology and Biochemistry* 33: 533-551.
- Grayston, S. J., G. S. Griffith, J. L. Mawdsley, C. D. Campbell, and R. D. Bardgett (2001). "Accounting for variability in soil microbial communities of temperate upland grassland ecosystems." *Soil Biology & Biochemistry* 33: 533-551.
- Green, R. H. (1979). *Sampling Design and Statistical Methods for Environmental Biologists*. New York, NY, John Wiley & Sons.
- Griffiths, R. I., A. S. Whiteley, A. G. O'Donnell, and M. J. Bailey (2003). "Influence of depth and sampling time on bacterial community structure in an upland grassland soil." *FEMS Microbiology Ecology* 43: 35-43.
- Gross, K. L., K. S. Pregitzer, and A. J. Burton (1995). "Spatial variation in nitrogen availability in three successional plant communities." *Journal of Ecology* 83: 357-367.
- Grundmann, G. L., and D. Debouzie (2000). "Geostatistical analysis of the distribution of NH_4^+ - and NO_2^- -oxidizing bacteria and serotypes at the millimeter scale along a soil transect." *FEMS Microbiology Ecology* 34: 57-62.
- Grundmann, G. L., and F. Gourbiere (1999). "A micro-sampling approach to improve the inventory of bacterial diversity in soil." *Applied Soil Ecology* 13: 123-126.

- Guggenberger, G., and K. M. Haider (2002). Effect of mineral colloids on biogeochemical cycling of C, N, P, and S in soil. *Interactions between Soil Particles and Microorganisms*. P. M. Huang, J.-M. Bollag and N. Senesi. Chichester, England, John Wiley & Sons.
- Haack, S. K., L. R. Fogarty, T. G. West, E. W. Alm, J. T. McGuire, D. T. Long, D. W. Hyndman, and L. J. Forney (2004). "Spatial and temporal changes in microbial community structure associated with recharge-influenced chemical gradients in a contaminated aquifer." *Environmental Microbiology* 6: 438-448.
- Hale, M. G., L. D. Moore, and G. J. Griffin (1978). Root exudates and exudation. *Interactions between non-pathogenic soil microorganisms and plants*. Y. R. Dommergues and S. V. Krupa. New York, NY, Elsevier. 163-203.
- Hassink, J., L. A. Bouwman, K. B. Zwart, and L. Brussaard (1993). "Relationships between habitable pore space, soil biota and mineralization rates in grassland soils." *Soil Biology & Biochemistry* 25: 47-55.
- Hattori, T., and R. Hattori (1976). "The physical environment in soil microbiology: an attempt to extend principles of microbiology to soil micro-organisms." *CRC Critical Review in Microbiology* 4: 423-461.
- Head, I. M., J. R. Saunders, and R. W. Pickup (1998). "Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms." *Microbial Ecology* 35: 1-21.
- Heijnen, C. E., C. Chenu, and M. Robert (1993). "Micro-morphological studies on clay-amended and unamended loamy sand, relating survival of introduced bacteria and soil structure." *Geoderma* 56: 195-207.
- Hengen, P. N. (1997). "Optimizing multiplex and LA-PCR with betaine." *Trends in Biochemical Science* 22: 225-226.
- Heuer, H., and K. Smalla (1997). Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. *Modern Soil Microbiology*. J. D. v. Elsal, J. T. Trevors and E. M. H. Wellington. New York, NY, Marcel Dekker. 353-373.
- Hiltner, L. (1904). "Uber neuere erfahrungen und probleme auf dem gebiet der boden bakteriologie und unter besonderer berucksichtigung der grundung und brache." *Arb. Deut. Landw. Ges.* 98: 59-78.
- Hobbie, S. E., and P. M. Vitousek (2000). "Nutrient regulation of decomposition in Hawaiian montane forests: Do the same nutrients limit production and decomposition?" *Ecology* 81.
- Hojberg, O., N. P. Revsbech, and J. M. Tiedje (1994). "Denitrification in soil aggregates analyzed with microsensors for nitrous-oxide and oxygen." *Soil Science Society of America Journal* 58: 1691-1698.
- Holben, W. E. (1997). Isolation and purification of bacterial community DNA from environmental samples. *Manual of environmental microbiology*. C. J. Hurst, G. R.

- Knudsen, M. J. McInerney, L. D. Setzenbach and M. V. Walter. Washington, DC, American society for microbiology. 431-436.
- Horsch, P., J. Leve, and F. H. Frimmel (2004). "Effect of an industrial waste water on the nitrification in fixed-bed biofilm reactors - use of fluorescence in-situ hybridization (FISH)." *Water Science & Technology* 49: 91-97.
- Hrselova, H., I. Chvatalova, M. Vosatka, J. Klir, and M. Gryndler (1999). "Correlation of abundance of arbuscular mycorrhizal fungi, bacteria and saprophytic microfungi with soil carbon, nitrogen and phosphorus." *Folia Microbiologica* 44: 683-687.
- Huang, P. M., and J. J. Germida (2002). *Chemical and biological processes in the rhizosphere: metal pollutants. Interactions between Soil Particles and Microorganisms*. P. M. Huang, J.-M. Bollag and N. Senesi. Chichester, England, John Wiley & Sons. 381-438.
- Hurlbert, S. H. (1984). "Pseudoreplication and the design of ecological field experiments." *Ecological Monographs* 54: 187-211.
- Hutchinson, G. L., and A. R. Mosier (1979). "Nitrous oxide emissions from an irrigated cornfield." *Science* 205: 1125-1127.
- Innes, L., P. J. Hobbs, and R. D. Bardgett (2004). "The impacts of individual plant species on rhizosphere microbial community in soils of different fertility." *Biology and Fertility of Soils* 40: 7-13.
- Isaaks, E. H., and R. M. Srivastava (1989). *An Introduction to Applied Geostatistics*. New York, NY, Oxford University Press.
- Jensen, M. A., and N. Straus (1993). "Effect of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplifications of bacterial ribosomal DNA spacer regions." *PCR Methods and Applications* 3: 186-194.
- Jocteur Monrozier, L., J. N. Ladd, R. W. Fitzpatrick, R. C. Foster, and M. Raupach (1991). "Components and microbial biomass content of size fractions in soils of contrasting aggregation." *Geoderma* 50: 37-62.
- Joergensen, R. G., and X. Castillo (2001). "Interrelationships between microbial and soil properties in young volcanic ash soils of Nicaragua." *Soil Biology & Biochemistry* 33: 1581-1589.
- Johnson, G. A., D. A. Mortensen, and C. A. Gotway (1996). "Spatial and temporal analysis of weed seedling populations using geostatistics." *Weed Science* 44: 704-710.
- Johnson, N. C., and D. A. Wedin (1997). "Soil carbon, nutrients, and mycorrhizae during conversion of dry tropical forest to grassland." *Ecological Applications* 7: 171-182.
- Judo, M. S. B., A. B. Wedel, and C. Wilson (1998). "Stimulation and suppression of PCR-mediated recombination." *Nucleic Acids Research* 26: 1819-1825.

