This is to certify that the

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The Diversity and Dynamics of Microbial Groups in Soils from Agroecosystems

presented by

Daniel Hezekiah Buckley

has been accepted towards fulfillment
of the requirements for

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Major professor

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THE DIVERSITY AND DYNAMICS OF MICROBIAL GROUPS IN SOILS FROM AGROECOSYSTEMS

By

Daniel Hezekiah Buckley

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

2000

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ABSTRACT

THE DIVERSITY AND DYNAMICS OF MICROBIAL GROUPS IN SOILS FROM AGROECOSYSTEMS

By

Daniel Hezekiah Buckley

Soil microbial communities are integrally involved in biogeochemical cycles, and their activities are crucial to the productivity and health of terrestrial ecosystems. Despite their relative importance, little is known about how microorganisms are distributed in the soil or the manner in which these organisms respond to environmental changes. To investigate the structure of microbial communities in soil, molecular techniques were used to determine the distribution and abundance of select microbial groups in soil. The influence of environment on microbial abundance was observed over a period of two years in a series of replicated plots that included agriculturally managed fields, fields abandoned from agriculture, and fields with no history of agriculture. Microbial community structure was characterized by using molecular phylogenetic techniques to monitor the relative abundance of eight of the most numerically abundant microbial groups in soil (the Alpha and Beta Proteobacteria, Actinobacteria,

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The data described in this dissertation reveal that soil microbial communities are dynamic, capable of changing significantly at temporal scales relevant to seasonal events. In this background of temporal change, however, the relative abundance of particular microbial groups remains constrained by localized environmental characteristics resulting in reproducible patterns of community structure. Microbial community structure was observed to be remarkably similar among fields that shared a long-term history of agricultural management despite the differences in plant community composition and land management practices that had been maintained on the plots in recent years. In contrast, the microbial communities in fields that had never been cultivated differed significantly from those in fields that shared a long-term history of cultivation. These data indicate that the long-term effects of agricultural management on the soil alter microbial community structure and that these changes continue to be evident in fields abandoned from cultivation for as long as a decade. This dissertation provides insight into the structure of soil microbial communities and reveals that while soil microbial communities are dynamic, alterations in the soil environment can influence them fundamentally.

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ACKNOWLEDGEMENTS

I count the six years that I've spent at Michigan State as the happiest that I have known. That I value this time in my life so dearly I attribute directly to the many people that I've had the great fortune of meeting while in graduate school. The friends I've made have contributed to my development both as a scientist and as a person and I owe them a debt of gratitude. My appreciation first goes to my advisor, Tom Schmidt for the guidance and encouragement he has provided over the years, for his curiosity and enthusiasm for science, and for his love of teaching. His example will guide me throughout my career. Next I would like to thank all of the members of the Schmidt and Breznak Labs, past and present, for their assistance and camaraderie. I'm especially grateful to Bonnie Bratina, Paul Lepp, and Brad Stevenson for showing me the ropes, and for making the lab such a fun place to be. I would also like to thank Deb Hogan, Mary Ellen Davey, and Mark and Carol Johnson for the experiences that we've shared from orientation all the way through graduation. Finally I must thank all of my friends in the Micro Department and in the Center for Microbial Ecology who've made my graduate school experience so memorable. I could never have completed my research without the guidance of my committee and I

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am grateful to John Breznak, Kay Gross, and Mike Klug for their assistance in designing and completing my research objectives. Likewise, I could never have completed my graduate studies without the support and love of my family. All that I am, that I have done, I owe to them. My deepest gratitude goes to the Micro Department softball team, because it was at a softball game that I met Merry, my soon to be wife. The greatest measure of my happiness I owe to Merry, for the times we've shared, and for the promise of our future. Thank you.

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CHAPTER 1

MICROBIAL DIVERSITY IN SOIL

INTRODUCTION

Although apparently dominated by plants, terrestrial ecosystems are estimated to contain 26 x 10^{28} prokaryotic microbes that harbor 26 Pg of carbon, 6.2 Pg of nitrogen and 0.65 Pg of phosphorus (75). When compared to the total amount of these elements estimated to be present in the Earth's terrestrial plants (559 Pg carbon, 10 Pg nitrogen, 1.05 Pg phosphorus), the importance of soil microbes as a component of terrestrial ecosystems is obvious.

The importance of microbes as a component of the Earth's terrestrial biomass and their power to influence terrestrial ecosystems is derived from their sheer numbers and from the diverse array of biochemical reactions they catalyze in soil. While it is generally understood that microbial communities in soil are of central importance to the productivity and health of terrestrial ecosystems (20), these communities remain largely unexplored. In addition to their impact on terrestrial ecosystems, microbial communities in soil also

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have a measurable impact on atmospheric chemistry and global climate by influencing budgets of gases such as CO_2, CH_4, H_2, N_2O, and NO (19).

Since the advent of agriculture, the human race has inadvertently undertaken a global ecological experiment whose outcome is far from certain. Extensive regions of land have been converted for agricultural use, leading to massive changes in soil nutrient cycles. For instance, the use of synthetic nitrogen fertilizers now doubles the rate of nitrogen input into the bioshpere (71). This tremendous influx of nitrogen has had and will continue to have widespread consequences for both terrestrial and aquatic ecosystems, since nitrogen from agricultural fields leaches into surface and ground waters. The full consequences of these large-scale alterations to the ecosystem are not yet known, but as we struggle to assess the impact of these changes, an improved understanding of the microbial communities that drive biogeochemical cycles on Earth becomes essential.

This chapter will review some of the processes mediated by soil microorganisms, provide background information on the composition of soil microbial communities and the methods used to study them, and discuss environmental factors that may influence microbial community structure and the distribution of microorganisms in the environment. The end of this chapter provides an overview of the questions that I have sought to address in this dissertation and the observations and experiments I have performed in

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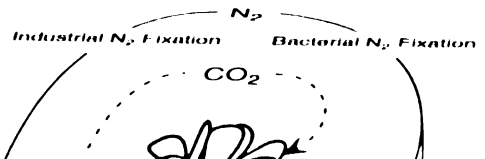
my attempts to answer them.. Although our understanding of the composition and functioning of microbial communities in soil is meager, we now have the tools and conceptual framework to begin an expansive exploration of this seldom appreciated natural resource.

AN OVERVIEW OF MICROBIAL PROCESSES IN SOIL

The linkage of carbon and nitrogen seen in global biogeochemical cycles is based in part on the activity of microbes in soil (Figure 1.1). Soil microbes breakdown and utilize a wide spectrum of organic compounds from natural sources like plant litter or animal feces to man-made sources including pesticides and herbicides. The products of microbial decomposition and microbial biomass itself contribute to soil organic matter and are important factors in determining the fertility of soils (2, 78). Microbes also catalyze the tranformation of many inorganic nitrogenous compounds in soil, beginning with the fixation of nitrogen - a process carried out exclusively by Bacteria and Archaea. Because of the central importance of carbon and nitrogen in terrestrial ecosystems, this chapter will focus on the interaction of microbes with these elemental cycles.

Soils are typically considered to be carbon-limited environments for microbes, with a majority of carbon input coming from the decomposition of plant litter. Plant polymers are degraded through the activity of fungi as well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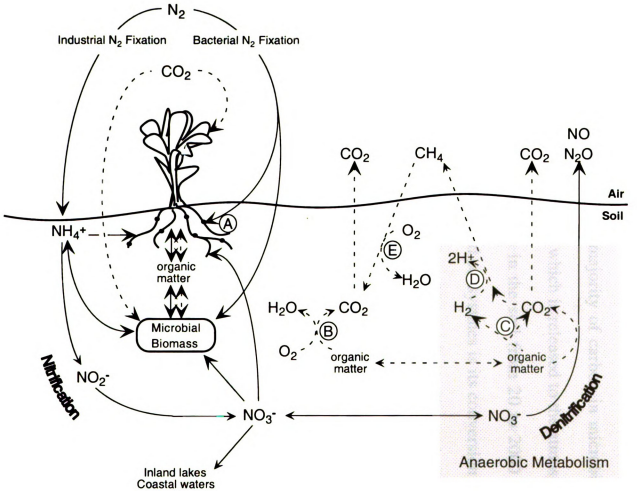

Figure 1.1. Major transformations of nitrogen (—) and carbon (- - -) in soil. A) nitrogen fixation by plant-associated bacteria, B) heterotrophic respiration, C) fermentation, D) methanogenesis, E) methane oxidation.

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as by both aerobic and anaerobic heterotrophic bacteria. Nutrients mineralized by microbial decomposition are then utilized by plants, scavenged by other microorganisms and maintained in microbial biomass, or released to the atmosphere. The majority of carbon in microbial biomass eventually ends up as either CO_2 - which is released to the atmosphere, or as humic compounds which persists in the soil from 20 to 2000 years (78). Another fate of carbon in terrestrial ecosystmes is its conversion to methane by methanogenic Archaea (Figure 1.1). This biologically produce methane is either released to the atmosphere, or consumed by the aerobic, methylotrophic bacteria in soil. The balance between these two processes has a major impact on the global methane budget (19).

Microbial transformations of organic compounds also contribute to the physical and chemical structure of soil. Humic compounds and polysaccharides produced by microorganisms interact with particulates in soil to form soil aggregates. Microbial activity in soil aggregates can then influence oxygen distribution in soils, creating habitats for microaerophillic and anaerobic microbes that catalyze a variety of important reactions in soils from methane production to denitrification (see below). The abundance and composition of aggregates also influences water infiltration rates and water holding capacity, which in turn alter microbial process involved in the cycling of other nutrients in soil, especially nitrogen (31, 66, 67).

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Nitrogen availability is a major determinant of the primary productivity of many terrestrial ecosystems. The largest natural input of combined nitrogen in soils is generated by the bacterial fixation of atmospheric nitrogen (2). This fixation is carried out by bacteria in symbiotic associations with certain plants, as well as by free living soil bacteria. Microbes also influence nitrogen cycling in soils through the decomposition of dead plant and animal matter, resulting in the release of ammonia to the soil or the immobilization of nitrogen in microbial biomass (2, 70). Nitrogen in microbial biomass is released by the activities of microbial predators including protozoans, nematodes, and microarthropods. This nitrogen is then available for uptake by other microbes or by plants. The release and uptake of nutrients during the turnover of microbial biomass creates a "microbial loop" analogous to that seen in aquatic ecosystems.

Microbes also impact nitrogen cycling in soils through the sequential activities of nitrification and denitrification. Nitrification is a type of aerobic metabolism that results in the sequential oxidation of ammonia (NH_3) to nitrite (NO_2^-) and then to nitrate (NO_3^-). As opposed to the ammonium ion (NH_4^+) which is retained well by soils, nitrate is readily leached from soils. The leaching of nitrate from soil has been observed to increase nitrate levels in ground water, making this water hazardous for human consumption (64), and has led to the eutrophication of lakes and coastal waters. The nitrate that

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remains in soil can act as a terminal electron acceptor for the type of anaerobic respiration known as denitrification. Denitrification occurs in anoxic environments in soil (e.g. the center of soil aggregates), and results in the reduction of nitrate to nitrous oxide (N_2O), nitric oxide (NO), or dinitrogen (N_2). The processes of nitrification and denitrification are ecologically relevant because of their role in determining the fate of nitrogen in soils, and because these processes yield gases that influence atmospheric chemistry and contribute to the greenhouse effect (21, 64). The potential influence of soil microbes on global scale processes is immense and warrants continued study of the structure and function of these microbial communities.

MICROBIAL DIVERSITY IN SOIL

Assessing microbial diversity

Although most microbiologists would agree that the diversity of microbial communities in soil is extraordinary, there would likely be less agreement as to how that diversity is best measured. Diversity is composed of two elements: richness and evenness, so that the highest diversity occurs in communities with many different species present (richness) in relatively equal abundance (evenness) (37). However, there are fundamental difficulties associated with determining the richness and evenness of

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communities composed of microbes whose morphological traits generally convey little physiological or phylogenetic information. The traditional way to study microbes is to grow and study them in pure culture, however this approach has severe limitations for studying soil microbial communities since less than 1% of the bacteria present in the soil can be readily grown on standard laboratory media (68). As a result, soil microbial communities are usually studied by examining the presence of microbial biomarkers in the soil. These biomarkers are frequently molecules such as lipids, proteins, or nucleic acids that convey either phenotypic or genotypic information about the microorganisms from which they originate. Phospholipid fatty acids (PLFA) comprise one group of biomarkers that is commonly used to study changes in microbial community structure in soil. Because the PLFA composition of membranes changes in response to the physiological condition of the cell, these markers provide phenotypic information about microbial communities. PLFA profiles have been used to determine whether microbial communities are similar or different, but generally it is difficult to identify the organisms that account for the similarities or differences between communities (83).

The genes that encode for ribosomal RNA (rRNA) have a low rate of evolutionary change, and are conserved among all cellular life forms, making them useful in examining phylogenetic relationships among

organisms (77).

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organisms (77). The nucleotide sequences and secondary structures of rRNAs consist of conserved domains found in all living organisms and variable domains which contain sequence motifs specific for groups of related organisms or even individual species. As a result, the rRNA-encoding genes have proved to be a valuable biomarker for studying the richness and evenness of microbial communities in natural environments. Figure 1.2 provides an overview of rRNA-based methods used to study microbial ecology and indicates which methods are preferred for assessment of richness or evenness of microbial communities. Although methods that rely on the Polymerase Chain Reaction (PCR) to amplify nucleic acids can be used to examine the richness of microbial communities, their ability to accurately represent the evenness of species in the community may be limited by biases imposed during amplification and cloning steps (76). The evenness of microbial communities can be measured by using DNA probes to determine the abundance of specific microbes or microbial groups within the community by using either nucleic acid hybridization or Fluorescent In Situ Hybridization (FISH) techniques (Figure 1.2). Nucleic acid hybridization is used to measure the abundance of either DNA or RNA from microorganisms in a community, while FISH allows specific microbial cells to be identified by using epifluorescent microscopy.