- Kaiser, E. A., and O. Heinemeyer (1993). "Seasonal variations of soil microbial biomass carbon within the plough layer." *Soil Biology and Biochemistry* 25: 1649-1655.
- Kanagawa, T. (2003). "Bias and artifacts in multitemplate polymerase chain reactions (PCR)." *Journal of Bioscience and Bioengineering* 96: 317-323.
- Kang, S., and A. L. Mills (2004). "Soil microbial community structure changes following disturbance of the overlying plant community." *Soil Science* 169: 55-65.
- Katznelson, H., A. G. Lochhead, and M. I. Timosin (1948). "Soil microorganisms and the rhizosphere." *Botanical Reviews* 14: 487-543.
- Kaye, J. P., and S. C. Hart (1997). "Competition for nitrogen between plants and soil microorganisms." *Trends in Ecology & Evolution* 12: 139-143.
- Kiem, R., and E. Kandeler (1997). "Stabilization of aggregates by the microbial biomass as affected by soil texture and type." *Applied Soil Ecology* 5: 221-230.
- Kiers, E. T., R. A. Rousseau, S. A. West, and R. F. Denison (2003). "Host sanctions and the legume-rhizobium mutualism." *Nature* 425: 78-81.
- Kim, G., and A. P. Barros (2002). "Space-time characterization of soil moisture from passive microwave remotely sensed imagery and ancillary data." *Remote Sensing of the Environment* 81: 393-403.
- Kjoller, A., and S. Struwe (1982). "Microfungi in ecosystems - fungal occurrence and activity in litter and soil." *OIKOS* 39: 391-422.
- Knezovich, J. P., F. L. Harrison, and R. G. Wilhelm (1987). "The bioavailability of sediment-sorbed organic chemicals: a review." *Water, Air and Soil Pollution* 32: 233-245.
- Kostov, K. G. (1993). "Passive Microwave Remote-Sensing of Soil-Moisture - Experimental and Modeling Results." *Advances in Space Research* 13: 105-114.
- Kowalchuk, G. A., P. L. E. Bodelier, G. H. J. Heilig, J. R. Stephen, and H. J. Laanbroek (1998). "Community analysis of ammonia-oxidising bacteria, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation." *FEMS Microbiology Ecology* 27: 339-350.
- Kowalchuk, G. A., D. S. Buma, W. de Boer, P. G. L. Klinkhamer, and J. A. van Veen (2002). "Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms." *Antonie van Leeuwenhoek* 81: 509-520.
- Kowalchuk, G. A., D. S. Buma, W. de Boer, P. G. L. Klinkhamer, and J. A. van Veen (2002). "Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms." *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology* 81: 509-520.

- Kowalchuk, G. A., G. J. v. Os, J. v. Aartrijk, and J. A. v. Veen (2003). "Microbial community responses to disease management soil treatment used in flower bulb cultivation." *Biology and Fertility of Soils* 37: 55-63.
- Kowalchuk, G. A., J. R. Stephen, W. de Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp (1997). "Analysis of ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments." *Applied and Environmental Microbiology* 63: 1489-1497.
- Kowalchuk, G. A., A. W. Stienstra, G. H. J. Heilig, J. R. Stephen, and J. W. Woldendorp (2000). "Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of clacareous grasslands." *Environmental Microbiology* 2: 99-110.
- Kozdroj, J., and J. D. van Elsas (2000). "Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches." *Soil Biology & Biochemistry* 32: 1405.
- Krasil'nikov, N. A. (1954). *Soil microorganisms and higher plants*. Moscow, Academy of Science, USSR.
- Lamboy, W. F. (1994). "Computing genetic similarity coefficients from RAPD data: the effects of PCR artifacts." *PCR Methods and Applications* 4: 31-37.
- Lame, F. P. J., and P. R. Defize (1993). "Sampling of contaminated soil: sampling error in relation to sample size and segregation." *Environmental Science & Technology* 27: 2035-2044.
- Lancini, G., and F. Parenti (1982). *Antibiotics, An Integrated View*. New York, NY, Springer-Verlag.
- LaPara, T. M., T. Zakharova, C. H. Nakatsu, and A. Konopka (2002). "Functional and structural adaptations of bacterial communities growing on particulate substrates under stringent nutrient limitation." *Microbial Ecology* 44: 317-326.
- Legendre, P., and L. Legendre (1998). *Numerical Ecology*. New York, NY, Elsevier.
- Legendre, P., and M. Troussellier (1988). "Aquatic heterotrophic bacteria: Modeling in the presence of spatial autocorrelation." *Limnology and Oceanography* 33: 1055-1067.
- Liebhold, A. M., and A. A. Sharov (1998). *Analysis of insect count data: testing for correlation in the presence of spatial autocorrelation. Population and Community Ecology for Insect Management and Conservation*. J. Braumgartner, P. Brandmayer and B. F. J. Manly. Balkema, Rotterdam, Swets & Zeitlinger Publishers. 111-117.
- Lilleskov, E. A., T. D. Bruns, T. R. Horton, D. L. Taylor, and P. Grogan (2004). "Detection of forest stand-level spatial structure in ectomycorrhizal fungal communities." *FEMS Microbiology Ecology* 49: 319-332.

- Lindstrom, E. S. (2001). "Investigating influential factors on bacterioplankton community composition: Results from a field study of five mesotrophic lakes." *Microbial Ecology* 42: 598-605.
- Ling, L. L., P. Keohavong, C. Dias, and W. G. Thilly (1991). "Optimization of the polymerase chain reaction with regard to fidelity: modified T7, *Taq* and Vent DNA polymerase." *PCR Methods and Applications* 1: 63-69.
- Ludwig, W., and K.-H. Schleifer (1999). "Phylogeny of *bacteria* beyond the 16S rRNA standard." *65: 752-757*.
- Lundberg, K. S., D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge, and E. J. Mathur (1991). "High-Fidelity Amplification Using a Thermostable DNA-Polymerase Isolated from *Pyrococcus-Furiosus*." *Gene* 108: 1-6.
- Lynch, J. M., and L. M. Panting (1982). "Effects of season, cultivation and nitrogen fertiliser on the size of the soil microbial biomass." *Journal of the Science of Food and Agriculture* 33: 249-252.
- Lynch, J. M., and J. M. Whipps (1990). "Substrate flow in the rhizosphere." *Plant & Soil* 129: 1-10.
- Mackas, D. L. (1984). "Spatial autocorrelation of plankton community composition in a continental shelf ecosystem." *Limnology and Oceanography* 29: 451-471.
- Mahbubani, M. H., and A. K. Bej (1994). Applications of polymerase chain reaction methodology in clinical diagnostics. *PCR technology: current innovations*. H. G. Griffin and A. M. Griffin. Boca Raton, FL., CRC Press. 307-326.
- Malmivaara-Lamsa, M., and H. Fritze (2003). "Effects of wear and above ground forest site type characteristics on the soil microbial community structure in an urban setting." *Plant and Soil* 256: 187-203.
- Marilley, L., and M. Aragno (1999). "Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots." *Applied Soil Ecology* 13: 127-136.
- Marschner, P., E. Kandeler, and B. Marschner (2003). "Structure and function of the soil microbial community in a long-term fertilizer experiment." *Soil Biology & Biochemistry* 35: 453-461.
- Marschner, P., G. Neumann, A. Kania, L. Weiskopf, and R. Lieberei (2002). "Spatial and temporal dynamics of the microbial community structure in the rhizosphere of cluster roots of white lupin (*Lupinus albus L.*)." *Plant and Soil* 246: 167-174.
- Marsh, T. L., W.-T. Liu, L. J. Forney, and H. Cheng (1998). "Beginning a molecular analysis of the eukaryal community in activated sludge." *Water Science & Technology* 37: 455-460.
- McGarigal, K., S. Cushman, and S. Stafford (2000). *Multivariate statistics for wildlife and ecology research*. New York, NY, Springer.