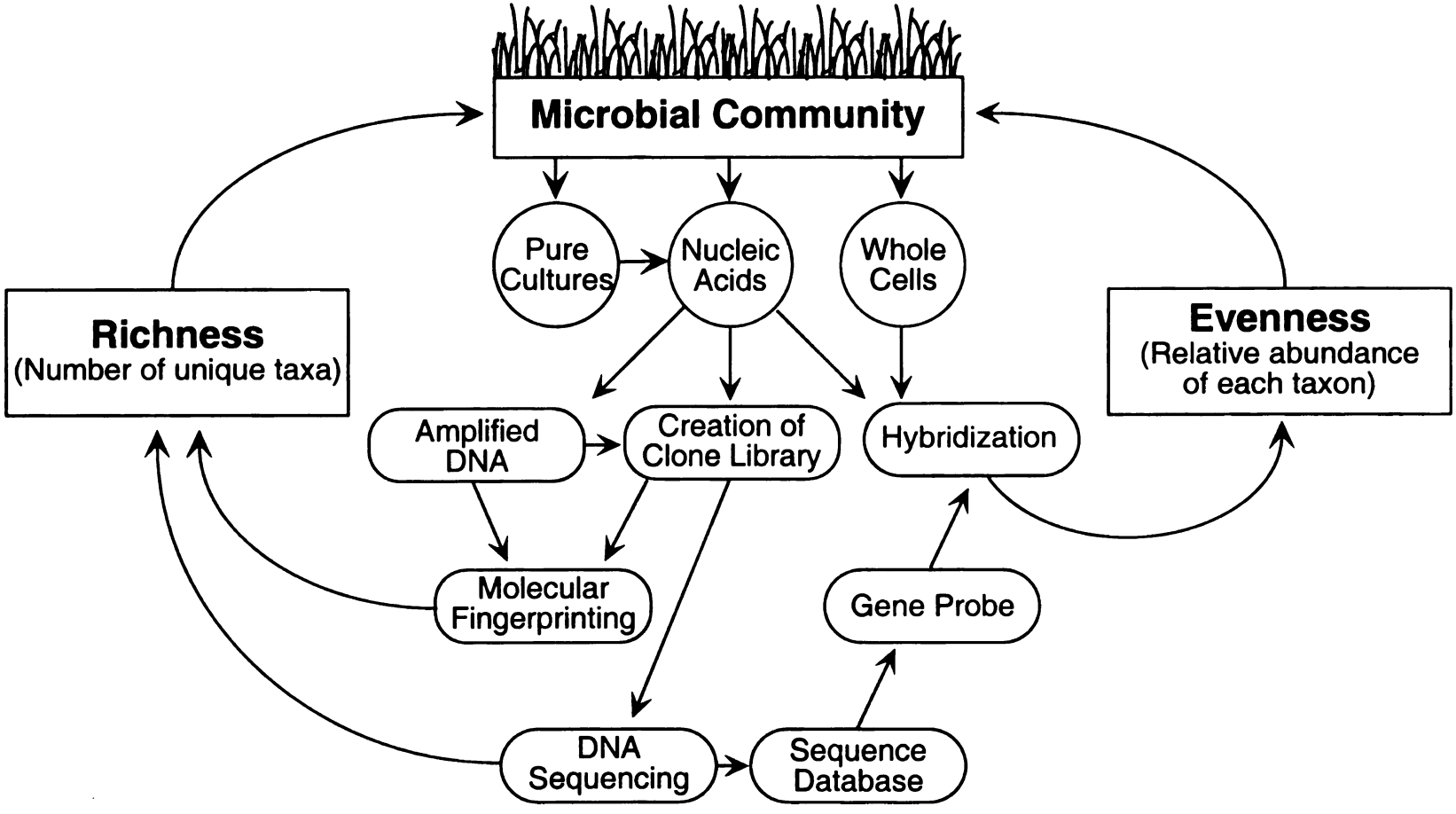

Figure 1.2. Nucleic acid-based approaches to study the diversity of natural microbial communities.

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The scale of diversity measurements

The phylogenetic scale used to measure microbial diversity is no less important a consideration than the physical scale at which diversity is measured. For example, since the sequence of the 16S rRNA changes very slowly over time relative to the rate at which microbes evolve new traits, organisms with similar 16S rRNA genes can have different genetic characteristics encoding distinct phenotypic traits. As a result, studies of microbial diversity that focus solely on the 16S rRNA gene can underestimate community richness. This does not mean that the 16S rRNA gene is a poor choice of a molecule to use in evaluating diversity in microbial communities, on the contrary its slow rate of change is what makes it a useful measure of the evolutionary history of an organism. Rather physiological traits predicted by differences in the 16S rRNA gene would be those that take a long time to develop. In contrast, the nucleotide sequence of a protein-encoding gene generally changes more rapidly and will tend to reveal higher levels of diversity. However, any measure of diversity based on a single functional gene will still underestimate the actual diversity present in a soil community because organisms that have identical DNA sequences at one locus can have multiple differences in other loci. As a result of these considerations, the extent of microbial diversity that is measured in any system will be proportional to the phylogenetic resolution

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of the method used. The most comprehensive analysis of the genetic diversity of a microbial community would require characterization of every distinct genome present in the community. Recent advances have made possible the analysis of large portions of the genomes of soil organisms (62). Further development in the analysis and interpretation of such data could reveal valuable insights into the diversity of microbial communities in the soil.

The extent of microbial diversity in soil

On the scale of a prokaryotic cell, a single gram of soil is an extremely complex ecosystem characterized by overlapping gradients of moisture, oxygen, organic compounds and other chemical constituents. If we were to think of a single bacterial cell as being the size of a human, then the total surface area in a single gram of soil (approximately 2.8×10^5 m^2 for a typical clay loam) is greater than the area of all of the Earth's continents combined. It is perhaps not surprising then that a gram of soil can contain as many as 1×10^{10} organisms representing at least 4,000 different microbial species (68). As a result, the potential biodiversity harbored in the soils of the Earth is staggering. Certain groups of microorganisms are commonly found in soils all over the world suggesting that microbes must have been dispersed among these sites. However, the rate at which indigenous

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microbes in a particular soil are replaced by microbes from other soils may be slower than the potential rate of evolution within the microbial community. Consequently the degree of spatial isolation between sites within a continuous landscape can be sufficient to permit the diversification of microbial populations as these populations adapt to local environmental conditions through natural selection (29, 46, 48).

Microbial groups commonly present in soil

The identity and abundance of microbes in the soil is currently being explored through the use of molecular techniques. Certain patterns have emerged from these analyses, with 7 of the 36 recognized bacterial divisions regularly found in soil samples (Table 1.1). These bacterial lineages diverged from one another hundreds of millions or perhaps even billions of years ago, and each accounts for a tremendous amount of genetic diversity (Figure 1.3). Although the microorganisms detected in soil most frequently fall into one of these bacterial divisions, so much diversity is present within each of these divisions that surveys of the rRNA genes present in a single soil sample rarely recover the exact same gene sequence twice. Despite the enormous genetic diversity within each of the bacterial divisions, the high frequency with which these groups are recovered from soils of diverse origin

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Group Name
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Acidobacteria
Verrucomicrobia
Cytophagales
Actinobacteria
Firmicutes
Planctomycetes

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Table 1. The relative abundance of bacterial groups commonly detected in soil microbial communities as determined by different techniques. [a]

Group Name	rDNA [b]	rRNA [c]	FISH [d]	Plate Counts [e]
Proteobacteria				
Alpha Subdivision	18 ± 18	25 ± 13	5 - 22	12 ± 12
Beta Subdivision	5 ± 5	2 ± 2	0 - 2	12 ± 13
Gamma Subdivision	3 ± 3	3 ± 1	< 1	10 ± 5
Delta Subdivision	4 ± 8	na	3 - 5	0
Acidobacteria	19 ± 19	4 ± 6	na	0
Verrucomicrobia	11 ± 12	2 ± 2	na	0
Cytophagales	7 ± 6	< 1	< 1	10 ± 13
Actinobacteria	7 ± 7	11 ± 8	na	15 ± 14
Firmicutes	6 ± 9	na	na	41 ± 37
Planctomycetes	3 ± 5	7 ± 5	4 - 7	0

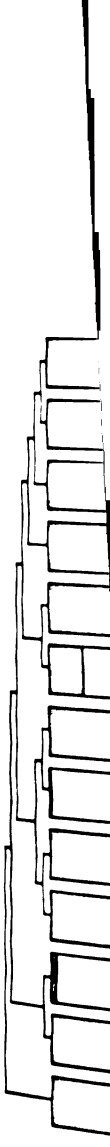
[a] Values are presented as means with standard deviations.

[b] Data calculated for 733 16S rDNA sequences in clone libraries obtained by PCR amplification of DNA from 11 distinct soils (7, 8, 43-45, 47, 69, 84).

[c] Average rRNA abundance of 85 soil samples taken from cultivated and grassland fields located in Southwestern Michigan.

[d] Data indicate the range of values obtained for three different European forest soils (18, 82).

[e] Data are calculated for soil isolate collections obtained on complex media under aerobic conditions (33, 34).



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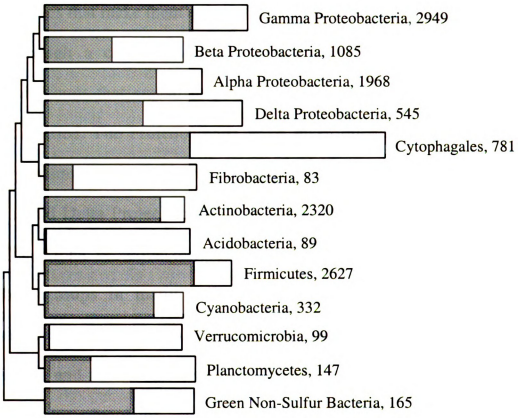

Figure 3. Phylogenetic tree showing the microbial groups that are most commonly recovered from soils. The length of the bar associated with each microbial group is equal to the amount of 16S rRNA sequence divergence that is encompassed by each microbial group (a measure of the potential diversity of the group), while the shaded regions portray the proportion of sequences within each group that represent cultivated strains. The total number of sequences that are known for each microbial group is listed to the right of the group name.

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argues that many bacteria within each of these groups have ancestral characteristics that make them especially suited to life in the soil.

Through a combination of techniques currently available, the microbial groups that appear to dominate soil microbial communities are being identified. The Proteobacteria are a metabolically diverse group of microorganisms subdivided into five groups, four of which, the Alpha, Beta, Gamma, and Delta Proteobacteria, are commonly detected in soils (Table 1.1). The Alpha Proteobacteria appear to be one of the most abundant microbial groups in many soils as assessed by both molecular and cultivation-dependent methods. This diverse microbial group contains the *Rhizobium* and many other nitrogen-fixing bacteria, as well as certain methylotrophic organisms among its members. Members of the Beta and Gamma Proteobacteria, though generally not as abundant as the Alpha Proteobacteria, are also commonly detected in the soil. Microbes known to mediate nitrification are found among the Beta proteobacteria, while the Gamma Proteobacteria contain organisms such as the fluorescent pseudomonads that are well known for their ability to metabolize a diverse array of carbon compounds. The Delta Proteobacteria are comprised mainly of sulfate- and iron-reducing bacteria; these organisms are commonly found in the soil although, because of their intolerance for atmospheric oxygen

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Though not as well known as the Proteobacteria, Acidobacteria are detectable in a wide variety of environmental samples and have been detected in nearly all soil samples analyzed (5). This bacterial group contains at least six phylogenetic subgroups and may be as genetically diverse as the Proteobacteria (5). Although Acidobacteria are widespread and abundant in soils, very little is known about these microbes. Currently, only three strains of Acidobacteria have been cultivated under laboratory conditions, providing few insights as to the metabolic capabilities of this diverse group of microorganisms.

Like the Acidobacteria, the Verrucomicrobia constitute a phylogenetically diverse group of bacteria commonly detected in the soil by molecular techniques, but rarely represented in soil isolate collections. Currently only six strains from this group have been characterized, four of which are prosthecate organisms and the other three of which are ultramicrobacteria. Thus, all of the isolated strains seem to have cell shapes that maximize surface to volume ratios. The cultivated strains have been isolated from aquatic systems or saturated soils and seem to specialize in the degradation of carbohydrates. It is difficult to speculate on the specific function of Verrucomicrobia in soils, but observations that this group is

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widespread and abundant in diverse soils indicates that these organisms are an important component of soil microbial communities.

Cytophagales are commonly detected in soil rDNA clone libraries and are frequently isolated from soil samples (Table 1.1). Many of these organisms are involved in the aerobic degradation of cellulose or chitin and so are suspected to be of importance in the decomposition of plant materials. Despite their widespread distribution and ease with which many of the members of this group have been obtained in pure culture, there have been few studies addressing the diversity or ecological significance of these microbes.

Microorganisms that possess tough gram-positive cell membranes tend to be abundant in soil microbial communities. Gram positive organisms fall into one of two phylogenetic groups, the Firmicutes and the Actinobacteria. The Actinobacteria tend to have genomes with high mole percent G + C contents and so are commonly referred to as High GC Gram-Positive Bacteria. These bacteria are well represented in pure cultures and are metabolically diverse. The coryneform bacteria and the filamentous actinomycetes are the Actinobacteria most commonly recovered in soil isolate collections. The Firmicutes are commonly known as the Low GC Gram-Positive Bacteria because of the tendency for their genomes to have a low mole percent G + C content. The Firmicutes group contains the

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endospore-forming bacteria, the lactic acid bacteria and the gram-positive cocci. It is interesting to note that the Actinobacteria and Firmicutes are recovered less frequently in rDNA clone libraries collected from soil than in soil isolate collections. This observation may be due to the overrepresentation of these organisms in culture collections or their under-representation in clone libraries due to difficulty extracting nucleic acid from these resilient gram-positive cells.

Planctomycetes are commonly found in aquatic systems and tend to be aerobic organisms that grow best in dilute media. These organisms are prosthecate, divide by budding, and are one of the only bacterial groups that lack peptidoglycan in their cell walls. Though a number of strains are present in culture collections, few planctomycetes have been obtained from soil samples. Molecular methods reveal that Planctomycetes are both diverse and abundant members of soil microbial communities (Table 1.1), though nothing is known about the role these organisms may be playing in soil systems.

While the bacterial groups mentioned above are those that are most consistently found in soils, they are far from the only microbial groups present. Figure 1.4 shows the relative abundance of the bacterial groups in soil in relation to the abundance of the Eukarya, the Archaea, and the portion of the microbial community that remains unknown. Eukaryotic

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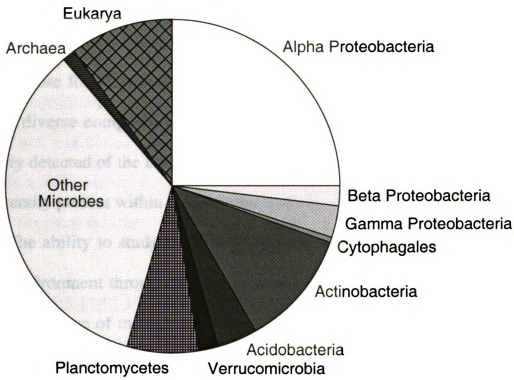

Figure 4. Data from the Kellogg Biological Station Long Term Ecological Research site located in southwestern Michigan that shows the average abundance of rRNA from microbial groups in the soil expressed as a proportion of the total amount of rRNA present.

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microorganisms in the soil such as Fungi, Protozoa, and numerous soil microfauna compose an enormous portion of the Earth's biodiversity, and even the Archaea, which comprise a small fraction of the total biodiversity in soils, contain a diverse array of organisms. For example, an ever increasing diversity of archaeal organisms belonging to the Crenarchaeota, a microbial group once thought only to exist in high temperature environments such as those found at deep-sea hydrothermal vents, have been detected in soils of diverse composition in sites all over the world (13). However, the diversity detected of the Eukarya and the Archaea in soils is just a fraction of the diversity present within the bacterial domain.

The ability to study the distribution and diversity of microorganisms in the environment through the use of molecular techniques has identified a central challenge of microbial ecology. Microbial physiology and behavior is most easily studied with microbial cultures that can be grown under laboratory conditions, however, many of the organisms that are abundant in the environment prove difficult to cultivate in the lab (36). Additionally, microbial groups once thought to dominate soil microbial communities because of the ease with which they could be cultivated, are now known to be less abundant than previously thought (26). Microorganisms from groups such as the Beta and Gamma Proteobacteria, the Cytophagales, and the Firmicutes are all over-represented in collections of cultivated isolates

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relative to their actual abundance in the soil (Table 1.1). A more formal understanding of the importance of microbial biodiversity will require both the developments of new techniques to study the physiology of cells *in situ* and new methods for cultivating recalcitrant organisms in the laboratory.

The enormous richness of bacterial species in soil suggests that there are numerous niches to exploit in soil. Sites that have distinct edaphic and climatic conditions might be expected to favor the growth of microbial groups not mentioned above. The groups described, however, have been found in a wide variety of soils, and their consistent presence and abundance in soils suggests that they occupy niches common in soil.

FACTORS INFLUENCING MICROBIAL DIVERSITY IN SOIL

The diversity and distribution of microorganisms present in soil are influenced by environmental factors that vary over space and time. However, relationships between environmental characteristics and microbial community structure remain poorly understood. The following sections describe the dynamic influence that environmental factors can have on microbial community structure from the scale of single soil aggregates to entire landscapes.