- Merz, B., and E. J. Plate (1997). "An analysis of the effects of spatial variability of soil and soil moisture on runoff." *Water Resources Research* 33: 2909-2922.
- Miller, D. N., J. E. Bryant, E. L. Madsen, and W. C. Ghiorse (1999). "Evaluation and optimization of DNA extraction and purification procedures for soils and sediment samples." *Applied and environmental microbiology* 65: 4715.
- Mills, A. L. (1999). The role of bacteria in environmental geochemistry. *Reviews in Economic Geology*. G. S. Plumlee and M. H. Logsdon, Society of Economic Geologists. 125-132.
- Miya, R. K., and M. K. Firestone (2001). "Enhanced phenanthrene biodegradation in soil by slender oat root exudates and root debris." *Journal of Environmental Quality* 30: 1911-1918.
- Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann, and D. A. Stahl (1996). "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria." *Applied and Environmental Microbiology* Appl. Environ. Microbiol. 62: 2156-2162.
- Morita, R. Y. (1990). "The starvation-survival state of microorganisms in nature and its relationship to the bioavailable energy." *Experientia* 46: 813-817.
- Morse, R., M. D. Collins, K. Ohanlon, S. Wallbanks, and P. T. Richardson (1996). "Analysis of the beta subunit of DNA-dependent RNA polymerase does not support the hypothesis inferred from 16S rRNA analysis that *Oenococcus oeni* (formerly *Leuconostoc oenos*) is a teychytelic (fast-evolving) bacterium." *International Journal of Systematic Bacteriology* 46: 1004-1009.
- Mummey, D. L., and P. D. Stahl (2003). "Spatial and temporal variability of bacterial 16S rDNA-based T-RFLP patterns derived from soil of two Wyoming grassland ecosystems." *FEMS Microbiology Ecology* 46: 113-120.
- Murray, A. E., J. T. Hollibaugh, and C. Orrego (1996). "Phylogentic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rRNA fragments." *Applied and Environmental Microbiology* 62: 2676-2680.
- Muyzer, G., S. Hotentr?er, A. Teske, and C. Wawer (1996). Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA - A new molecular approach to analyse the genetic diversity of mixed microbial communities. *Molecular Microbial Ecology Manual: Supplement 1*. A. D. L. Akkermans, J. D. van Elsas and F. J. De Bruijn. Dordrecht, The Netherlands, Kluwer Academic Publishers. 3.4.4:1-23.
- Muyzer, G., and K. Smalla (1998). "Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology." *Antonie van Leeuwenhoek* 73: 127-141.
- Muyzer, G., E. C. d. Waal, and A. G. Uitterlinden (1993). "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of

- polymerase chain reaction-amplified genes coding for 16S rRNA." *Applied and Environmental Microbiology* 59: 695-700.
- Myers, R. M., N. Lumelsky, L. S. Lerman, and T. Maniatis (1985). "Detection of single base substitutions in total genomic DNA." *Nature* 313: 495-498.
- Nantel, P., and P. Neumann (1992). "Ecology of Ectomycorrhizal-Basidiomycete communities on a local vegetation gradient." *Ecology* 73: 99-117.
- Nester, E. W., C. E. Roberts, N. N. Pearsall, D. G. Anderson, and M. T. Nester (1998). *Microbiology: A human perspective*. Boston, MA, WCB/McGraw-Hill.
- Newman, E. I. (1985). The rhizosphere: carbon sources and microbial populations. *Ecological Interactions in Soil*. A. H. Fitter. Oxford, UK, Blackwell Scientific Publishers. 107-121.
- Nicolaisen, M. H., N. Risgaard-Petersen, N. P. Revsbech, W. Reichardt, and N. B. Ramsing (2004). "Nitrification-denitrification dynamics and community structure of ammonia oxidizing bacteria in a high yield irrigated Philippine rice field." *FEMS Microbiology Ecology* 49: 359-369.
- Niemi, R. M., I. Heiskanen, K. Wallenius, and K. Lindstr? (2001). "Extraction and purification of DNA rhiosphere soil samples for PCR-DGGE analysis of bacterial consortia." *Journal of Microbiological Methods* 45: 155-165.
- NRCS (1982). *Soil Survey of Clarke County, Virginia, USDA NRCS / VA Tech*.
- Nunan, N., K. Wu, L. M. Young, J. W. Crawford, and K. Ritz (2002). "*In situ* spatial patterns of soil bacterial populations, mapped at multiple scales, in an arable soil." *Microbial Ecology* 44: 296-305.
- Odum, E. P. (1971). *Fundamentals of Ecology*. Philadelphia, PA, Saunders.
- Ogram, A. (1998). Isolation of nucleic acids from environmental samples. *Techniques in microbial ecology*. R. S. Burlage, R. Atlas, D. Stahl, G. Geessey and G. Sayler. Oxford, UK, Oxford University Press. 273-288.
- Olea, R., Ed. (1991). *Geostatistical Glossary and Multilingual Dictionary*. New York, NY, Oxford University Press.
- Ovreas, L., L. Forney, F. L. Daae, and V. Torsvik (1997). "Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA." *Applied and Environmental Microbiology* 63: 3367-3373.
- Ovreas, L., and V. Torsvik (1998). "Microbial diversity and community structure in two different agricultural soil communities." *Microbial ecology* 36: 303.
- Owen, A. G., and D. L. Jones (2001). "Competition for amino acids between wheat roots and rhizosphere microorganisms and the role of amino acids in plant N acquisition." *Soil Biology & Biochemistry* 33: 651-657.
- Paerl, H. W., J. Dyble, P. H. Moisaner, R. T. Noble, M. F. Piehler, J. L. Pinckney, T. F. Steppe, L. Twomey, and L. M. Valdes (2003). "Microbial indicators of aquatic

- ecosystem change: current applications to eutrophication studies." *Fems Microbiology Ecology* 46: 233-246.
- Pannatier, Y. (1996). *VARIOWIN: Software for spatial data analysis in 2D*. New York, NY, Springer-Verlag.
- Peixoto, R. S., H. L. D. Coutinho, N. G. Rumjanek, A. Macrae, and A. S. Rosado (2002). "Use of *rpoB* and 16S rRNA genes to analyse bacterial diversity of a tropical soil using PCR and DGGE." *Letters in Applied Microbiology* 35: 316-320.
- Pennanen, T., S. Caul, T. J. Daniell, B. S. Griffiths, K. Ritz, and R. E. Wheatley (2004). "Community-level responses of metabolically-active soil microorganisms to the quantity and quality of substrate inputs." *Soil Biology & Biochemistry* 36: 841-848.
- Pennock, D. J., B. J. Zebarth, and E. Dejong (1987). "Landform classification and soil distribution in hummocky terrain, Saskatchewan, Canada." *Geoderma* 40: 297-315.
- Pepper, I. L., C. P. Gerba, and J. W. Bredecke (1995). *Environmental Microbiology: A Laboratory Manual*. San Diego, CA, Academic Press.
- Polyanskaya, L. M., M. K. Orazova, O. A. Burkanova, and D. G. Zvyagintsev (2000). "Microsampling of rhizosphere soil and laboratory artifacts." *Microbiology* 69: 581-585.
- Polz, M. F., and C. M. Cavanaugh (1998). "Bias in template-to-product ratios in multitemplate PCR." *Applied and Environmental Microbiology* 64: 3724-3730.
- Postma, J., C. H. Hokahin, and J. A. v. Veen (1990). "Role of microniches in protecting introduced rhizobium-leguminosarum *Biovar trifolii* against competition and predation in soil." *Applied and Environmental Microbiology* 56: 495-502.
- Powell, W., M. Morgante, C. Andre, M. Hanafey, J. Vogel, S. Tingey, and A. Rafalski (1996). "The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis." *Molecular Breeding* 2: 225-238.
- Pray, L. A. (2004). Consider the Cyclor. *The Scientist*. 18: 34-37.
- Prosser, J. I. (1997). 7. Microbial Processes within the Soil. *Modern Soil Microbiology*. J. D. van Elsas, J. T. Trevors and E. M. H. Wellington. New York, NY, Marcel Dekker. 183-213.
- Pyper, B. J., and R. M. Peterman (1998). "Comparison of methods to account for autocorrelation in correlation analyses of fish data." *Canadian Journal of Fisheries and Aquatic Sciences* 55: 2127-2140.
- Ranjard, L., D. P. H. Lejon, C. Mougél, L. Schehrer, D. Merdinoglu, and R. Chaussod (2003). "Sampling strategy in molecular microbial ecology: influence of soil sample size on DNA fingerprinting analysis of fungal and bacterial communities." *Environmental Microbiology* 5: 1111-1120.