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A microenvironment is defined by the chemical and physical characteristics in the immediate surroundings of a microbe. The microenvironment is the scale most pertinent to the survival and activity of individual microorganisms because ultimately it is at this scale that microbes interact with their environment. Soils, because of their physical and chemical complexity, have an enormous number of potential microenvironments. Many of the microenvironments in soil are formed within soil aggregates that vary in diameter from a few dozen microns to many millimeters. Larger soil aggregates are composed of numerous microaggregates (< 50 micrometers in diameter) that provide additional habitats for microbial growth. Aggregates can provide bacteria a refuge from bactivorous protozoa and nematodes as these aggregates posses pores accessible to bacteria but small enough to exclude larger bacterial predators. The composition and activity of microbial communities change in relation to location within soil aggregates, consistent with the notion that soil aggregates are composed of numerous distinct microenvironments that are home to distinct microbial populations (55).

Biological activity can alter the chemical composition within soil aggregates. For example, aerobic organisms present at the surface of a large aggregate can consume oxygen and produce carbon dioxide (57). As a result

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the interiors of aggregates frequently have reduced oxygen and increased carbon dioxide levels relative to those of air, and the centers of aggregates can be completely anoxic. Soil moisture and temperature influence both gas diffusion and microbial activity so that over time the concentrations of oxygen and carbon dioxide at any given location in the aggregate will be a function of these environmental variables (57). Overlay onto this complex picture the heterogeneous distribution of resources within any given aggregate, and the large number of microbial process that are able to influence the chemistry of the aggregate and we begin to appreciate the potential number of microenvironments that can co-exist even in a single soil aggregate.

Factors that influence the distribution and type of microenvironments that are present in a particular environment may also influence patterns of microbial diversity at larger scales, such as individual fields. For example, the genetic diversity of *Pseudomonas cepacia* strains isolated from fields was strongly correlated with the degree of microenvironment heterogeneity at each field located within a landscape gradient of soil habitat variability (46). Microbial community structure at the scale of a field is composed of a dynamic mosaic of microenvironments each inhabited by a smaller microbial community whose composition may be sensitive to physiochemical changes that occur in the microenvironment over time.

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The influence of plants

There are many ways that plants influence the structure of microbial communities in the soil. One way is through root exudates that can influence the composition of microbial communities associated with the plant rhizosphere (30). Microbial communities in rhizosphere soil have been shown to differ significantly from those in non-rhizosphere soil by analysis of amplified 16S rDNA through denaturing gradient gel electrophoresis (25, 79), and through analysis of both FAME (38) and PLFA (50) profiles. One of the fundamental differences between rhizosphere microbial communities and non-rhizosphere communities seems to be an increase in the numbers of gram-negative organisms present in rhizosphere systems (38). Less clear is whether the rhizosphere effect is a general phenomenon resulting in similar patterns of community structure in all plant rhizospheres or a specific phenomenon with each plant rhizosphere tending to favor a particular set of microorganisms. Recent evidence indicates that microbial community structure in the rhizosphere can be significantly altered by changes in plant nutritional status (79). Additionally, studies of cultivated isolates suggest that plants can influence the microbial community composition in the rhizosphere (74). These observations are consistent with the idea that

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individual plants may either actively or passively alter the composition of microbial communities associated with their root systems.

Plants also influence the structure of soil microbial communities both by consuming resources and through the addition of root exudates and dead plant matter to the soil. Microbial biomass in the soil displays a positive linear relationship with annual net primary productivity, demonstrating that the growth of certain organisms within microbial communities are likely to be controlled by plant derived carbon inputs to the soil (81). Plant community composition and spatial distribution within fields can influence the composition of microbial communities, but the dynamics of this relationship are poorly defined. Experiments involving the short-term manipulation of plant community composition rarely demonstrate an influence on microbial community composition. Instead, most of the evidence for a relationship between plant and microbial communities comes from the observation of sites established for many years or decades. As a result, the influence of plants on microbial community structure in non-rhizosphere soil is likely to be indirect, caused by the long-term impact that plants have on local soil characteristics.

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Soil characteristics

Soil type, surface topography, and water distribution are all factors that have an influence on microbial communities (53). Organic matter content, texture, pH, and nutrient status all vary with soil type and are influenced by past and current management practice. Analysis of DNA complexity and 16S rDNA clone libraries from soil (51) as well as soil PLFA profiles (10) reveal that soil type can affect microbial community structure. Soil topography primarily influences patterns of water distribution in fields, and both of these characteristics in turn influence soil microbes. The impact of water distribution and soil elevation on microbial communities is indicated by relationships between these variables and both soil microbial biomass (60) and denitrification rates (54, 59). The significance of soil water distribution on microbial community composition is further emphasized by the sensitivity of microbial PLFA profiles to soil flooding (9).

Temporal variability in the environment

Changes in microbial activity can occur within a few hours or days in response to sudden changes in soil moisture content. A sudden increase in soil moisture is typically followed by rapid increases in soil respiration, nitrification, and nitrogen mineralization rates, as well as an increase in the

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concentration of extractable ammonium (11, 21, 39). The magnitude of these fluctuations in soil is proportional to the magnitude of the change in water potential caused by the wetting event (39). Wetting events lead to temporary increases in the availability of nutrients in soil by causing the release of nutrients from microbial biomass and by increasing the capacity for diffusion of these nutrients within the soil matrix (15). The release of nutrients following a wetting event occurs as some proportion of the soil microbes explode as a result of their inability to cope with the sudden change in osmotic stress and others release solutes into the soil to balance osmotic stresses (39). The activity of microbial predators may also to the nutrient pulse as their activity increases following a wetting event causing the turnover of nutrients from microbes susceptible to predation. Though less rapid in its effects than wetting, desiccation of the soil can also influence community structure as the movement of nutrients through soil decreases and microbes must cope with lowered intracellular water potentials (11). Wetting/drying cycles influence microbial communities in soil by causing a susceptible portion of the community to be killed, and allowing a resistant portion of the community to benefit from the nutrients released.

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these changes have been poorly characterized. A positive correlation between latitude and the temporal variability of microbial biomass reveals that variation in microbial communities occurs in response to seasonal changes in the environment (72). Additionally, significant changes in microbial community structure have been shown to occur at time scales that are consistent with seasonal changes in the environment (14). Though the seasonal dynamics of soil microbial communities have been poorly explored, there is reason to believe that cyclical changes in these communities may occur in response to seasonal changes in the environment. In early spring, before plants begin uptake of water and nutrients, microbial communities in soil achieve high rates of nitrogen mineralization, nitrification, and denitrification (31). During summer months competition between plants and microbes for available nutrients and increased soil temperatures may influence microbial community composition. While in the fall microbial communities in soil are likely to be influenced by the senescence of plant roots and an increased deposition of plant litter. Finally, the seasonal changes that occur in microbial communities should differ both qualitatively and quantitatively in response to land-use patterns and plant community composition (32).

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Alteration of the physical or chemical characteristics of the soil can lead to changes in the structure and function of microbial communities. Severe changes in habitat such as those caused by the presence of chemical pollutants in the soil can cause significant reductions in the diversity of microbial communities (3). The structure of microbial communities can also be influenced by agricultural practices. In cultivated fields changes in tillage and cropping practices, as well as fertilizer and herbicide addition have been shown to alter both microbial community composition and function (10, 23, 27, 38, 61). These management-induced changes in microbial community composition are usually a consequence of changes in habitat and substrate availability experienced by the microorganisms in soil (52).

The microbial communities associated with historically disturbed sites can take years to attain the characteristics of microbial communities in non-disturbed plots (28, 40). The Kellogg Biological Station Long Term Ecological Research site in southwestern Michigan contain fields under different types of agricultural practices as well as fields that have abandoned from cultivation and fields that have never been cultivated. At this site, microbial community structure is not discernibly different between actively cultivated fields and fields abandoned from cultivation for as long as a decade. At the same time the microbial community structure in both

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cultivated and abandoned fields is significantly different from that in fields that have never been cultivated (14). Similar observations have been made at this site at different levels of phylogenetic resolution with the autotrophic ammonia-oxidizing community in the soil (12). While at other sites analyses of autotrophic ammonia-oxidizer communities reveal that the succession of these communities in disturbed fields proceeds over a span of decades (42).

That microbial communities require long periods of time to recover from the effects of cultivation likely indicates that soil microbial communities are sensitive to soil characteristics that require long periods of time to recover from the effects of cultivation. Long-term cultivation of the soil can significantly deplete soil carbon and nitrogen levels (24), and can cause major changes in the distribution of soil resources and soil structure (58). The depletion of nitrogen and organic matter in agricultural fields can influence microbial activity in soil for many years after abandonment (1). While soil carbon and nitrogen levels can impact microbial community composition as determined by both PLFA profiles (73, 80) and catabolic diversity (22). Recovery of microbial communities from disturbance is likely dependent on recovery of soil characteristics to pre-disturbance levels. As a result microbial succession may require long periods of time as the natural recovery of soil carbon and nitrogen pools to their pre-agricultural levels can require hundreds of years (41).

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MICROBIAL COMMUNITIES AND ECOSYSTEM FUNCTION

The issue of functional redundancy

Microbial communities in soil possess enormous genetic diversity encoding for a diverse range of physiological activities. In many cases two or more genetically distinct organisms may have the capacity to mediate a single process in soil. When multiple organisms mediate a single process, these organisms are functionally redundant so that the removal of one of the species has no appreciable effect on ecosystem function. The idea of functional redundancy provides the intellectual underpinnings for the hypothesis that biodiversity leads to the stability of ecosystem function (17, 49). Certain processes in the soil are mediated by so many different types of microorganisms that changes in the community composition have little or no effect on the activity of the community as a whole. Functionally redundant organisms may be able to co-exist within the soil matrix due to spatial isolation or due to subtle physiological differences that permit them to exploit slightly different niches. One such process that is widespread among physiologically diverse microbes within soil is the aerobic respiration of carbon compounds. As a result of the redundancy in this metabolism, there can be wholesale changes in community composition that have negligible effects on rates of carbon respiration or the incorporation of carbon into

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microbial biomass (63). The tremendous diversity of soil microbes buffers the community as a whole against changes in activity. Obviously, the degree of functional redundancy should dictate the sensitivity of a given process to changes in the environment. Ecosystem processes mediated by a less diverse set of microorganisms than are seen for the aerobic heterotrophs, such as the processes of nitrification and denitrificantion, are more likely to be influenced by changes in the microbial diversity of an environment.

Soil biodiversity and the carbon cycle

Whole ecosystem models used to predict carbon flows in terrestrial ecosystems do not consider the composition of soil microbial communities, which has led to the hypothesis that microbial community composition has no consequence on the flow of carbon through terrestrial ecosystems (63). In late successional grasslands, for example, changes in microbial biomass and respiration of labile organic carbon compounds are directly related to climate and measures of annual net primary productivity regardless of differences in microbial community composition (81). However, there is still reason to believe that microbial community composition can have significant impacts on global carbon cycles. Litter bag experiments, in which the decomposition of plant residues can be monitored in different ecosystems, indicate that the community composition of soils can both quantitatively and

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qualitatively influence the breakdown of plant materials (63). Also, the examination of microbial community changes that occur in soils over a successional gradient encompassing sites abandoned for as many as 100 years indicate that late successional microbial communities are more metabolically efficient (in regards to respiration per unit biomass) than more recently disturbed sites (50). As a result, late successional microbial communities should tend to store more carbon in microbial biomass than communities in disturbed or recently (< 20 years) abandoned sites. Considerations of changes in the carbon storage potential of soil microbial communities in relation to changing patterns of land usage could have implications for global climate models that predict atmospheric carbon dioxide levels.

The composition of methane producing and consuming microbial communities might also impact global climate models. The known organisms that consume methane also require oxygen and are restricted to a few microbial groups while the methane-producing microbes grow in the absence of oxygen and are members of the Archaea. Though the link between the diversity and function of methane producing and methane consuming microbes is even less well characterized than that for the microbial groups mentioned previously, the restriction of these activities to a

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limited number of phylogenetic groups may lower the potential for functional redundancy.

Soil biodiversity and the nitrogen cycle

Microbial community composition has a major impact on the fate of nitrogen in the soil. Nitrogen-fixing organisms in association with plants account for the majority of the natural inputs of nitrogen into terrestrial ecosystems, however, there are also free-living microorganisms that are capable of fixing atmospheric nitrogen. These diazotrophic (i.e. N_2-fixing) organisms can account for a considerable amount of the fixed nitrogen in many environments. Diazotrophic bacteria that live in association with the roots of *Spartina* plants in coastal salt marshes have been shown to be of sufficient diversity to make them functionally redundant (4). Despite environmental fluctuations, the rate of nitrogen fixation remains constant in these systems because of the buffering influence of biodiversity. In contrast, reductions in the diversity of nitrogen fixing bacteria may have a major impact on certain ecosystems as has been suggested in the litter of clearcut forest patches. The soils under clearcut forest patches have a decreased rate of nitrogen fixation relative to those in undisturbed forest patches, this decrease in nitrogen fixation could be associated with a reduction in the diversity of diazotrophic microbes observed in the clearcut sites (65).

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The processes of nitrification and denitrification can also be influenced by changes in the composition of microbial communities in the soil. Autotrophic ammonia-oxidizing bacteria such as *Nitrosomonas* are responsible for the first step in the conversion of ammonia, a common component of fertilizer, into nitrate which is easy leached from the soil into groundwater, lakes and rivers (Figure 1). In fields recovering from disturbance, the composition of the autotrophic ammonia-oxidizing community in soil has been shown to change, accompanied by reductions in the rate of nitrification, though total numbers of autotrophic ammonia-oxidizing bacteria remains constant (12, 42). In addition, changes in autotrophic ammonia-oxidizing community composition can affect the response of these organisms to low oxygen tensions in the soil thereby influencing nitrification in fields that undergo varying degrees of water saturation (6).

Changes in the community composition of denitrifying microbes can influence the rate at which nitrate is converted into atmospheric nitrogen and nitrous oxide thereby influencing nitrogen loss from soil and the production of nitrous oxide, a powerful greenhouse gas (16, 35). The composition of denitrifying communities in soil can influence the total amount of denitrification and the ratio of nitrous oxide to nitrogen gas produced by soils (16). In addition, microbial community composition can influence the

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The influence of microbes on plant community composition

Microbial communities control the cycling and availability of soil nutrients and as a result they may influence the composition and structure of overlying plant communities. More directly, microorganisms are capable of forming both beneficial and deleterious associations with plants. These relationships, in many cases, involve specific associations between a microorganism and its plant host, such that the distribution of individual microbial species in the soil can profoundly affect the survival of plant species in the environment.

The root systems of most if not all, terrestrial plants possess some sort of mycorhizal association. Mycorhizal fungi are microorganisms that form associations with plant roots thereby increasing the transport of moisture and nutrients to plant roots and also increasing the ability of a plant to resist infection. The association of mycorhizal fungi with plant roots is largely nonspecific so that many different fungi may be able to colonize the roots of a particular plant, however, different species of mycorhizal fungi can vary in the effects that they have on different plants (56). In this way the diversity of

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mycorhizal fungi present in the soil can influence plant nutrition and influence the composition of plant communities.

The diversity of nitrogen-fixing organisms in the soil can also have a profound influence on the composition of plant communities. Many nitrogen-fixing microbes enter into symbiotic relationships with plants and can contribute significantly to the nutrition and productivity of their hosts. The most notable examples involve symbioses that form between microbes and either leguminous plants (*Rhizobium*-legume symbioses) or angiosperms (*Frankia*-actinorhizal symbioses). These nitrogen-fixing symbioses involve specific interactions between the microbes and plant roots so that a particular plant strain can only enter into symbioses with a select set of microbes. As a result of this specificity, the diversity of nitrogen-fixing organisms in the soil can significantly influence the productivity of both legumes and angiosperms and should influence the relative fitness of these plants in the environment.

The capacity of some microbes to cause plant disease is an obvious means by which the composition of microbial communities in the soil can influence plant communities. As with symbiotic organisms, plant pathogens tend to form fairly specific associations with susceptible plant hosts so that the distribution of a particular pathogen in the soil can influence the distribution of susceptible plant species. Plants that co-evolve in the

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presence of pathogenic microbes typically develop resistance mechanisms that minimize the impact of local diseases. However, under conditions of stress or when non-native plants or microbes are introduced to an environment plant pathogens can have tremendous impact on plant communities.

SUMMARY

Microbes are abundant in the soils of the Earth and their activities have far-reaching consequences for all of Earth's ecosystems. Soil microorganisms sequester nitrogen and phosphorus in quantities similar to that of terrestrial plants and the processes they mediate have a fundamental impact on the productivity of terrestrial ecosystems. Microbial communities in soil also impact the entire planet by acting as both a source and sink of atmospheric gases that affect the global climate - from the formation of clouds to the greenhouse effect (61). As we struggle to understand the complex interactions between microorganisms in soils and the function of terrestrial ecosystems, the face of the planet is changing. Increasing amounts of the Earth's surface are being converted to agricultural uses as anthropogenic nitrogen inputs to terrestrial systems escalate. A better understanding of the function and composition of microbial communities in

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soils will be needed to predict the consequences that these ecosystem changes will have on the biosphere.

THESIS OVERVIEW

Objectives

Very little is currently known about the composition of microbial communities in the soil, the manner in which community structure changes in response to the environment, and the importance of community structure as a factor influencing ecosystem function. The physiology of the microorganisms that dominate soil microbial communities for the most part remains a mystery as many of these microorganisms continue to resist attempts at enrichment and cultivation. Methods that allow for the study of microorganisms in the soil independent of cultivation provide us insight into their environmental distribution and allow us to observe the dynamic interactions between these organisms and the environments they inhabit. The research described in this dissertation has been designed to focus on the most abundant microbial groups in the soil and to identify environmental characteristics that influence their abundance. The hypothesis tested by this research is as follows:

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Microbial community structure in the soil varies in response to changes in the environment that occur as a result of agricultural management practices.

In addressing this hypothesis I have addressed the following questions:

1) Are microbial communities influenced by field level characteristics or are these changes solely influenced by landscape level characteristics such as climate or soil classification?
2) Do microbial communities vary in relation to changes in the composition of overlying plant communities?
3) Do microbial communities respond to changes in fertilization or tillage regime?
4) How much time is required for microbial communities to change in response to changes in environmental variables or field management?

These questions were addressed by focusing both on specific microbial groups and on whole communities in order to understand both how individual groups respond to environmental change and the importance of certain environmental variables to the community as a whole.

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Experimental approach

To answer the questions posed above and to validate the stated hypothesis I used rRNA-based methods to characterize soil microbial communities at the Long Term Experimental Research (LTER) site located at Michigan State University's W. K. Kellogg Biological Station (KBS). The KBS LTER site was established in 1988 as part of the U.S. National Science Foundation's LTER network. The KBS LTER main includes seven different agricultural treatments each replicated in six one-hectare blocks. The treatments include four annual (wheat/corn/soybean) crop rotations that vary in tillage type and fertilizer input, two perennial systems (poplar trees and alfalfa), and successional fields. These seven treatments were established in 1989 on a site that was historically (for > 50 yr.) under cultivation. An additional set of fields that have never been cultivated are located nearby the main site. The availability of data concerning soil resources, heterogeneity, treatment effects, climate, and many other aspects of the site, make the LTER site ideal for the study of agricultural ecosystems.

Microbial community structure was assessed as a function of microbial group abundance and richness in several of the KBS-LTER fields sampled over a period of two years. An initial survey was performed in October 1996 to determine if microbial community structure differed among the replicated field-level treatments at the KBS-LTER site and to try to

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understand the causes of this variation. In this initial survey, the relative abundance of rRNA from five microbial groups was examined in the soil along with whole community profiles generated by Terminal Restriction Fragment Length polymorphisms in PCR amplified 16S rDNA. This research, described in Chapter 2, revealed that the microbial communities in fields abandoned from cultivation for 7 years still resembled those in currently managed agricultural fields but differed significantly from those in fields that had never been cultivated. This result was intriguing but having only analyzed samples from the Fall of 1996 I could not be sure if this observation resulted from the long-term impact of agricultural management on the soil or was rather the result of some sort of seasonal anomaly.

This initial survey included an examination of a particularly interesting microbial group that has been observed in numerous soils but has been poorly characterized due to a lack of cultivated isolates. In Chapter 3, I provide a description of the phylogeny and abundance of the nonthermophilic members of the Crenarchaeota as they occur in the soil at the KBS-LTER site. The Crenarchaeota have been observed in soil samples all over the world, but their activity and abundance in the soil has not been previously characterized. In Chapter 3, I summarize the phylogeny of these organisms, demonstrate that members of this group are present in KBS-LTER soils, and describe the abundance of these organisms in soil samples.

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In Chapter 4, I examine the rRNA abundance of Verrucomicrobia in the KBS-LTER fields. Like the Crenarchaeota, the Verrucomicrobia comprise a diverse group of organisms that have been found to be abundant in soils from all over the globe. Also, like the Crenarchaeota, these organisms are poorly represented in culture collections. In Chapter 4, I examine the distribution of Verrucomicrobia in soil at the KBS-LTER site and examine the possibility of a relationship between soil moisture content and the abundance of Verrucomicrobia in soil.

Finally, in Chapter 5 I return to my analysis of the effects of agricultural management on microbial community structure in the soil. In this chapter I analyze the abundance of rRNA from eight of the most common microbial groups in the soil in samples taken over a period of two years from agricultural and successional fields at the KBS-LTER site. This chapter confirmed the results from my initial analyses and revealed that the changes in microbial community structure that result from long-term agricultural management can persist for many years after a change in land management. In addition, this analysis revealed that microbial communities are capable of responding to environmental change over seasonal time scales. As a result I conclude that the environmental variables that are driving the patterns I observe in microbial community structure must require

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long periods of time to recover from agricultural management such as has been observed for soil carbon and nitrogen content.

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CHAPTER 2

THE STRUCTURE OF MICROBIAL COMMUNITIES IN SOIL AND THE LASTING IMPACTS OF CULTIVATION

These results will be published in the article: Buckley, D. H. and T. M. Schmidt. 2001. The structure of microbial communities in soil and the lasting impacts of cultivation. Microb. Ecol. *In Press*.

INTRODUCTION

Soil microbial communities regulate nutrient cycles in terrestrial ecosystems, yet there remains a scarcity of basic knowledge about the structure of soil microbial communities and the factors that influence it in soils. This lack of knowledge arises, in part, from the extraordinary complexity of soil microbial communities, estimated to contain over 4,000 different genomic equivalents in a single gram of soil (39). Further complicating matters is the observation that the organisms isolated from soil represent only a portion of the microbial groups present *in situ*, while the vast majority of soil microorganisms have yet to be cultivated (22). Recently, cultivation-independent approaches utilizing 16S rRNA genes have been used to explore the taxonomic diversity of soil microbial

communities (5, 12, 16, 17, 25, 26, 30, 31, 37, 40, 44). These 16S rRNA-based techniques can also be exploited to examine the abundance and distribution of specific microbial groups in relation to environmental characteristics.

There is little doubt that microbial communities are sensitive to changes in the surrounding soil. Comparative studies have documented that microbial communities can change in response to soil disturbance (2, 13, 23, 30, 31), and differences have been observed between microbial communities in fields with different histories of soil amendment, irrigation, tillage, and plant community structure (3, 4, 7, 8, 19, 20, 41). Though there is evidence that components of community structure vary at small spatial scales in plots having uniform treatment regimens (11, 36), it appears that overall patterns of community structure may be remarkably conserved (18). Analyses of microbial community structure are commonly restricted to a determination of whether microbial communities are similar or different and do not permit any examination of how the abundance of specific microbial groups vary in the environment or the scale at which variation in microbial abundance is significant. By examining how specific microbial groups respond to environmental manipulation, it should be possible to identify environmental factors that influence the structure of microbial communities and the scale at

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which these environmental factors influence the distribution of individual microbial groups in the soil.

Microbial communities in plots at the W. K. Kellogg Biological Station Long Term Ecological Research (KBS-LTER) site were analyzed using both 16S ribosomal RNA and DNA extracted from soils. The KBS-LTER site includes a large-scale experiment with replicated plots under distinct management regimes ranging from conventionally tilled, annual cropping systems to abandoned fields. The site provided an opportunity to evaluate the effects of tillage, fertilization, and plant community composition on the structure of microbial communities. In addition, since both cultivated fields and fields abandoned from cultivation at the KBS-LTER site are present on a contiguous parcel of land that had been uniformly cultivated for greater than 50 years prior to 1989 it is possible to evaluate the lasting impact of agricultural management on soil microorgansisms. The relative abundance of microbial groups was determined by extracting total RNA from soils and challenging the extracted RNA with oligonucleotide probes specific for rRNA from the Alpha, Beta and Gamma Proteobacteria, the Actinobacteria (Gram positive bacteria with high mol % G+C content), the Bacteria and the Eukarya. In addition, microbial communities were compared on the basis of patterns generated

from 16S rDNA Terminal Restriction Fragment Length Polymorphisms (T-RFLP) (28).

MATERIALS & METHODS

Site description and soil sampling

Soil samples were taken in October 1996 from the KBS-LTER site located at the Michigan State University W. K. Kellogg Biological Station (Hickory Corners, Michigan). The KBS-LTER site, established in 1989 to study ecological processes in agroecosystems, includes a large-scale replicated field experiment with seven treatments representing different cropping systems and types of management (For a more detailed site description see http://lter.kbs.msu.edu). The main site is located on 48 hectares of land that had been uniformly farmed for over fifty years prior to establishment (44). Soil was sampled from five of the main site treatments and from a field area that had never been cultivated but was adjacent to the LTER experimental site (Table 2.1). The conventional till (CT), no till (NT), and no input (NI) treatments received a corn/soybean/wheat crop rotation that was in corn at the time of sampling. These treatments were maintained with or without chemical inputs, tillage, and the presence of cover crops (Table 2.1). The alfalfa treatment (AF) received fertilization but no tillage and differed from the previous three treatments because the plant community

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TABLE 2.1. Codes and descriptions of experimental treatments and reference communities on the KBS-LTER site.

Name	Chemical Inputs [a]	Current Tillage	Management History	NPP (g/m^2) [b]	Plant Community
conventional till (CT)	yes	yes	cultivated >50 yr	929 ± 104	annual rotation, corn/soyben/wheat
no till (NT)	yes	no	cultivated >50 yr	1082 ± 184	annual rotation, corn/soyben/wheat
no input (NI)	no	yes	cultivated >50 yr	1017 ± 75	annual rotation, corn/soyben/wheat with cover crop
alfalfa (AF)	yes	no	cultivated >50 yr	959 ± 39	perennial crop
successional field, historically cultivated (HCS)	no	no	cultivated >50 yr, in succession since 1989	634 ± 38	herbaceous perennials
successional field, never cultivated (NCS)	no	no	never cultivated	460 ± 47	herbaceous perennials

[a] Chemical additions to CT and NT consisted of standard agronomic inputs of fertilizer and herbicide, while AF received fertilizer and insecticide. More specific information may be found at http://lter.kbs.msu.edu.

[b] Values for aboveground net primary productivity in 1996 were obtained with permission from http://lter.kbs.msu.edu.

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was dominated by perennial instead of annual crops. Following abandonment from cultivation in 1989 the plant communities in the historically cultivated successional fields (HCS) had progressed from initial dominance by annual species to dominance by biennials and herbaceous forbs which dominated for three years prior to sampling (25). The plant communities in the never cultivated successional field (NCS) were also dominated by herbaceous forbs and closely resembled the plant communities in the HCS fields.

Soil was sampled from three of the six replicate plots (KBS-LTER field replicates 2, 3, and 4) from each main site treatment and from three replicate plots within the HCS field area. Soils at the site were Typic Hapludalfs, sandy to silty clay loam and were of moderate fertility (44). Plots were sampled by taking a soil core (2.5 cm diameter, 10 cm depth) from each of the five permanent sampling locations in each replicate plot. The soil cores from each replicate plot were pooled, sieved (4 mm mesh), frozen in liquid nitrogen and stored at -80°C.

Nucleic acid extraction

DNA suitable for use in PCR amplification was purified from 1 g of soil using the method of Purdy *et al.* (33). RNA for use in hybridization experiments was extracted as previously indicated (10). Briefly, 10 g of soil

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was suspended in a homogenization buffer containing guanidium isothiocyanate to prevent RNA degradation and bead milling was used to disrupt cells (Beadbeater, Biospec Products, Inc.). After solids were removed by centrifugation, purification of RNA was achieved by precipitation with polyethelene glycol followed by an organic extraction and passage through both hydroxyapetite and sephadex G-75 spin-columns. The total RNA concentrations of samples were estimated by using an orcinol reaction to determine ribose concentration (15).

Quantitative filter hybridization

Quantitative filter hybridizations were performed as previously described with minor modifications (46). Nucleic acids from soil samples and cultures were denatured with 0.5% glutaraldehyde-50 mM Na_2HPO_4, serially diluted to provide a range of sample concentrations, blotted onto nylon membranes using a 96 well dot blot manifold, and immobilized by UV crosslinking. RNA isolated from pure cultures (*Ketogulonogenium vulgare* DSM 4025, *Nitrosomonas europaea* ATCC 25978, *Pseudomonas aeruginosa* ATCC 10145, *Cytophaga johnsonae* ATCC 17061, *Arthrobacter globiformis* ATCC 8010, *Bacillus subtilis* ATCC 6051, and *Saccharomyces cerevisiae* American Ale Yeast 1056 (Wyeast Labs, Inc.)) were included on all filters for use as positive and negative controls. Hybridization protocols

were used for ^{32}P-5'-labeled oligonucleotide probes as previously described (46). Replicate filters were prepared and used for hybridization with the following probes: Univ1390, Eub338, Euk1195, Alf1b, Bet42a, Gam42a, and HGC69a (1). All filters were hybridized for >12 h at 45°C, washed for 30 min at 45°C and then washed for an additional 30 min to provide stringency (45 °C for Univ1390, Eub338, Euk1195; 50 °C for HGC69a; 55 °C for Alf1b; and 62 °C for Bet42a and Gam42a). Specifically bound probe was quantified using a radioanalytic imaging system (AMBIS, Inc).

Within a soil sample, the relative abundance of rRNA derived from a specific group was measured as the ratio of the signal derived from a group-specific probe to the signal derived from the universal probe. This approach for determining microbial rRNA abundance has been used previously to describe aspects of microbial community structure (46). Relating specific probe binding to universal probe binding controls for variability in the total amount of RNA recovered from each soil sample, and also controls for the presence of hybridization inhibitors that may co-purify with RNA from soil. Positive controls were included on each membrane to correct for variations in the labeling efficiency of different oligonucleotide probes while negative controls were used to correct for the possibility of non-specific probe binding. Every RNA sample was represented by five aliquots in a dilution series to examine potential differences in signal intensity due to inhibition or

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membrane saturation. The ratio of signal intensities obtained for specific and universal probe binding to an RNA sample was defined as $R = \Sigma^{n}_{i=1}[G_i(U_i)^{-1}]n^{-1}$, where G_i and U_i represent, respectively, the corresponding signal intensities obtained for group specific and universal probe binding to each aliquot representing the sample, and n equals the total number of aliquots representing the RNA sample. The value R was calculated for each soil RNA sample (R_s), and a mean value of R was determined for all positive (R_p) and negative (R_n) controls present on each membrane. The relative abundance of rRNA from a specific microbial group was then defined as $(R_s - R_n)(R_p - R_n)^{-1}$ x 100. To calculate the amount of 16S rRNA g^{-1} of soil, the relative abundance determined for samples was multiplied by the total amount of 16S rRNA present in soil samples as estimated from measurements of soil RNA content.