- Ranjard, L., F. Poly, J. Combrisson, A. Richaume, F. Gourbiere, J. Thioulouse, and S. Nazaret (2000). "Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA)." *EMicrobial Ecology* 39: 263-272.
- Ranjard, L., and A. Richaume (2001). "Quantitative and qualitative microscale distribution of bacteria in soil." *Research in Microbiology* 152: 707-716.
- Ravenschlag, K., K. Sahn, and R. Amann (2000). "Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine arctic sediments." *Applied and Environmental Microbiology* 66: 3592-3602.
- Ravit, B., J. G. Ehrenfeld, and M. M. Haggblom (2003). "A comparison of sediment microbial communities associated with *Phragmites australis* and *Spartina alterniflora* in two brackish wetlands of New Jersey." *Estuaries* 26: 465-474.
- Rayner, A. D. M., and L. Boddy (1988). *Fungal Decomposition of Wood: Its Biology and Ecology*. Chichester, England, John Wiley & Sons.
- Renganayaki, K., J. C. Read, and A. K. Fritz (2001). "Genetic diversity among Texas bluegrass genotypes (*Poa arachnifera* Torr.) revealed by AFLP and RAPD markers." *Theoretical and Applied Genetics* 102: 1037-1045.
- Ritz, K., J. W. McNicol, N. Nunan, S. Grayston, P. Millard, D. Atkinson, A. Gollotte, D. Habeshaw, B. Boag, C. D. Clegg, B. S. Griffiths, R. E. Wheatley, L. A. Glover, A. E. McCaig, and J. I. Prosser (2004). "Spatial structure in soil chemical and microbiological properties in an upland grassland." *FEMS Microbiology Ecology* 49: 191-205.
- Robertson, G. P., and K. L. Gross (1994). Assessing the heterogeneity of below ground resources: quantifying pattern and scale. *Exploitation of Environmental Heterogeneity by Plants: Ecophysiological Processes Above and Below Ground*. M. M. Caldwell and R. W. Pearcy. New York, NY, Academic Press. 237-253.
- Robertson, G. P., K. M. Klingensmith, M. J. Klug, E. A. Paul, J. R. Crum, and B. G. Ellis (1997). "Soil resources, microbial activity, and primary production across an agricultural ecosystem." *Ecological Applications* 7: 158-170.
- Rodriguez, H. G., I. C. Silva, M. V. G. Meza, and R. G. R. Lozano (2004). "Plant water relations of thornscrub shrub species, north-eastern Mexico." *Journal of Arid Environments* 58: 483-503.
- Rovira, A. D. (1959). "Root excretions in relation to the rhizosphere effect; IV. Influence of plant species, age of plant, light, temperature, and calcium nutrition on exudation." *Plant & Soil* 11: 53-64.
- Rovira, A. D. (1965). "Interaction between plant roots and soil microorganisms." *Annual Review of Microbiology* 19: 241-266.
- Ryu, C.-M., M. A. Farag, C.-H. Hu, M. S. Reddy, H.-X. Wei, P. W. Pare, and J. W. Kloepper (2003). "Bacterial volatiles promote growth in *Arabidopsis*."

- Proceedings of the National Academy of Sciences of the United States of America 100: 4927-4932.
- Saetre, P., and E. Baath (2000). "Spatial variation and patterns of soil microbial community structure in a mixed spruce-brich stand." *Soil Biology & Biochemistry* 32: 909-917.
- Sait, M., P. Hugenholtz, and P. H. Janssen (2002). "Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys." *Environmental Microbiology* 4: 654-666.
- Sambrook, J., and D. W. Russell (2001). *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press.
- Sampson, P. H., P. M. Treitz, and G. H. Mohammed (2001). "Remote sensing of forest condition in tolerant hardwoods: An examination of spatial scale, structure and function." *Canadian Journal of Remote Sensing* 27: 232-246.
- Sanyal, D., and G. Kulshrestha (1999). "Effects of repeated metolachlor applications on its persistence in field soil and degradation kinetics in mixed microbial cultures." *Biology and Fertility of Soils* 30: 124-131.
- Schimel, J. P., and F. S. Chapin, III (1996). "Tundra plant uptake of amino acid and NH₄⁺ nitrogen *in situ*: plants compete well for amino acid N." *Ecology* 77: 2142-2147.
- Schimel, J. P., J. M. Gullledge, J. S. Clein-Curley, J. E. Lindstrom, and J. F. Braddock (1999). "Moisture effects on microbial activity and community structure in decomposing birch litter in the Alaskan taiga." *Soil Biology and Biochemistry* 31: 831-838.
- Schmidt, N., and M. Bolter (2002). "Fungal and bacterial biomass in tundra soils along an arctic transect from Taimyr Peninsula, central Siberia." *Polar Biology* 25: 871-877.
- Schnurer, J., M. Clarholm, and T. Rosswall (1985). "Microbial biomass and activity in an agricultural soil with different organic matter contents." *Soil Biology & Biochemistry* 17: 611-618.
- Schuetz, C., J. Bogner, J. Chanton, D. Blake, M. Morcet, and P. Kjeldsen (2003). "Comparative oxidation and net emissions of methane and selected non-methane organic compounds in landfill cover soils." *Environmental Science & Technology* 37: 5150-5158.
- Schulze, E. D., H. A. Mooney, O. E. Sala, E. Jobbagy, N. Buchmann, G. Bauer, J. Canadell, R. B. Jackson, J. Loreti, M. Oesterheld, and J. R. Ehleringer (1996). "Rooting depth, water availability, and vegetation cover along an aridity gradient in Patagonia." *Oecologia* 108: 503-511.
- Shabana, Y. M., R. Charudattan, J. T. Devalerio, and M. A. Elwakil (1997). "An evaluation of hydrophilic polymers for formulating the bioherbicide agents *Alternaria cassiae* and *A. eichhorniae*." *Weed Technology* 11: 212-220.

- Shabana, Y. M., and M. E. Ragab (1997). "*Alternaria infectoria*, a promising biological control agent for the fig wax scale, *Ceroplastes rusci* (Homoptera:Coccidae), in Egypt." *Biocontrol Science and Technology* 7: 553-564.
- Sheffield, V. C., D. R. Cox, L. S. Lerman, and R. M. Myers (1989). "Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes." *Proceedings of the National Academy of Sciences of the United States of America* 86: 232-236.
- Shristie, P., and J. A. M. Beattie (1987). "Significance of sample size in measurement of soil microbial biomass by the chloroform fumigation-incubation method." *Soil Biology & Biochemistry* 19: 149-152.
- Shuldiner, A. R., A. Nirula, and J. Roth (1989). "Hybrid DNA artefact from PCR of closely related target sequences." *Nucleic Acids Research* 17: 4409.
- Smalla, K., G. Wieland, A. Buchner, A. Zock, J. Parzy, S. Kaiser, N. roskot, H. Heuer, and G. Berg (2001). "Bulk and rhizosphere soil bacterial communities studies by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed." *Applied and Environmental Microbiology* 67: 4742-4751.
- Smit, E., P. Leeftang, S. Gommans, J. van den Broek, S. van Mil, and K. Wernars (2001). "Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods." *Applied and Environmental Microbiology* 67: 2284-2291.
- Smith, K. T., C. M. Long, B. Bowman, and M. M. Monor (1990). "Using cosolvents to enhance PCR amplification." *Amplifications* 5: 16-17.
- Sobieraj, J. A., H. Elsenbeer, R. M. Coelho, and B. Newton (2002). "Spatial variability of soil hydraulic conductivity along a tropical rainforest catena." *Geoderma* 108: 79-90.
- Soderberg, K. H., A. Probanza, A. Jumponen, and E. Baath (2004). "The microbial community in the rhizosphere determined by community-level physiological profiles (CLPP) and direct soil- and cfu-PLFA techniques." *Applied Soil Ecology* 25: 135-145.
- Sokal, R. R., and F. J. Rohlf (1995). *Biometry*. New York, NY, Freeman.
- Sokal, R. R., and F. J. Rohlf (1995). *Biometry*. New York, NY, Freeman and Company.
- Speksnijder, A. G. C. L., G. A. Kowalchuk, S. D. Jong, E. Kline, J. R. Stephen, and H. J. Laanbroek (2001). "Microvariation artifacts introduced by PCR and cloning of closely related 16S rRNA gene sequences." *Applied and Environmental Microbiology* 67: 469-472.
- Spósito, G., and R. J. Reginato (1992). *Opportunities in Basic Soil Science Research*. Madison, WI, SSSA.
- Starkey, R. L. (1929). "Some influences of the development of higher plants upon the microorganisms in soil. I. Historical and introductory." *Soil Science* 27.