16S rDNA T-RFLP analysis

Bacterial community composition was investigated in fields from the treatments CT, HCS and NCS on the basis of T-RFLP analysis of 16S rDNA amplified from soil DNA extracts. Bacterial 16S rDNA from soil extracts was PCR amplified using the oligonucleotide primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3'), labeled at the 5' end with the phosphoramidite dye 5-hexachorofluorescein, and 1492R (5'-

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GGTTACCTTGTTACGACTT-3') (33). PCR was carried out in a volume of 50 µl with 50 ng template DNA, 0.05% Nonidet P-40, 0.05% bovine serum albumin, 1.5 mM $MgCl_2$, 200 nM of each dNTP, 0.5 µM primer 8F, 0.3 µM primer 1492R, and 1.25 U Taq polymerase with 1x concentration of the supplied buffer (Gibco BRL). Reactions were performed in a Gene Amp 9600 thermocycler (Perkin-Elmer) for 30 cycles (1 min at 92°C, 1 min at 37°C, and 1 min at 72°C). Amplified 16S rDNA was purified using Ultrafree-MC (30,000 NMWL) filtration units (Millipore) according to the manufacturer's specifications. After purification, amplified 16S rDNA was separately digested with the restriction endonucleases *Msp*I (Boehringer Mannheim), *Rsa*I (Gibco BRL), or *Hae*III (Gibco BRL) according to the manufacturer's instructions. As a result each field replicate was represented by three distinct T-RFLP profiles. The exact lengths of fluorescently labeled terminal restriction fragments from each restriction digestion were determined by electrophoresis of 50 ng sample through a 36 mm 6% polyacrylamide gel on a model 373A automated sequencer (Applied Biosystem Instruments, Inc.).

Community T-RFLP profiles were compared solely on the basis of fragment size, and without respect to band intensity. The number of bands shared between any two T-RFLP profiles was calculated for all pairwise comparisons of samples using the Sorenson index of Similarity: S =

$2ab/(a+b)$, where a and b are the number of bands in any two samples and ab is the number of bands shared between those samples (34). Bands were considered identical provided that their calculated fragment sizes differed by less than one base pair.

Data analysis

Measurements of 16S rRNA relative abundance were analyzed using nonparametric statistical tests to compensate for heteroscedasticity (inequality of variance among samples) observed in these data. The effects of the five main site treatments (CT, NT, NI, AF, and HCS) on microbial group abundance were analyzed using MANOVA by ranks for all groups simultaneously and by using the Kruskal-Wallis test independently on each group of organisms. Mann-Whitney U tests were used to examine relationships in microbial group abundance between specific pairs of treatments and between the historically cultivated fields of the main site and the NCS fields. In addition, MANOVA by ranks was used to examine differences in microbial community structure between the historically cultivated fields and NCS fields.

The average similarity in microbial community structure between treatments CT, HCS, and NCS was also estimated based on the Sorenson index calculated from T-RFLP patterns. These similarity values were

compared using ANOVA following arcsine data transformation. Between-treatment similarities in T-RFLP patterns were compared using a mixed factorial ANOVA where each comparison was represented by nine measurements made with each of the three restriction enzymes. Comparisons of within-treatment variability and numbers of discrete T-RFLP bands were analyzed using a mixed factorial ANOVA where each treatment was represented by three measurements made with each restriction enzyme. Post hoc analyses were performed using the Scheffe test to identify differences between specific treatments. All statistical analyses were performed using StatView v5.0 (SAS Institute, Inc.).

RESULTS

Relative abundance of microbial groups in soil samples

Ribosomal RNA was readily detected from all of the microbial groups surveyed in the soils examined (Table 2.2). The ratios of HGC69a/Univ1390 probe binding are displayed for CT, NCS, HCS samples as well as for relevant controls in order to represent the manner in which 16S rRNA relative abundance was calculated for all other samples and probes (Figure 2.1). Negative controls are used to adjust for nonspecific binding, and positive controls are used to adjust for differences in probe specific activities as discussed in the methods. ANCOVA revealed that differences between

TABLE 2.2. Relative abundance of rRNA from specific groups of microorganisms in the KBS LTER plots

		Mean % rRNA Abundance (± s.d.)[b]					
Treatment	µg RNA [a]	Alf1b	Bet42a	Gam42a	HGC69a	Euk1195	Eub338
CT	3.53 ± 1.31	28.8 ± 8.8	6.2 ± 2.8	3.8 ± 0.3	5.8 ± 4.7	13.1 ± 4.5	59.0 ± 10.0
NT	4.39 ± 0.58	30.8 ± 3.4	5.1 ± 2.3	3.3 ± 0.9	10.9 ± 9.0	9.7 ± 8.5	51.8 ± 7.2
NI	3.99 ± 1.35	26.1 ± 3.7	4.3 ± 1.0	3.3 ± 0.4	6.8 ± 2.4	12.3 ± 6.7	51.0 ± 5.0
AF	4.29 ± 1.04	26.3 ± 5.1	3.7 ± 0.9	3.0 ± 0.5	7.0 ± 2.3	7.7 ± 5.2	52.3 ± 4.4
HCS	4.50 ± 1.32	26.6 ± 2.9	5.2 ± 2.3	3.3 ± 0.5	9.0 ± 5.0	9.5 ± 8.9	53.4 ± 3.1
NCS	7.41 ± 2.07	38.6 ± 4.1	8.5 ± 1.5	3.6 ± 0.7	17.1 ± 2.3	14.3 ± 3.2	62.8 ± 4.1

[a] µg/g soil dry weight

[b] Values for each treatment determined from three replicate plots and values for each replicate plot were determined from five separate measurements.

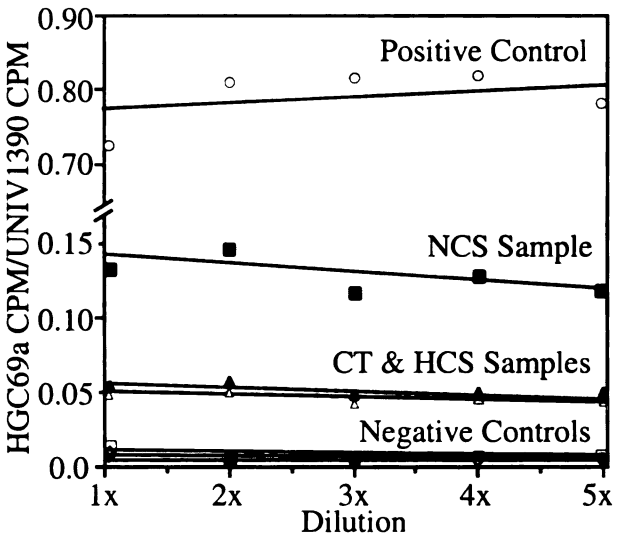

Figure 2.1. Data generated from hybridization experiments with the 32P-labeled oligonucleotide probes HGC69a and Univ1390 reveal the characteristic linear response of the ratio of specific to universal probe binding over a range of sample concentrations. The lack of any significant slope indicates that differences in sample RNA concentration and the possible presence of hybridization inhibitors in soil RNA extracts will not affect calculations of rRNA relative abundance. The symbols represent values obtained for RNA from A. globiformis (m), B. subtilis (o), K. vulgarum ( ), N. europaea (), P. aeruginosa (◊), and RNA from CT (▲), HCS (Δ), and NCS (n) soil samples.

the slopes of the probe binding ratios for samples and controls are not significant. The homogeneity of slopes for the probe binding ratios indicates that differences in sample RNA concentration and the possible presence of hybridization inhibitors in soil RNA extracts will not affect calculations of rRNA relative abundance. Of the bacterial groups surveyed, the Alpha Proteobacteria composed the largest fraction of community rRNA in all plots (29.8% ± 1.6%; mean ± standard error)). The Actinobacteria (9.7% ± 1.5%) were the second most abundant group surveyed, followed by the Beta Proteobacteria (5.5% ± 0.6%), and the Gamma Proteobacteria (3.3% ± 0.2%) (Table 2.2). The bacterial groups examined in this study represent 88.0% ± 9.7% of the total bacterial signal as measured by the probe Eub338.

Effects of environmental characteristics on microbial community structure

Microbial community structure was remarkably similar among the fields of the five historically cultivated treatments at the main experimental site (CT, NT, NI, AF, HCS), despite the wide variation in plant community composition that existed between the fields in these treatments at the time of sampling (Table 2.1, 2.2). In addition, analysis of RNA yields indicated that the total amount of RNA present in the soil did not vary appreciably among the historically cultivated treatments (Table 2.2). In contrast, differences in

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microbial group rRNA abundance were readily observed when the historically cultivated fields were compared to the NCS fields (Figure 2.2). The rRNA relative abundance of the Alpha Proteobacteria, the Beta Proteobacteria and the Actinobacteria were significantly higher in the NCS fields than in the fields that shared a history of cultivation (Figure 2.2A). In addition, the total amount of 16S rRNA for all of the microbial groups examined was significantly higher in the NCS fields than in the historically cultivated fields (Figure 2.2B). MANOVA revealed that the differences in microbial community structure between the historically cultivated fields and the NCS fields were significant (Roy's Greatest Root, $P < 0.0001$). Also, RNA yields in the fields that had never been cultivated differed significantly (t-test, $P < 0.05$) from the RNA yields obtained from the historically cultivated main site treatments (Table 2.2).

Analysis of T-RFLP profiles

Relationships between bacterial communities in the never cultivated reference fields (NCS), the historically cultivated successional fields (HCS), and the conventionally managed agricultural fields (CT) were explored using T-RFLP analysis of amplified 16S rDNA (Figure 2.3). By comparing the microbial community composition in the HCS fields relative to those in the

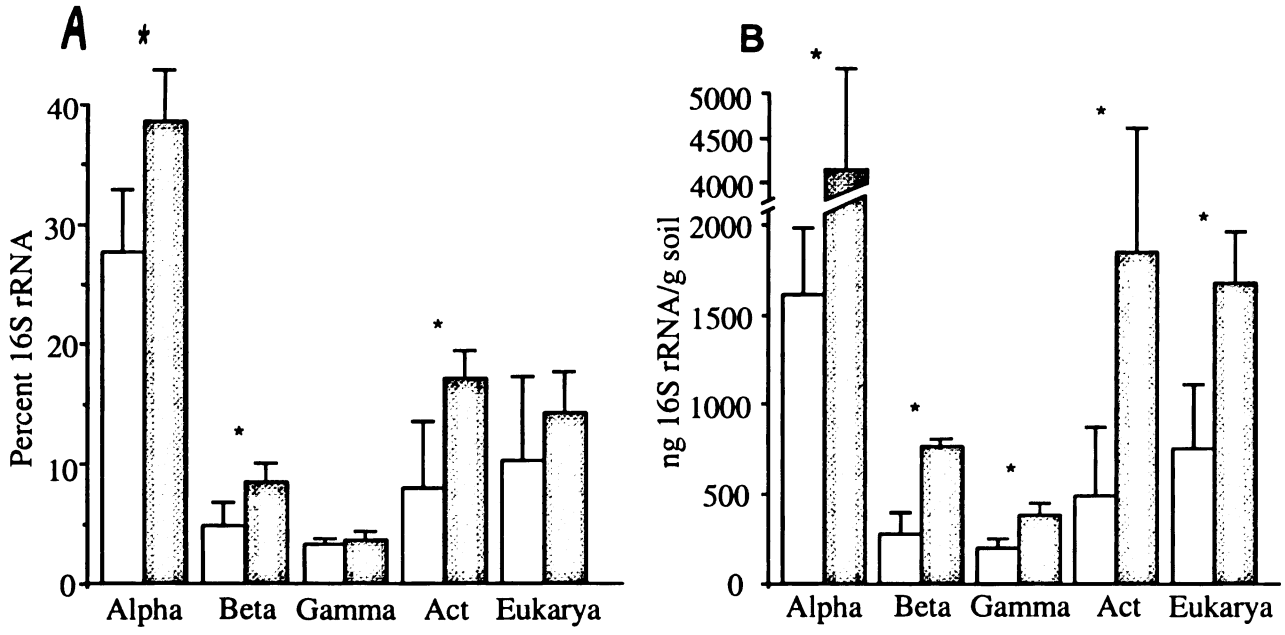

Figure 2.2. Relative abundance of 16S rRNA (A) and quantity of 16S rRNA (per gram dry weight of soil) (B) for microbial groups in fields sharing a history of agricultural disturbance (CT,NT,NI,AF,HCS; open bars) and in fields that have never been cultivated (NCS; shaded bars). Groups shown in the figure are the Alpha, Beta and Gamma Proteobacteria, the Actinobacteria (Act), and the Eukarya. The differences between historically cultivated and never cultivated fields that are indicated by an asterisk were found to be significant by Mann-Whitney U-tests ($p < 0.05$). Error bars indicate one standard deviation from the mean.

	CT-R2	CT-R3	CT-R4	HCS-R2	HCS-R3	HCS-R4	NCS-R2	NCS-R3	NCS-R4
CT-R2	-								
CT-R3	0.69 ± 0.04	-							
CT-R4	0.64 ± 0.17	0.49 ± 0.21	-						
HCS-R2	0.57 ± 0.14	0.48 ± 0.28	0.50 ± 0.16	-					
HCS-R3	0.53 ± 0.10	0.35 ± 0.14	0.62 ± 0.19	0.48 ± 0.11	-				
HCS-R4	0.59 ± 0.05	0.55 ± 0.03	0.49 ± 0.11	0.54 ± 0.22	0.59 ± 0.22	-			
NCS-R2	0.37 ± 0.19	0.36 ± 0.18	0.34 ± 0.21	0.29 ± 0.13	0.31 ± 0.15	0.35 ± 0.06	-		
NCS-R3	0.32 ± 0.14	0.34 ± 0.15	0.37 ± 0.16	0.30 ± 0.04	0.33 ± 0.15	0.38 ± 0.17	0.38 ± 0.25	-	
NCS-R4	0.31 ± 0.03	0.27 ± 0.10	0.38 ± 0.02	0.36 ± 0.19	0.37 ± 0.14	0.41 ± 0.14	0.31 ± 0.17	0.46 ± 0.21	-

Figure 2.3. Pairwise comparisons of T-RFLP patterns representing soil communities from three field replicates of the treatments CT, HCS and NCS. The values were calculated using the Sorenson index and represent the mean and standard deviation of comparisons made with T-RFLP patterns generated from digesting 16S rDNA with the enzymes *Msp* I, *Hae* III, and *Rsa* I. The shaded regions along the diagonal of the matrix contain the data for the Within-Treatment comparisons in Figure 2B while the open and shaded blocks in the body of the matrix contain the data for the Between-Treatment comparisons in Figure 2A.

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CT and NCS fields it is possible to assess the lasting impact of cultivation on the microbial communities in fields that have been abandoned from cultivation for seven years. T-RFLP profiles reveal a different level of community structure than rRNA probing, providing a broader view of the phylogenetic diversity within microbial communities while sacrificing the ability to quantify individual microbial groups. Analysis of Sorenson Similarity values calculated from 16S rDNA T-RFLP profiles revealed that there are significant differences in the composition of bacterial communities between the treatments CT, HCS, and NCS (ANOVA; $F(2,24) = 35.43$, $P < 0.01$; Figure 2.4). On average the bacterial communities in CT and HCS were more similar to each other (0.53 ± 0.15) than those in CT and NCS (0.34 ± 0.13), or HCS and NCS (0.34 ± 0.12) (Sheffe test, $P< 0.01$). An analysis of variance for within-treatment community similarity in CT (0.61 ± 0.16), HCS (0.54 ± 0.17), and NCS (0.38 ± 0.19) also revealed a significant treatment effect ($F(3, 6) = 6.015$, $P < 0.05$), and additional tests revealed that the T-RFLP patterns from CT were significantly less variable than that those in NCS (Scheffe test, $P < 0.05$).