- Steer, J., and J. A. Harris (2000). "Shifts in the microbial community in rhizosphere and non-rhizosphere soils during the growth of *Agrostis stolonifera*." *Soil Biology & Biochemistry* 32: 869-878.
- Stendell, E. R., T. R. Horton, and T. D. Bruns (1999). "Early effects of prescribed fire on the structure of the ectomycorrhizal fungus community in a Sierra Nevada ponderosa pine forest." *Mycology Research* 103: 1353-1359.
- Tate, R. L. (2000). *Soil Microbiology*. New York, NY, John Wiley & Sons, Inc.
- Tatineni, V., R. G. Cantrell, and D. D. Davis (1996). "Genetic diversity in elite cotton germplasm determined by morphological characteristics and RAPDs." *Crop Science*. 36: 186-192.
- Taylor, J. P., B. Wilson, M. S. Mills, and R. G. Burns (2002). "Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques." *Soil Biology & Biochemistry* 34: 387-401.
- Terry, T. A., D. K. Cassel, and A. G. Wollum II (1981). "Effects of soil sample size and included root and wood on bulk density in forested soil." *Soil Science Society of America Journal* 45: 135-138.
- Thomas, G. W. (1996). 16. Soil pH and soil acidity. *Methods of Soil Analysis, Part 3. Chemical Methods*. D. L. Sparks, A. L. Page, P. A. Helmke et al. Madison, WI, Soil Science Society of America. 475-490.
- Tindall, K. R., and T. A. Kunkel (1988). "Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase." *Biochemistry* 27: 6008-6013.
- Tiquia, S. M., J. H. C. Wan, and N. F. Y. Tam (2002). "Microbial population dynamics and enzyme activities during composting." *Compost Sciences & Utilization* 10: 150-161.
- Tisdall, J. M., and J. M. Oades (1982). "Organic matter and water-stable aggregates in soils." *Journal of Soil Science* 33: 141-163.
- Tolley, P. M., and R. R. Christian (1999). "Effects of increased inundation and wrack deposition on a high salt marsh plant community." *Estuaries* 22: 944-954.
- Trumbore, S. (2000). "Age of soil organic matter and soil respiration: radiocarbon constraints on belowground C dynamics." *Ecological Applications* 10: 399-411.
- Ueda, K., T. Seki, T. Kudo, T. Yoshida, and M. Kataoka (1999). "Two distinct mechanisms cause heterogeneity of 16S rRNA." *Journal of Bacteriology* 181: 78-82.
- Vainio, E. J., and J. Hantula (2000). "Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA." *Mycological Research* 104: 927-936.
- van Gestel, M., J. N. Lad, and M. Amato (1992). "Microbial biomass responses to seasonal change and imposed drying regimes at increasing depths of undisturbed topsoil profiles." *Soil Biology & Biochemistry* 24: 103-111.

- van Gestel, M., R. Merckx, and K. Vlassak (1996). "Spatial distribution of microbial biomass in microaggregates of a silty-loam soil and the relation with the resistance of microorganisms to soil drying." *Soil Biology & Biochemistry* 28: 503-510.
- Webster, R., and M. A. Oliver (2001). *Geostatistics for Environmental Scientists*. West Sussex, UK, John Wiley & Sons.
- Welhan, J. (2004). *Geostatistics and Spatial Modeling, Lecture Notes*. Idaho State University, Department of Geosciences.
- Westover, K. M., A. C. Kennedy, and S. E. Kelley (1997). "Patterns of rhizosphere microbial community structure associated with co-occurring plant species." *Journal of Ecology* 85: 863-873.
- White, J. G., R. M. Welch, and W. A. Norvell (1997). "Soil zinc map of the USA using geostatistics and geographic information systems." *Soil Science Society of America Journal* 61: 185-194.
- Wikstrom, P., A.-C. Andersson, and M. Forsman (1999). "Biomonitoring complex microbial communities using random amplified polymorphic DNA and principal component analysis." *FEMS Microbiology Ecology* 28: 131-139.
- Wilson, J. M., and D. M. Griffin (1975). "Water potential and the respiration of microorganisms in the soil." *Soil Biology & Biochemistry* 7: 199-204.
- Wintzingerode, F. v., U. B. Gobel, and E. Stackebrandt (1997). "Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis." *FEMS Microbiology Review* 21: 213-229.
- Woese, C. R., and G. E. Fox (1977). "Phylogenetic structure of the prokaryotic domain: the primary kingdoms." *Proceedings of the National Academy of Sciences of the United States of America* 74: 5088-5090.
- Yang, C.-H., D. E. Crowley, and J. A. Menge (2001). "16S rDNA fingerprinting of rhizosphere bacterial communities associated with healthy and *Phytophthora* infected avocado roots." *FEMS Microbiology Ecology* 35: 129-136.
- Yang, J., R. D. Hammer, and R. W. Blanchar (1995). "Microscale pH spatial-distribution in the Ap horizon of Mexico silt loam." *Soil Science* 160: 371-375.
- Yang, J. C., and H. Insam (1991). "Microbial biomass and relative contributions of bacteria and fungi in soil beneath tropical rain forest, Hainan Island, China." *Journal of Tropical Ecology* 7: 385-393.
- Yi, S., J. H. Tay, A. M. Maszenan, and S. T. L. Tay (2003). "A culture-independent approach for studying microbial diversity in aerobic granules." *Water Science & Technology* 47: 283-290.
- Yoshitomi, K. J., and J. R. Shann (2001). "Corn (*Zea mays* L.) root exudates and their impact on ¹⁴C-pyrene mineralization." *Soil Biology & Biochemistry* 33: 1769-1776.

- Young, I. M., A. Roberts, B. S. Griffiths, and S. Caul (1994). "Growth of a ciliate protozoan in model ballotini systems of different particle sizes." *Soil Biology & Biochemistry* 26: 1173-1178.
- Zak, D. R., P. M. Groffman, K. S. Pregitzer, S. Christensen, and J. M. Tiedje (1990). "The vernal dam: plant-microbe competition for nitrogen in northern hardwood forests." *Ecology* 71: 651-656.
- Zak, D. R., W. E. Holmes, D. C. White, A. D. Peacock, and D. Tilman (2003). "Plant diversity, soil microbial communities, and ecosystem function: Are there any links?" *Ecology* 84: 2042-2050.
- Zhou, J., M. A. Bruns, and J. M. Tiedje (1996). "DNA recovery from soils of diverse composition." *Applied and Environmental Microbiology* 62: 316-322.
- Zhu, H. C., J. M. Charlet, and P. Doremus (1996). "Kriging radon concentrations of groundwaters in western ardennes." *Environmetrics* 7: 513-523.