DISCUSSION

Hybridization of extracted RNA with 16S rRNA-targeted oligonucleotide probes provides a quantitative measurement of the protein

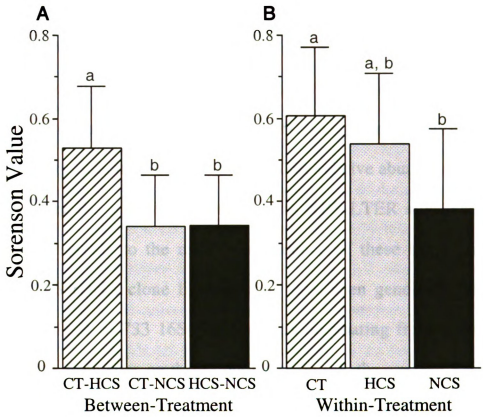

Figure 2.4. Sorenson similarity indices generated from T-RFLP patterns reveal the relative similarity in community structure between and within treatments CT, HCS, and NCS. Bars with different letters were revealed to be significantly different (Scheffe test, $p < 0.05$), error bars indicate one standard deviation from the mean.

synthetic capacity of microorganisms in the environment, which, since cellular rRNA concentrations increase with growth rate, is influenced by both the number and metabolic activity of cells in the environment (50). The relative abundance of rRNA for a microorganism in a microbial community may differ from the relative abundance of rDNA for that organism if there are large differences in the growth rates (in the case of rRNA) or in the rRNA gene copy number (as in the case of rDNA) of the microorganisms in that community. It is interesting to note that the relative abundance of rRNA determined for the microbial groups at the KBS-LTER site (Table 2.2) roughly corresponds to the relative abundance of these same microbial groups in 16S rDNA clone libraries that have been generated from soil samples. Analysis of 733 16S rDNA clones originating from diverse soil samples taken from sites on three continents reveals the average abundance of clones from the Alpha, Beta, and Gamma Proteobacteria to be 16%, 4%, 3% respectively, while Actinobacteria compose 9% of clones (5, 6, 30-32, 35, 48, 53). That the relative abundance of microbial groups as reflected by representation in clone libraries and rRNA probing is roughly similar in many diverse soil samples may reveal that there are certain characteristics of soil environments that lead to overall similarities in microbial community structure.

While there may be similarities among microbial communities from different soils, we have shown that community structure can change significantly in a contiguous landscape as the result of changes in the soil environment brought about by the long-term impacts of cultivation (Figure 2.2). Fields that had been cultivated prior to 1989 had significantly lower proportions of rRNA from the Alpha Proteobacteria, Beta Proteobacteria, and the Actinobacteria, and had 16S rDNA T-RFLP profiles that were significantly different from those in the NCS fields (Figure 2.2, Figure 2.4). In addition, the total amount of 16S rRNA from each of the microbial groups and the total amount of RNA g^{-1} soil was significantly lower in fields that had been cultivated relative to NCS fields (Table 2.2, Figure 2.2). These differences in the total amount of RNA g^{-1} soil between the historically cultivated and NCS fields are most likely a reflection of similar differences observed in the size of the total microbial biomass between these fields (39). Differences observed between the CT and NCS fields in the composition of both the denitrifying and the autotrophic ammonia oxidizing microbial communities are also consistent with the conclusion that microbial community structure differs significantly as a result of the lasting impact of cultivation (10, 13).

In this study the Eub338 and Euk1195 probes together accounted for only 66.0% ± 13.9% of the community rRNA detected with the universal

probe (Univ1390). Archaeal rRNA in these soils was previously measured to be 1.5% ± 0.6% (11). While the Eub338 probe is generally specific for the majority of the Bacteria and the Euk1195 probe is specific for the majority of the Eukarya, both probes will miss a portion of the sequence diversity within their respective groups. For example, the Eub338 probe does not recognize two groups of Bacteria known to occur in soil, the Planctomycetes and the Verrucomicrobia (16). These two microbial groups accounted for 9.6% ± 3.5% of the Univ1390 signal in KBS-LTER plots (Buckley and Schmidt, unpublished data). Even if the results are adjusted to include the Planctomycetes and Verrucomicrobia, approximately 23% of the Univ1390 signal remains unidentified. While experimental error could account for a portion of the discrepancy from 100% coverage, a more likely explanation is that the unaccounted portion of the microbial community is composed of rRNA from the Bacteria and Eukarya that bind the Univ1390 probe but are not recognized by the Eub338 and Euk1195 probes. As a result, there are certainly microorganisms in the soil that have not been targeted by the probes used in this study, but it is clear that the microorganisms that have been detected by these probes are influenced by the lasting impact of cultivation on the soil.

The bacterial probe, Eub338, accounted for 55% of the rRNA molecules extracted from soil microbial communities. As mentioned above,

this estimate is likely an underestimate of the actual bacterial contribution to community rRNA (16). It is interesting to note that fluorescent *in situ* hybridization (FISH) studies in soil have found that cell counts made using the fluorescently labeled Eub338 probe detect only 40% to 45% of the total DAPI stained cells (14, 52). The low ratio of Eub338 FISH stained cells to DAPI stained cells could be interpreted as evidence for low permeability of cells to flourescently labeled oligonucleotide probes. However, in light of the fact that Eub338 identified on average only 55% of community rRNA in this hybridization analysis, it is possible that discrepancies between counts of FISH stained cells and DAPI stained cells may be due to limitations in the specificity of the Eub338 probe.

Microbial community structure, as assessed by rRNA probing, did not vary significantly across the historically cultivated fields at the KBS-LTER site (CT, NT, NI, AF, HCS) despite differences in chemical inputs, tillage, plant community composition and productivity that existed in these fields at the time of sampling. It is possible that slight differences exist in community structure between these fields, and that these differences were not detected because the number of samples analyzed was low relative to the natural variability in the microbial communities. However, differences between microbial community structure in the historically cultivated fields and the NCS fields were readily detected. These observations indicate that any

differences that exist among the historically cultivated fields are small in comparison to differences between these fields and the fields that had never been cultivated.

Probing of rRNA provides a quantitative view of a very broad level of microbial community structure. A great deal of biological diversity can exist within each of the microbial groups examined in this study. Analyses of 16S rDNA T-RFLPs were performed to assess changes in community composition that were not detected by quantitative probing of rRNA. T-RFLP analysis of amplified 16S rDNA can be used to provide a general comparison of the overall phylogenetic similarity between microbial communities at a finer level of resolution than is provided through quantitative analysis of microbial group rRNA abundance. T-RFLP analyses supported the results obtained from probing rRNA as similarities in bacterial community T-RFLP profiles among the historically cultivated plots (CT and HCS) were significantly higher than those between the historically cultivated plots and the NCS plots (Figure 2.4). As measured by T-RFLP, variability in bacterial community structure was lowest among the CT replicates, while such variability was highest among the NCS plots. The low number of replicates and high variability made it difficult to determine if the T-RFLP variability among the HCS replicates was either significantly different from

the other treatments or truly occupies an intermediate level of variability between the two.

These data allowed us to assess the influence of plant community composition, fertilization, tillage, and the effect of historical cultivation on microbial community structure across different treatments at the KBS-LTER site. At the time of sampling, despite maintenance for seven years under several different management practices, the microbial community structure was not appreciably different in fields sharing a common long-term history of cultivation. In addition, while the plant community composition and productivity in the HCS fields closely resembled those of fields that had never been cultivated, the microbial communities in the HCS fields were still indistinguishable from the microbial communities found in active agricultural fields. Previous studies have also identified patterns of microbial community structure that are consistent across sites that vary in plant composition and agricultural treatment (9, 22, 28, 49). It is clear that plants influence microbial community structure in soil immediately adjacent to plant roots (20, 27, 36, 37, 51), but there is conflicting evidence as to whether plant communities influence microbial distribution across individual fields (9, 12, 22, 40). This study does not provide any evidence that plant community composition is influencing soil microbial community structure at

the KBS-LTER site, though any plant effects may be masked by the overwhelming influence of past agricultural practices.

It is important to note that the two methods of community analysis that were employed both have a fairly coarse level of resolution. Microbial communities whose overall structure appears similar by rRNA probing and T-RFLP analyses may still possess ecologically significant differences in community composition as these methods are insensitive to changes in community composition that may occur at the level of individual strains or even species. Such strain or species level changes in community composition could be responsible for differences in the physiological capacity of microbial communities whose overall structure is very similar. Though these data are unable to account for absolute differences in community composition between the fields examined it is clear that there are surprising similarities in community structure between the CT and HCS fields. At some level there are probably differences between the composition of the microbial communities in the CT and HCS fields, however, the fact that the communities in these fields are still more similar to each other than to the communities in NCS fields suggests strongly that after seven years of abandonment the microbial communities in the HCS fields have still not recovered from the effects of cultivation.

Microbial communities can respond rapidly to changes in their local environment, so it may seem odd that the microbial communities in abandoned fields remain similar to those in agricultural fields. A possible explanation of this observation is that soil microbial communities respond to soil characteristics that require long periods of time to recover from disturbance. The soil organic carbon, and total soil nitrogen pools are examples of soil characteristics that can be depleted by long-term agricultural practices and can require decades or even centuries to recover to pre-agricultural levels (18, 29, 39). In addition, studies of spatial variability in soil resources indicate that the distribution of soil nutrients in post-agricultural fields can require decades to recover from the homogenizing effects of tillage (42, 43). Consistent with these observations total carbon and nitrogen content of soil were significantly lower in the historically cultivated fields than in NCS fields at the KBS-LTER site (13, 39). Further study, leading to the identification of specific soil characteristics that influence the dynamics and spatial variability of microbial community structure, should aid in understanding the long term effects of disturbance on microbial communities and on ecosystem function.

In this study rRNA-based phylogenetic probes were used to characterize the abundance of specific microbial groups in the soil and to determine the relative importance of certain environmental variables in

influencing patterns of community structure across a replicated field site. Patterns of microbial community structure, as assessed by both quantitative rRNA probing and by analysis of 16S rDNA T-RFLP profiles, revealed similarities in microbial community structure among fields sharing a history of cultivation, despite differences in chemical inputs, tillage, plant community composition and productivity. Microbial communities in fields abandoned from agriculture for seven years retained the characteristics of contemporary agricultural fields. Meanwhile community structure in those fields sharing a history of cultivation was shown to differ significantly from that in fields that had never been cultivated. Additional studies are currently underway to assess specific factors that may influence soil microbial community structure, and to determine if the patterns observed continue to hold true during different times of the year and with increasing time since abandonment.

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CHAPTER 3

PHYLOGENETIC ANALYSIS OF NONTHERMOPHILIC MEMBERS OF THE KINGDOM *CRENARCHAEOTA* AND THEIR DIVERSITY AND ABUNDANCE IN SOILS

These results have been published in the article: Buckley, D. H., J. R. Graber, and T. M. Schmidt 1998. Phylogenetic analysis of nonthermophilic members of the Kingdom *Crenarchaeota* and their diversity and abundance in soils. Appl. Environ. Microbiol. **64:**4333-4339.

INTRODUCTION

The kingdom *Crenarchaeota* are is one of the two kingdoms that comprise the archaeal domain. The members of the *Crenarchaeota* that have been isolated to date are extreme thermophiles that have optimal growth temperatures of more than 80°C. With certain exceptions, these extreme thermophiles are obligate anaerobes with sulfur-dependent metabolisms. Within the last several years, however, an ever increasing diversity of crenarchaeotal 16S rRNA gene (rDNA) sequences have been recovered from low to moderate temperature environments. These

sequences represent a unique lineage of the *Crenarchaeota* and have been obtained from environments that include Pacific and the Atlantic oceans (10-13, 22, 28, 34), freshwater sediments of North American lakes (16, 19, 31), the gut of a sea cucumber (24), the tissues of a sponge (28), agricultural soils from North America and Japan (5, 37), and forest soils from Europe and South America (7, 17). This collection of more than 100 16S rDNA sequences represents a diverse and globally distributed group of organisms that belong to the kingdom *Crenarchaeota*, but are phylogenetically distinct from the thermophilic *Crenarchaeota*.

No member of the nonthermophilic *Crenarchaeota* group has isolated and cultivated; therefore, the physiological characteristics of these organisms and their roles in ecosystems are unknown. It is presumed that these members of the *Crenarchaeota* are nonthermophillic based on the environments in which they have been found (temperatures, -1.5°C to 32°C), their phylogenetic distance from the thermophilic members of the *Crenarchaeota*, and the low G+C contents of their 16S rDNA (51% - 58%) compared to the G + C contents of the thermophilic organisms (60% - 69%) (10, 16, 28). In addition, the abundance of nonthermophilic *Crenarchaeota* in the marine water column and in the oxic region of freshwater sediments suggests that certain members of the nonthermophilic *Crenarchaeota* are tolerant to oxygen (11, 19). The abundance of nonthermophilic

crenarchaeotal rRNA found in picoplankton from cold ocean waters suggests that these organisms are ecologically relavent members of marine microbial communities (11, 19, 22). Members of the nonthermophilic *Crenarchaeota* have recently been identified in soils, but the abundance and significance of these organisms in soil microbial communities have not been assessed (5, 7, 17, 37).

In this paper we describe recovery, phylogenetic analysis, and quantification of crenarchaeotal 16S rRNA sequences in soil samples. Soil samples were taken from plots that historically had been cultivated with intensive agricultural practices or from nearby successional plots that had never been cultivated. Total community DNA was extracted from soil and 16S rDNA was amplified, cloned, and characterized by restriction fragment length polymorphism (RFLP) and sequence analysis. An oligonucleotide probe specific for all of the nonthermophilic *Crenarchaeota* was designed and tested. Total RNA was extracted from soils, and the relative abundance of crenarchaeotal rRNA was measured by quantitative hybridization.

MATERIALS & METHODS

Strains used

The microorganisms used in this study were *Arthrobacter globiformis* ATCC 8010, *Bacillus subtilis* ATCC 6051, *Cytophaga johnsonae* ATCC

17061, *Haloferax volcanii* ATCC 29605, *Methanobrevibacter* RFM-3 (18), *Nitrosomonas europeae* ATCC 25978, *Pseudomonas aeruginosa* ATCC 10145, and *Serratia marcesens* ATCC 13880. Most of the strains were cultivated by using the conditions recommended by the American Type Culture Collection (14); the only exception was *Methanobrevibacter* sp. strain RFM-3, which was grown as described by Leadbetter and Breznak (18).

Soil sampling

Soil samples were obtained in May 1997 from the Michigan State University W. K. Kellogg Biological Station (KBS) Long-Term Ecological Research (LTER) site located in Hickory Corners, Mich. Soil samples were obtained from both fields that had never been cultivated (NC) and conventionally tilled fields (CT) (complete plot descriptions may be accessed at http://www.kbs.msu.edu). NC fields have never been farmed and are generally covered with vegetation consisting of a variety of perennial herbs and grasses. CT fields have been historically farmed (>50 years), and since 1988 have been under a regime characterized by high levels of fertilization, herbicide addition, annual tillage, and a wheat/corn/soybean crop rotation. At the time of sampling, soybeans had been sown in cultivated fields but had not germinated. Soil cores (depth 10

cm, diameter 2.5 cm) were taken from three replicate plots for each of the two treatments.

Soil cores were homogenized by using a 4-mm sieve, immediately frozen in liquid nitrogen, and stored at -80°C. Portions of samples were saved at 4°C in order to determine moisture contents, microscopically visible cell numbers, and numbers of CFU per gram of soil. The moisture content of a sample was determined by baking 10 g of soil at 80°C for more than 48 hr and determining the decrease in mass due to desiccation. The total number of cells per gram of soil was determined by using the fluorescent stain 5-([4, 6-dichlorotriazin-2-yl]amino)-fluorescein (DTAF) (Sigma) as previously described (6). The number of CFU per gram of soil was determined by diluting and dispersing cells in a buffered salt solution (0.85% sodium chloride, 50 mM sodium phosphate; pH 8) and plating the solution on R2A agar medium (Difco). Plates were incubated at 30°C, and the colonies were counted after 48 hr.