APPENDIX A. EXPERIMENTAL EVALUATION OF SEMIVARIOGRAM ANALYSIS BASED ON RELATIVE DISSIMILARITY

As discussed several times throughout the dissertation, multivariate binary data of microbial community structure from DGGE were not the best format for semivariogram analysis. Instead, relative dissimilarity, based on Jaccard similarity coefficients, was calculated between pairs of samples and used to construct experimental semivariograms. Although this approach has historical background as similarity-distance relationship, there has not been any evaluation of usage for formal geostatistical analysis. Researcher did simple experimental evaluation by calculating continuous variables from current study (total nitrogen content and C/N ratio) in both formal semivariogram and ‘pseudo-semivariogram’ based on relative dissimilarities.

$$\gamma(\mathbf{h}) = \frac{1}{2N(\mathbf{h})} \sum_{i=1}^{N(\mathbf{h})} [z(\mathbf{u}_i) - z(\mathbf{u}_i + \mathbf{h})]^2, \quad \gamma'(\mathbf{h}) = \frac{1}{2N(\mathbf{h})} \sum_{i=1}^{N(\mathbf{h})} [a_i - a_{i+\mathbf{h}}].$$

All the conditions are same between two approaches that lag distances and scale settings are as they appeared in the table. Medium scale lag distance was differently set for comparisons that semivariogram used 25 cm and relative dissimilarity used 30 cm.

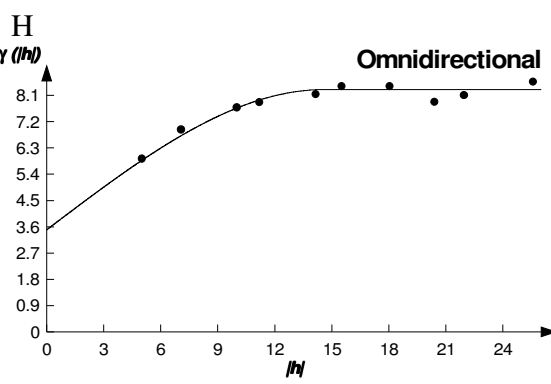
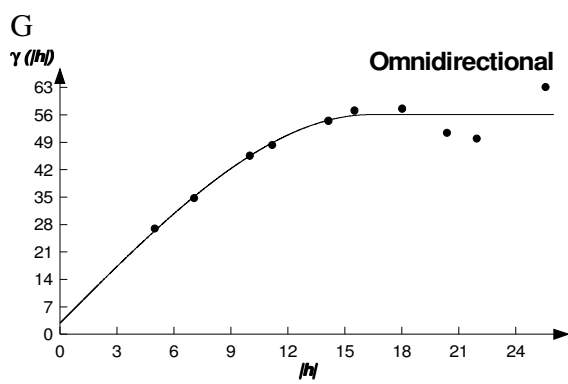
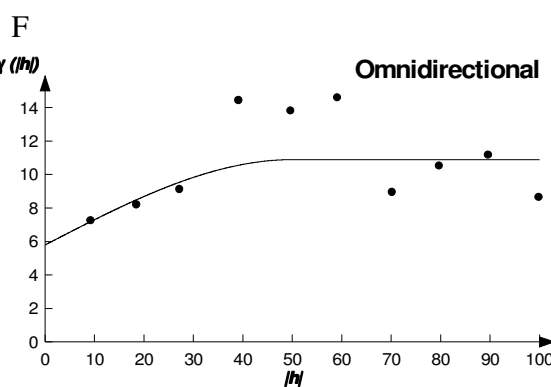
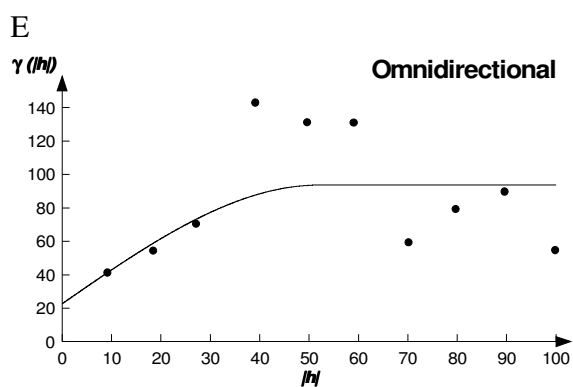
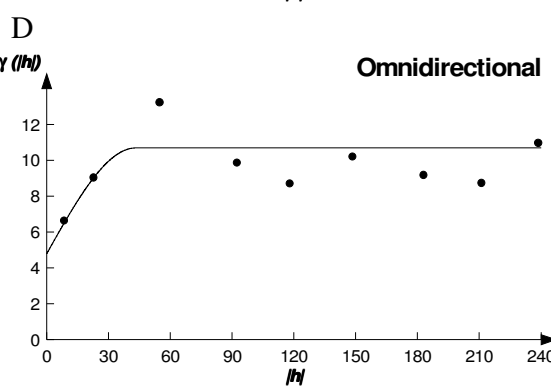
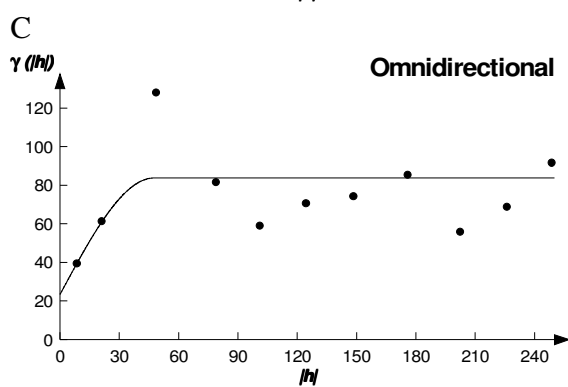
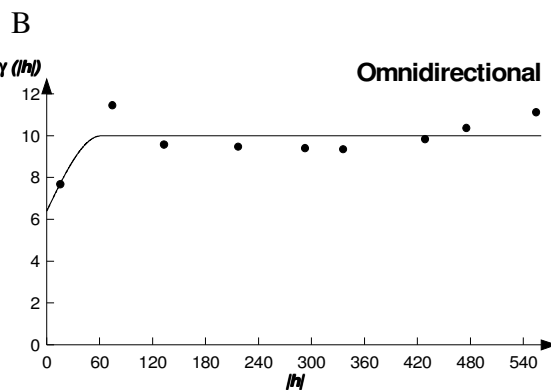
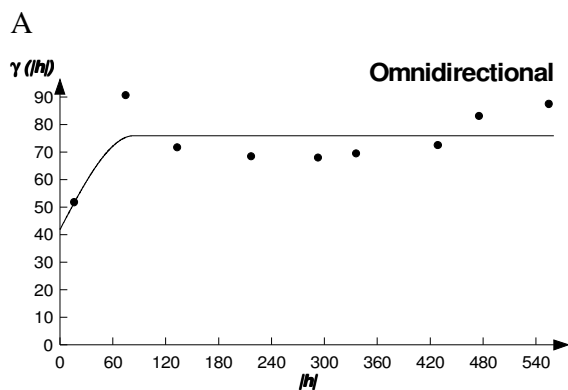
Table A1. Lag distances for different scale of variables N and C/N.

	Fine scale	Small scale	Medium scale	Plot scale
N	2 cm	10 cm	25 cm / 30 cm	70 cm
C/N	2 cm	10 cm	25 cm / 30 cm	50 cm

Lag tolerance is half of lags and angular tolerance is set to 90° because both variables are isotropy.

Table A2. Summary of results from semivariogram analysis and relative dissimilarity analysis for total nitrogen content and C/N ratio. All semivariograms were modeled as spherical model. Medium scale models were analyzed in different bin size and emphasized by bold characters.