Nucleic acid extraction and analysis

Sufficient quantities of DNA suitable for use in PCR amplification experiments were readily obtained from 1 g of soil by using the method of Purdy *et. al.* (29); however, this method did not provide sufficient amounts of nucleic acids for filter hybridization experiments. Therefore, a modified

method was used to obtain total nucleic acids from soils, as described below. Ten grams of soil was suspended in 20 ml of homogenization buffer (4 M guanidium isothiocyanate, 200 mM sodium phosphate (pH 8), 25 mM sodium citrate, 0.5% N-lauryl sarcosine) (26) and then combined with 20 g of 0.1 mm diameter zirconia/silica beads (Biospec Products). To lyse the soil microorganisms, samples were disrupted in a beadbeater (Biospec Products) for two 1 min cycles on ice. The particulate matter fraction was removed by centrifugation at 5,000 x *g* for 10 min. The supernatant fraction was collected, and the pellet was washed with 20 ml homogenization buffer. The supernatants were pooled and combined with 0.1 volume 5 M sodium chloride, and 0.5 volume 50% polyethelene glycol 8000, and incubated 2 hr at 4°C to precipitate the nucleic acids. The nucleic acids were recovered by centrifugation at 15,000 x *g* for 30 min. The pellet was washed with 70% ethanol and resuspended in 2 ml of 120 mM sodium phosphate buffer (pH 7.2). Then the nucleic acids were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (pH 4.7). Hydroxyapatite spin columns were used to remove humic acids by the method of Purdy *et al.* (29) with the following modifications: (I) 3-ml syringe barrels were used to provide a 2-ml hydroxyapatite bed volume; (ii) the columns were pre-washed three times with 1 ml of 120 mM sodium phosphate (pH 7.2); (iii) 2-ml aqueous samples were added to hydroxyapatite columns; (iv) the

loaded columns were then washed again as described above; and (v) the nucleic acids were eluted with 1 ml of 300 mM potassium phosphate (pH 7.2). The nucleic acids were desalted and precipitated (29) and then resuspended in 200 µl of Rnase-free water. RNA was extracted from cultures by using a conventional bead beating protocol (33).

Nucleic acids were analyzed with a Lambda 3B spectrophotometer (Perkin-Elmer). The absorption of light by samples was determined at wavelengths between 220 nm and 320 nm, and point measurements were taken at 230, 260, and 280 nm. Absorption of light by humic acids occurs throughout the UV spectrum but can be most conveniently measured at 230 nm; therefore, absorption at 260 nm $(A_{260})/A_{230}$ values provided an indication of humic acid contamination in nucleic acid samples (41). The total RNA concentrations of samples were estimated by using an orcinol reaction to determine ribose concentrations (9).

PCR amplification and cloning of *Crenarchaeota* 16S rDNA

DNA purified from soil was used as a template for PCR. The archaea-specific primers used in the PCR included primer 89Fb (5'-ACGGCTCAGTAACRC -3'), modified from the primer described by Hershberger *et al.* (16), and Arc915R (5'- GTGCTCCCCCGCCAATTCCT

-3') (32). This primer pair was designed to amplify DNA from the nonthermophilic *Crenarchaeota*, but may also amplify DNA from some members of the thermophilic *Crenarchaeota* and the *Euryarchaeota*. PCR were performed by using 100 µl mixtures containing 200 ng template DNA, each primer at a concentration of 30 pM, each deoxynucteoside triphosphate at a concentration of 50 µM (Boehinger Mannheim), 0.05% Nonidet P-40, 0.05% bovine serum albumin, 2.5 U *Taq* polymerase (Gibco), and 10 µl PCR buffer (supplied with enzyme). The reactions were performed with a Gene Amp model 9600 thermocycler (Perkin Elmer). Each PCR amplification included a 4-min hold at 94°C, followed by 30 cycles consisting of 1.5 min at 94°C, 1.5 min at 48°C, and 2 min at 72°C. After amplification, an additional extension step consisting of 15 min at 72°C was performed. Positive controls (mixtures containing 200 ng SCA1145 plasmid as the template (5)) and negative controls (mixtures containing no template) were included. Amplified products from environmental samples were directly cloned by using a TOPO-TA cloning kit (Invitrogen).

As a preliminary screening step to eliminate redundancy, the amplified 16S rDNA of 25 clones from each soil sample were digested with a pair of restriction enzymes to determine RFLP patterns. PCR amplification of clones was performed as described above except that an inoculating loopful of colony material from each clone was used as the

template. The amplified 16S rDNA fragments were digested with *Hin*P1I and *Msp*I (New England Biolabs) and resolved on a 2.5% NuSieve gel (FMC BioProducts). Six clones from each soil sample, representing unique restriction patterns, were selected for sequencing. The clones were sequenced with a model 373A DNA sequencer (Automated Biosystems Inc.) by using dideoxy dye terminator chemistry. The primers used for sequencing were primers 89Fb, Arc915R, and Cren745R (see below). The clones were screened for the presence of chimeras with the CHIMERA_CHECK algorithm (www.cme.msu.edu/RDP) (21).

Phylogenetic analyses

Phylogenetic analyses were performed by using the programs ARB (www.mikro.biologie.tu-muenchen.de) (35), PAUP (36), and MacClade (20). Previously published Crenarchaeotal clone sequences were obtained from public databases and were inserted with our cloned sequences into the ARB environment. The sequences were initially aligned by using the ARB automatic aligner and then verified and corrected manually. Regions of ambiguous alignment were identified and excluded from subsequent phylogenetic analyses. Phylogenetic trees were generated using neighbor joining (30), parsimony (33), and maximum likelihood analyses (27). Phylogenetic trees were assembled by using MacClade and rearranged

manually to generate the most parsimonious trees. In addition, transversion distance analyses were performed in ARB by considering only transversion events during construction of trees by the neighbor joining method. During tree construction the sequences belonging to the *Crenarchaeota*, and outgroup sequences were varied.

Oligonucleotide probe design and characterization

The oligonucleotide probe Cren745 was designed with the ARB program (35) to target 16S rRNA from members of the nonthermophilic *Crenarchaeota*. The Oligonucleotide Probe Database (www.cme.msu.edu/OPD) (1) designation for Cren745 is S-*-Cren-0745-a-A-19. The dissociation temperature of Cren745 was determined empirically by membrane hybridization as previously described (32) by using rRNA transcribed in vitro from the clone SCA1145 (5). To transcribe the SCA1145 clone rRNA, the pGEM-11ZF (Promega) backbone was cut by using *Eco*RI and *Hind*III (Broehinger Mannheim), and the rRNA was transcribed by using SP6 RNA polymerase as indicated by the Riboprobe system (Invitrogen). The specificity of Cren745 was determined empirically by hybridizing the probe to 100, 50, and 25 ng of either the transcribed SCA1145 target RNA or various nontarget RNA (see above) by using the hybridization conditions described below.

Quantitative filter hybridization

Quantitative filter hybridizations were performed as previously described, with certain exceptions (10). Nucleic acids from soil samples and cultures were denatured with 0.5% glutaraldehyde-50 mM Na_2HPO_4 and serially diluted to provide a range of sample concentrations for blotting. Nucleic acids were blotted onto nylon membranes with a dot blot device and were immobilized by using UV cross-linking (Stratalinker, Stratagene). The membranes were prehybridized and hybridized using ^{32}P-labeled oligonucleotide probes as previously described (10). Replicate filters were prepared and used for hybridization with either Univ1390 (2), Arc 915 (31) or Cren745. All hybridizations were carried out for more than 12 hr at 45°C; the filters were washed for 30 min at 45°C and then for an additional 30 min at 45°C for Univ1390, 56°C for Arc915, or at 60°C for Cren745 (10). Specifically bound probe was quantified by using a radioanalytic imaging system (AMBIS, Inc.).

To calculate the percent abundance of nonthermophilic *Crenarchaeota* in samples, probe binding was determined for serial dilutions of controls and environmental samples. The abundance was then calculated by determining the ratio of probe Cren745 binding to probe Univ1390 binding; controls were used to account for nonspecific binding and

differences in probe specific activities, as previously described (10, 15). To calculate the amount of *Crenarchaeota* 16S rRNA (in nanograms) per gram of soil, the relative abundance determined for samples was normalized to the estimated total amount of 16S rRNA present in soil samples.

Nucleotide sequence accession numbers

The nucleotide sequences of the KBS 16S rDNA clones have been deposited in the GenBank database under the accession no. AFO58719 through AFO58730.

RESULTS

Soil extraction protocol

Table 3.1 lists some of the characteristics of the soils and extracted nucleic acids analyzed in this study. Soil samples from NC fields possessed a notably higher moisture content than CT fields. This is not surprising as the NC fields had a dense vegetation cover capable of retaining moisture, while the CT fields were devoid of vegetation at the time of sampling. The NC and CT field samples supported similar numbers of microorganisms, as determined by microscopic counts and by CFU counts on R2A agar media. For samples from both sites the proportion of microscopically visible cells growing on plates was quite low (~0.33%), as demonstrated previously for

TABLE 3.1. Comparison of soils and nucleic acids extracted from native and cultivated plots[a]

Sample	H_2O (%)	CFU/g (10^6)[b]	Cells/g (10^9)[c]	RNA (μg/g)[d]	260/230[e]	260/280[e]
Native	9.2 ±0.2	6.70 ±0.90	2.37 ±0.38	3.00 ±0.62	1.71 ±0.04	1.95 ±0.07
Cultivated	5.9 ±0.7	7.77 ±2.27	2.02 ±0.23	0.96 ±0.06	1.89 ±0.05	1.52 ±0.11

[a] Values for each treatment represent the mean of multiple measurements from three replicate plots.

[b] Colony forming units per gram of soil (dry weight).

[c] Microscopic counts of DTAF stained cells per gram of soil (dry weight).

[d] RNA concentrations per gram of soil (dry weight) as estimated by orcinol reaction (9).

[e] Ratios of nucleic acid sample absorption at wavelengths indicated (in nm).

soil samples (2). Despite the similarities in population sizes, the total RNA yields from NC samples were considerably higher than the total RNA yields found in CT fields. The nucleic acids isolated were relatively free of proteins and humic acids, as demonstrated by high A_{260}/A_{280} and A_{260}/A_{230} values. A possible source of bias when nucleic acids are extracted from soil is the potential for differential cell lysis, which could lead to the misrepresentation of nucleic acid concentrations from certain populations. To assess the extent of this problem the lysis efficiency of the extraction procedure was measured. The bead beating protocol which we used disrupted 97.3% ± 0.8% of the cells present, as determined by microscopic counting of DTAF-stained cells before and after homogenization. The efficiency of RNA extraction from soil was estimated to be 19% ± 5.3%, as determined by spiking soil samples with known quantities of RNA and comparing actual RNA yields to expected yields.

Analysis of 16S rDNA clones

Analysis of 35 16S rDNA clones resulted in identification of seven unique RFLP patterns. A total of 12 clones were sequenced; these clones included representatives exhibiting all of the RFLP patterns obtained from each sampling site. The phylogenetic positions of clones from the KBS soils were determined relative to the positions of all previously described

crenarchaeotal clones (Figure 3.1). The environments in which the clones were found, the accession numbers, and references are presented in Table 3.2. Generation of the phylogenetic tree in Figure 3.1 was complicated by the fact that many of the crenarchaeotal clones have been sequenced only partially and many of the sequences do not overlap. To overcome this difficulty, maximum-likelihood analysis was used to construct a tree that included 67 clones for which sequence data between *E. coli* 16S rDNA positions 1 and 915 were available. Overlapping partial sequences were then added to this backbone tree by using the parsimony method and considering only regions in which there were sequence data for the clones. The validity of the tree was tested by generating alternative trees by the distance and parsimony analysis methods with various subsets of sequences that shared regions of sequence data. Parsimony and maximum-likelihood analyses were used to generate bootstrap values for the phylogenetic clusters within the nonthermophilic *Crenarchaeota* by using representative sequences from each group (Figure 3.2).

All trees supported the monophyletic grouping of clones within the FFSB, marine, and terrestrial clusters, as well as the relative branching order of these groups, as indicated by the robust bootstrap values associated with these groups (Figure 3.2). The phylogenetic position of the freshwater cluster and the group composed of clones pSL1, pSL69, pSL123, pJP44, and

Figure 3.1. Phylogenetic tree showing the relationships of nonthermophilic *Crenarchaoeta* 16S rDNA sequences. Symbols indicate thespecificity of the probes Cren667 (11) and GI-554 (22) (n), and Cren745 (this study) (l). Sequences determined in this study are indicated by bold type.

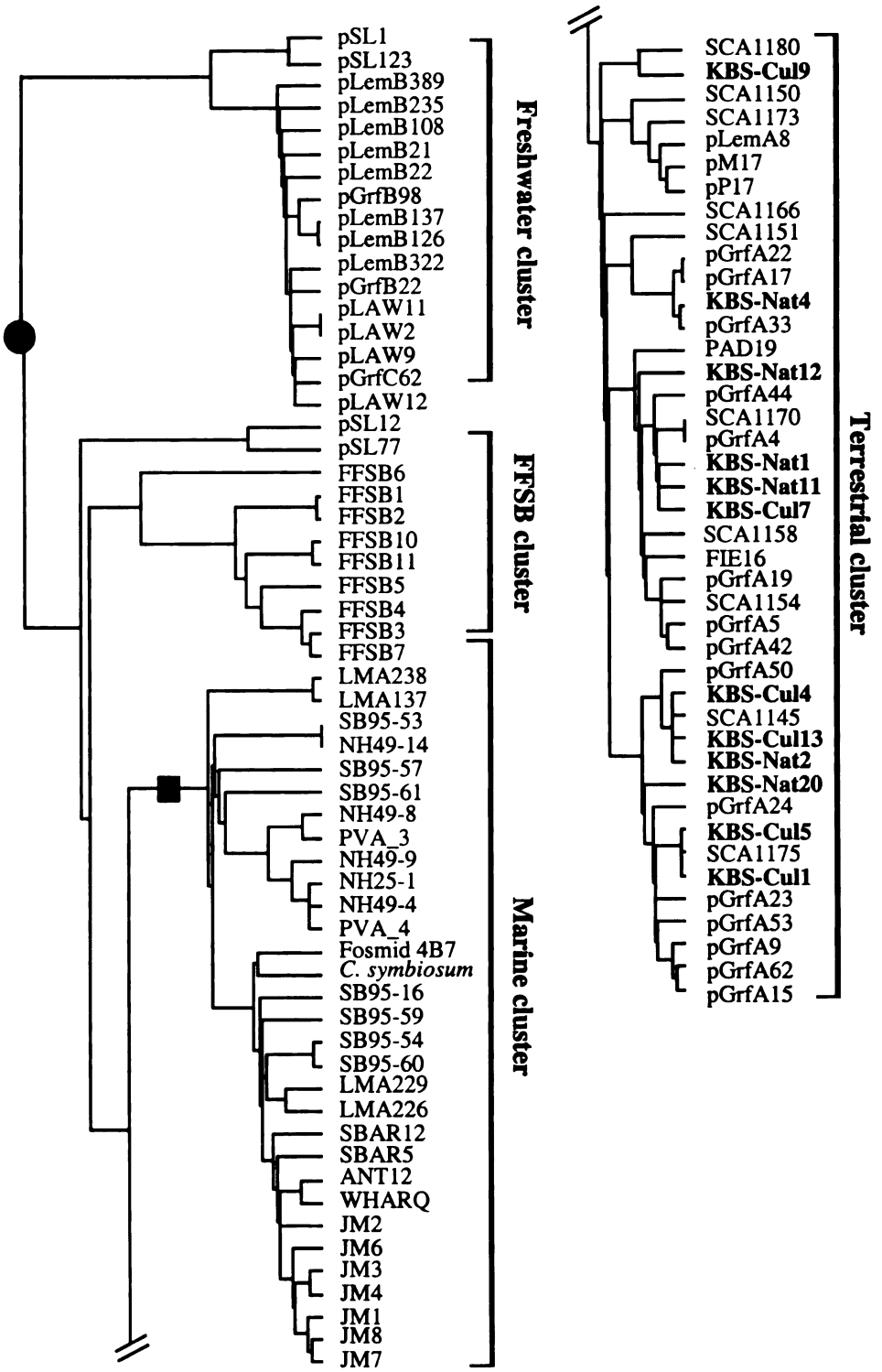
pSL1
pSL123
pLemB389
pLemB235
pLemB108
pLemB21
pLemB22
pGrfB98
pLemB137
pLemB126
pLemB322
pGrfB22
pLAW11
pLAW2
pLAW9
pGrfC62
pLAW12
Freshwater cluster
pSL12
pSL77
FFSB6
FFSB1
FFSB2
FFSB10
FFSB11
FFSB5
FFSB4
FFSB3
FFSB7
FFSB cluster
LMA238
LMA137
SB95-53
NH49-14
SB95-57
SB95-61
NH49-8
PVA_3
NH49-9
NH25-1
NH49-4
PVA_4
Fosmid 4B7
C. symbiosum
SB95-16
SB95-59
SB95-54
SB95-60
LMA229
LMA226
SBAR12
SBAR5
ANT12
WHARQ
JM2
JM6
JM3
JM4
JM1
JM8
JM7
Marine cluster
SCA1180
KBS-Cul9
SCA1150
SCA1173
pLemA8
pM17
pP17
SCA1166
SCA1151
pGrfA22
pGrfA17
KBS-Nat4
pGrfA33
PAD19
KBS-Nat12
pGrfA44
SCA1170
pGrfA4
KBS-Nat1
KBS-Nat11
KBS-Cul7
SCA1158
FIE16
pGrfA19
SCA1154
pGrfA5
pGrfA42
pGrfA50
KBS-Cul4
SCA1145
KBS-Cul13
KBS-Nat2
KBS-Nat20
pGrfA24
KBS-Cul5
SCA1175
KBS-Cul1
pGrfA23
pGrfA53
pGrfA9
pGrfA62
pGrfA15
Terrestrial cluster