Variable	Scale	Method	ID	IGF	Nugget (C_0)	Sill (C_1)	Range (a)	NMSD
Nitrogen	Field	Semi	A	3.10E-2	41.9	34.1	84 cm	0.45
		Dissim	B	1.31E-2	6.4	3.6	61.6 cm	0.36
	Medium	Semi	C	1.63E-1	23.43	60.35	47.5 cm	0.72
		Dissim	D	2.98E-2	4.8	5.9	43.2 cm	0.55
	Small	Semi	E	1.39E-1	22.7	71	52 cm	0.76
		Dissim	F	3.65E-2	5.8	5.1	50 cm	0.47
	Fine	Semi	G	2.73E-3	2.84	53.3	16.38 cm	0.95
		Dissim	H	6.03E-4	3.5	4.8	14.56 cm	0.58
C/N ratio	Field	Semi	I	1.95E-1	0	0.348	49.5 cm	1.00
		Dissim	J	2.65E-2	0.31	0.35	66 cm	0.53
	Medium	Semi	K	2.23E-1	0.044	0.352	70 cm	0.89
		Dissim	L	1.14E-2	0.26	0.43	60 cm	0.62
	Small	Semi	M	5.50E-2	0.024	0.344	61 cm	0.93
		Dissim	N	2.46E-2	0.28	0.42	61 cm	0.60
	Fine	Semi	O	4.44E-3	0.0040	0.136	13 cm	0.97
		Dissim	P	6.24E-4	0.17	0.26	12.48 cm	0.61



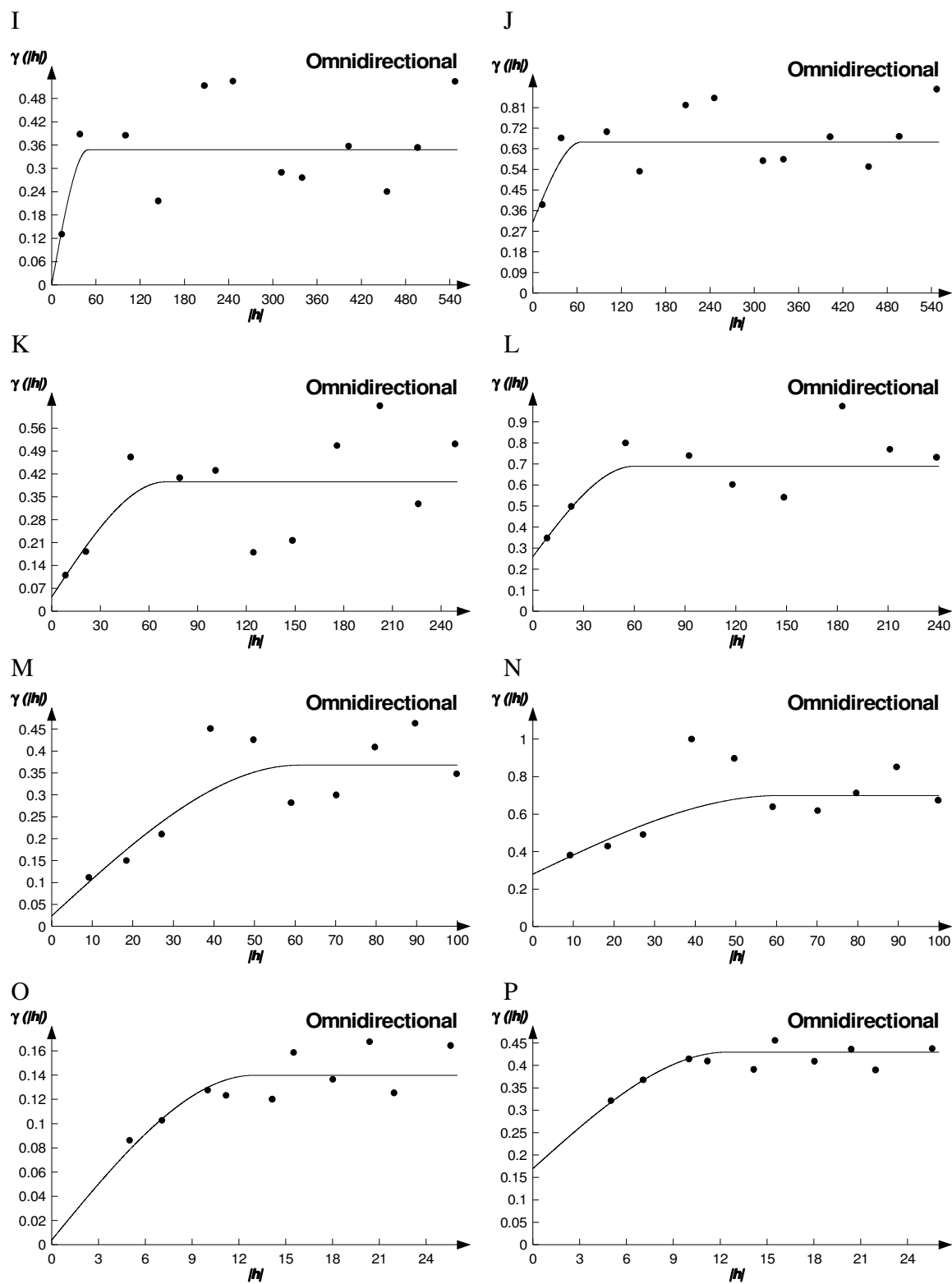


Figure A1. Comparisons of models from semivariogram and relative dissimilarity.

Patterns of semivariogram models between two methods were not conflicting from the first impression that all of them were spherical models, and ranges were compatible between methods even in medium scale, which was analyzed with different lag distances. However, substantial differences were identified in models of two methods in spatial dependence (NMSD) that all of the models based on relative dissimilarity were significantly less spatially structured than the semivariogram models. Between-method comparisons show that lower spatial dependence is generated from higher nuggets. The model fittings were better in the analysis based on relative dissimilarity. The overall trends were that the shapes of experimental semivariograms and models were very similar between two methods, but the locations of models were different in vertical position.

These results are confirmed the question rose from the analysis of bacterial and fungal community structures based on relative dissimilarity (4.5.2.). The extremely well fitting of the model and very low spatial dependence are two features found in the current evaluation. Therefore, at least certain degree of higher nuggets must be artificially generated from the spatial structure analysis based on relative dissimilarity.

APPENDIX B. PRACTICAL CHALLENGES OF GEOSTATISTICAL ANALYSIS USING VARIOWIN AND GSLIB TOGETHER.

As was mentioned in the Methods section, two different software packages were used for semivariogram modeling analysis (VARIOWIN) and estimation analysis (GSLIB).

GSLIB is a geostatistical software library that contains not only modules for estimation analysis (kriging and stochastic simulation) but also modules for semivariogram modeling. The freeware version of GSLIB is distributed in Fortran source code and compiled to DOS executables. Each module consists of DOS executable and parameter files. The latter are text files containing parameters for analysis and the data to be analyzed. In addition, the output is written in another text file, and needs another executable module to generate the graphs in postscript format. In semivariogram modeling, multiple runnings in both steps of experimental semivariogram and model fitting are needed. In experimental semivariogram, lag distance is determined by balancing between resolution (lag distance) and meaningful number of samples in each lag. As it is called 'experimental', this procedure is done by repeated redrawing with different lag distance (bin size) to determine best representing experimental semivariogram. Actual semivariogram modeling procedure requires even more extensive re-running to find best fitting model. Being working in DOS environment by changing two parameter files (analysis and postscript drawing) for each trial is not particularly efficient way to analyze large data sets. VARIOWIN is based on MS Windows 95 and possesses typical features of Windows-based software that multiple running for experimental semivariogram and actual semivariogram modeling are much more efficient.

One of the best features is found in Model module that modeling window and plot window are integrated to be simultaneously changed.

Since VARIOWIN is designed for only semivariogram modeling portion of geostatistics, its output includes both model in graph and parameters for estimation analysis in GSLIB in text file. Exponential and Gaussian models are defined more than one way due to the different conventions of range (a). This is because the fact that neither the exponential nor Gaussian models have true range, i.e., a lag distance at which the semivariance reaches constant. For those models, practical range is defined as distance at which semivariance reaches 95% of the sill (Isaaks and Srivastava, 1989). Geostatistical software chooses one of two conventions between theoretical range and practical range; therefore, it is critical to know the convention of software used and make adjustments for sharing between different software if necessary (Table 4.2.). VARIOWIN uses practical range in their model expression for both models. However, GSLIB is little bit confusing. The original version (GSLIB 1992 distributed in Fortran 77) used theoretical range in both model, so ranges obtained from VARIOWIN, which was distributed in 1997, automatically divided by 3 (exponential model) or $\sqrt{3}$ (Gaussian model) in output text file. The updated version (GSLIB 1998 distributed in Fortran 90) changed convention for both models that adjustment became unnecessary any more for the exponential model – range was multiplied by 3 from VARIOWIN output, since VARIOWIN has not been updated after 1998. But GSLIB's new convention for the Gaussian model uses a practical range as distance at which semivariance reaches 99.99% instead of 95% of the sill. Thus, for the Gaussian model, range was multiplied by 3 ($=\sqrt{3}\times\sqrt{3}$) before being fed into GSLIB (Mueller, 2004).

Table B1. Different conventions of exponential and Gaussian model between VARIOWIN and GSLIB.

	Exponential model	Gaussian model
VARIOWIN	$\gamma(\mathbf{h}) = C \left\{ 1 - \exp\left(-\frac{3\mathbf{h}}{a}\right) \right\}$	$\gamma(\mathbf{h}) = C \left\{ 1 - \exp\left(-3\left(\frac{\mathbf{h}}{a}\right)^2\right) \right\}$
GSLIB (1992)	$\gamma(\mathbf{h}) = C \left\{ 1 - \exp\left(-\frac{\mathbf{h}}{a}\right) \right\}$	$\gamma(\mathbf{h}) = C \left\{ 1 - \exp\left(-\left(\frac{\mathbf{h}}{a}\right)^2\right) \right\}$
GSLIB (1998)	$\gamma(\mathbf{h}) = C \left\{ 1 - \exp\left(-\frac{3\mathbf{h}}{a}\right) \right\}$	$\gamma(\mathbf{h}) = C \left\{ 1 - \exp\left(-\left(\frac{3\mathbf{h}}{a}\right)^2\right) \right\}$

C is structure variance and a is range.

There is another convention incompatibility problem in notation of direction.

Both uses degree to define directions but the beginning direction and the direction of rotation are different. VARIOWIN begins at East (0°) and rotates counterclockwise and GSLIB begins at North and rotates clockwise. For example, West is 180° from VARIOWIN convention and 270° from GSLIB convention. Again, VARIOWIN output text file contains automatically adjusted directions for GSLIB.

APPENDIX C. LIST OF GRASSES AND FORBS SOWN IN THE EXPERIMENTAL FIELD

Grasses:

Family: Poaceae

<i>Andropogon gerardi</i>	big bluestem
<i>Andropogon virginicus</i>	broom sedge
<i>Bouteloua curtipendla</i>	side-oats grama
<i>Panicum virgatum</i>	switch grass
<i>Schizachyrium scoparium</i>	little blue stem
<i>Sorghastrum nutras</i>	Indian grass

Forbs:

Genus and Species

Common Name

Monocots

Family: Commelinaceae

<i>Tradescantia virginiana</i>	Virginia spiderwort
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Family: Liliaceae

<i>Lilium philadelphicum</i>	wood lily
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Dicots

Family: Apiaceae

<i>Eryngium yuccifolium</i>	rattlesnake master
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Family: Apocynaceae

<i>Apocynum cannabinum</i> +	Indian hemp
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Family: Asclepiadaceae

<i>Asclepias incarnata</i> +*	swamp milkweed
<i>Asclepias syriaca</i> +	common milkweed
<i>Asclepias tuberosa</i> +	butterfly weed
<i>Asclepias verticillata</i>	whorled milkweed
<i>Asclepias viridifolia</i>	green-flowered milkweed

Family: Asteraceae

<i>Antennaria neglecta</i>	field pussytoes
<i>Antennaria. plantaginifolia</i>	plantainleaf pussytoes
<i>Aster novae-angliae</i> +*	New England aster
<i>Aster pilosus</i> +	heath aster
<i>Coreopsis lanceolata</i>	lance-leaved coreopsis
<i>Coreopsis verticillata</i>	whorled coreopsis
<i>Erigeron annuus</i> +	daisy fleabane
<i>Eupatorium fistulosum</i> *	hollow joe-pye weed
<i>Eupatorium maculatum</i> +*	joe-pye weed
<i>Eupatorium perfoliatum</i> *	boneset
<i>Helenium autumnale</i>	yellow sneezeweed
<i>Heliopsis helianthoides</i>	oxeye sunflower
<i>Kuhnia eupatorioides</i>	false boneset
<i>Liatris graminifolia</i>	grass-leaf blazing star
<i>Liatris scariosa</i>	large blazing star
<i>Liatris squarrosa</i>	scaly blazing star
<i>Parthenium integrifolium</i>	wild quinine
<i>Rudbeckia hirta</i> +	black-eyed Susan
<i>Rudbeckia fulgida</i>	orange coneflower
<i>Senecio pauperculus</i>	balsam ragwort
<i>Silphium trifoliatum</i>	whorled rosinweed
<i>Solidago altissima</i> +	tall goldenrod
<i>Solidago canadensis</i>	tall goldenrod
<i>Solidago gigantea</i> *	late goldenrod
<i>Solidago graminifolia</i>	lance-leaved goldenrod
<i>Solidago juncea</i> +	early goldenrod
<i>Solidago speciosa</i>	showy goldenrod
<i>Veronia noveboracensis</i> +*	New York ironweed

Family: Campanulaceae

<i>Campanula rotundifolia</i>	harebell
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Family: Euphorbiaceae

<i>Euphorbia corollata</i>	flowering spurge
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Family: Fabaceae

<i>Baptisia australis</i>	wild indigo
<i>Cassia fasciculata</i>	partridge pea
<i>Lespedeza capitata</i>	round-headed bush clover
<i>Lespedeza hirta</i>	hairy bush clover

Lupinus perennis wild lupine

Family: Gentianaceae

Sabatia angularis rose pink

Family: Lamiaceae

Monarda fitulosa + wild bergamot
Pycnanthemum tenuifolium * narrow-leaved mountain mint
Salvia lyrata lyre-leaved sage
Scutellaria integrifolia hyssop skullcap
Trichostema dichotomum blue curls

Family: Linaceae

Linum virginianum wild yellow flax

Family: Onagraceae

Ludwigia alternifolia * seedbox
Oenothera biennis + common evening primrose
Oenothera fruticosa narrow-leaved sundrops

Family: Plantaginaceae

Plantago aristata bracted plantain

Family: Polygalaceae

Polygala sanguinea rose polygala

Family Primulaceae

Lysimachia quadrifolia whorled loosestrife

Family: Rosaceae

Rosa carolina + pasture rose

Family: Scrophulariaceae

Chelone glabra * turtle head
Gerardia purpurea purple gerardia

<i>Mimulus ringens</i> +*	square-stemmed monkey flower
<i>Penstemon digitalis</i>	foxglove beard-tongue
<i>Penstemon laevigatus</i>	smooth beard-tongue

Family: Solanaceae

<i>Solanum carolinense</i> +	horse nettle
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Family: Verbenaceae

<i>Verbena hastata</i> +*	blue vervain
<i>Verbena simplex</i>	narrow-leaved vervain

+ Species previously existed in the field

* Species usually associated with wetter habitat