TABLE 3.2. Information concerning the crenarchaeotal clones in Figure 3.1.

Sequence Designation	Environment Found	Accession Number	Ref.
Uncertain Affiliation			
pSL	hot spring: Yellowstone National Park	U46338-71	3
pJP	hot spring: Yellowstone National Park	L25300-09	4
Freshwater cluster			
pLemB	freshwater sediment: Lake Lemon, In	U59968-99	16
pGrfB	freshwater sediment: Lake Griffy, In	U59968-99	16
pLAW	freshwater sediment: Lawerence Lake, MI	U77568-75	31
Marine cluster			
SB95	picoplankton: Santa Barbara Channel	U78195-206	22
SBAR	picoplankton: Santa Barbara Channel	M88057-58	10
NH	picoplankton: Pacific Ocean, San Diego	Z11569-73	12
PVA	picoplankton: Pacific Ocean, Hi	U46679-80	25
Fosmid 4B7	picoplankton: Pacific Ocean, Or	U39635	34
ANT12	picoplankton: Arthur Harbor, Antartica	U11043	11
WHARQ	picoplankton: Woods Hole, MA	M88079	10
C.symbiosum	marine sponge tissue: Pacific Ocean	U51469	28
JM	sea cucumber midgut: Atlantic Ocean	L24195-201	24
LMA	freshwater sediment: Lake Michigan	U87515-20	19
Terrestrial cluster			
KBS	agricultural soil: Mi	AFO58719-30	
PAD16/FIE16	agricultural soil: Japan	D26206, 66	37
SCA	agricultural soil: Wisconsin	U62811-20	5
pM17/pP17	forest soil: Eastern Amazon, Brazil	U68605, 54	7
pLemA	freshwater sediment: Lake Lemon, In	U59968-99	16
pGrfA	freshwater sediment: Lake Griffy, In	U59968-99	16
FFSB cluster			
FFSB	forest soil: Northern Finland	X96688-96	17

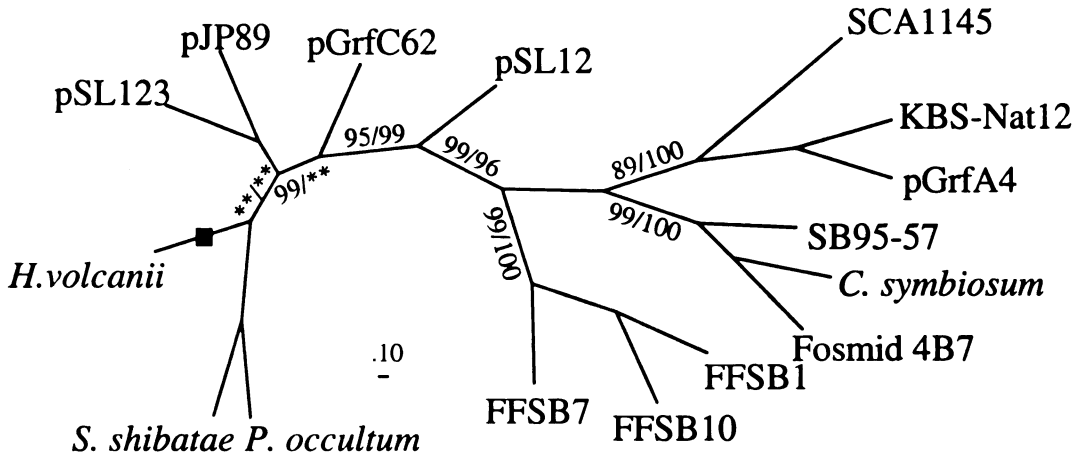

Figure 3.2. Phylogenetic tree generated using maximum likelihood analysis for 740 nucleotide positions between *E. coli* 16S rDNA positions 1-915. The bootstrap values to the left of the backslash were generated using maximum likelihood analysis and the values to the right were generated using parsimony analysis. Bootstrap values less than 50% are indicated (**). The scale bar represents a 10% difference between the nucleotide sequences presented in the tree. In the figure *S. shibatae* is *Sulfolobus shibatae* and *P. occultum* is *Pyrodictum occultum.*

pJP89 were somewhat variable, as reflected by the poor bootstrap values associated with the positions of these organisms (Figure 3.2). Although the members of these two groups typically exhibited close affiliations with one another, their ancestries alternated between affiliation with the thermophilic *Crenarchaeota* and affiliation with the nonthermophilic *Crenarchaeota*, depending on the method used to generate the tree. Crenarchaeotal clones pGrfB286, pSL4, pSL17, pSL22, pSL55, pSL78, pSL79 are not shown in Figure 3.1 as their phylogenetic positions were found to be highly variable, alternating between positions close to the freshwater cluster, positions within the thermophilic *Crenarchaeota*, and positions ancestral to the *Crenarchaeota* (3).

Cren745 design and characterization

Several 16S rRNA-targeted probes that are specific for certain crenarchaeotal taxa have been described (Figure 3.1). None of these probes, however, is complementary to crenarchaeotal sequences that have been found in the soil. The probe which we designed, Cren745, recognized more than 95% of the 16S rRNA sequences of members of the nonthermophilic *Crenarchaeota*, including sequences found in the soil (Figure 3.1). The melting profile of Cren745 hybridized to target RNA was empirically determined in order to determine the hybridization conditions required for

stringency. The temperature at which one-half of the bound probe was removed was found to be 61°C. Outside the nonthermophilic *Crenarchaeota* lineage, Cren745 is not complementary to any known rRNA sequence. Negative controls for hybridization with Cren745 were chosen to represent phylogenetically diverse microorganisms but included organisms (*H. volcanii*, *Methanobrevibacter* sp. strain RFM-3) that represented the most similar nontarget rRNA sequences known (Figure 3.3). Hybridization experiments in which Cren745 was tested against target and nontarget nucleic acids demonstrated that the probe provided the desired specificity when it was used as described above (Figure 3.3).

Quantification of nonthermophilic crenarchaeotal 16S rRNA in soil samples

Probe Cren745 was used to determine the contribution of nonthermophilic crenarchaeotal 16S rRNA to total community rRNA. The relative abundance of nonthermophilic crenarchaeotal rRNA was lower in the NC soils (0.37 ± 0.13%) than in the CT soils (1.42% ± 0.59%) (Figure 3.4A), though this difference was not found to be significant in an unpaired *t* test. In CT samples, *Archaea* 16S rRNA comprised 1.5% ± 0.59% of the total 16S rRNA as determined with the domain level archaeal probe Arc915

A

Cren745 Sequence	5' CCC	AGC	TTT	CAT	CCC	TCA	C 3'
a) Target sequence	3' GGG	UCG	AAA	GUA	GGG	AGU	G 5'
Controls							
b) *H. volcanii*	3' — A	—	—	– C–	—	—	– 5'
c) *M. RFM-3*	3' — A	—	—	– C–	—	—	– 5'
d) *A. globiformis*	3' — U	A—	—	– C G	A—	—	C 5'
e) *B. subtilus*	3' — U	G—	—	– C G	A—	—	C 5'
f) *P. aeruginosa*	3' — U	G—	—	– C G	U—	—	C 5'
g) *S. marcesens*	3' — U	G—	—	– C G	U—	– C–	C 5
h) *N. europaea*	3' — U	G—	—	– C –	C– U	– C–	C 5'
i) *C. johnsonae*	3' — U	G—	—	– C –	— U	—	C 5'

B

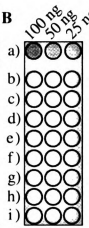

Figure 3.3. (A) Cren745 oligonucleotide probe sequence aligned with its target sequence from nonthermophilic crenarchaeotal 16S rRNA, and nontarget sequences used as negative controls. Bases not shared with the target sequence are indicated, while bases shared with the target sequence are nindicated by dashes. (B) To demonstrate specificity, Cren745 was hybridized to 100, 50, and 25 ng of total RNA from each of the controls. *M.* RFM-3, *Methanobrevibacter* sp. strain RFM-3.

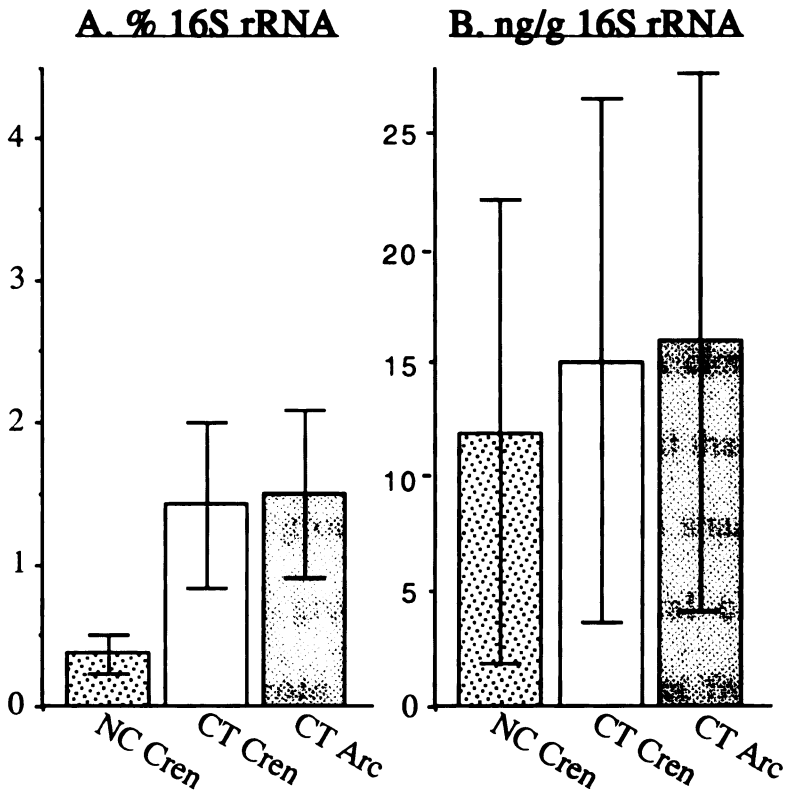

Figure 3.4. (A) Relative abundance of nonthermophilic crenarchaeotal 16S rRNA (Cren) in soil samples from NC or CT fields, as well as the abundance of archaeal 16S rRNA (Arc) in the CT field samples. (B) Amounts of crenarchaeotal 16S rRNA per gram of soil (dry weight) estimated by normalizing the percent abundance of 16S rRNA to the total amount of community 16S rRNA recovered from the soils. The error bars indicate sample standard errors; the sample sizes were 9 for NC samples and 8 for CT samples.

(Figure 3.4A). It should be noted, however, that the amounts of nonthermophilic crenarchaeotal 16S rRNA per gram (dry weight) of soil were practically the same in NC (12.1 ± 10.2) and CT fields (15.3 ± 11.7) (Figure 3.4B). This finding reflected the relationships between the relative abundance of 16S rRNA and total amount of rRNA in the soil samples.

DISCUSSION

Phylogenetic analysis of crenarchaeotal 16S rDNA clones recovered from low- to moderate-temperature environments revealed that these clones belong to at least four distinct groups which appear to share a common ancestry. We described this group of environmental clones as the nonthermophilic *Crenarchaeota* to distinguish them from the other members of the *Crenarchaeota*. The majority of the sequences used in this phylogenetic analysis have been described previously (Table 3.2). For the sake of consistency, the names of groups which contained sequences from independent studies were chosen based on the environments from which the majority of the clones were obtained. The terrestrial cluster contains sequences that were primarily recovered from soil samples, although it should be noted that some sequences found in freshwater sediments also fell within the terrestrial cluster (pGrfA, pLemA) . Most of the sequences in the marine cluster were found in marine systems; the only exceptions were four

sequences recovered from freshwater sediments (LMA137, LMA226, LMA229, LMA238). The FFSB cluster is limited to the sequences identified in a single study of boreal soil from Finland (17), and the freshwater cluster contains sequences found exclusively in freshwater sediments.

On the basis of a phylogenetic analysis that included the pLEM and pGrf environmental 16S rDNA clones, Hersberger et al. (16) proposed that the ability to grow at low to moderate temperatures arose independently at least three times in the *Crenarchaeota*. The results of analyses that included the additional rDNA sequences currently available are consistent with a monophyletic grouping of the nonthermophilic *Crenarchaeota* provided that the clones pSL12 and pSL77, which were recovered from a hot spring, represent allochtonous organisms washed into the hot spring from a moderate temperature environment. A finding which supports this hypothesis is the observation that the G+C contents of pSL12 and pSL77 16S rDNA (57% and 58% respectively) are similat to the G + C contents of the rDNAs of nonthermophilic *Crenarchaeota* (51% - 58%) and fall outside of the range of the G + C contents of the rDNAs of the thermophilic *Crenarchaeota* (60% - 69%). Another complication in resolving the evolution of low- to moderate-temperature growth in the *Crenarchaeota* is the uncertain placement of the freshwater cluster and the clone pGrfB286.

The relative positions of these sequences are dependent on the method and sequences used to generate phylogenetic trees. The phylogeny presented here indicates that members of the nonthermophilic *Crenarchaeota* are distinct from members of the thermophilic *Crenarchaeota*, but the data are not sufficient to conclude whether the ability to grow in low- to moderate-temperature environments has evolved once or multiple times in the crenarchaeotal lineage.

In soil samples taken from fields with distinct treatment histories, amplification, cloning, and RFLP screening of 16S rDNA resulted in the identification of 12 unique crenarchaeotal 16S rDNA sequences. Phylogenetic analysis of the KBS sequences revealed that they were associated with the sequences in the terrestrial cluster (Figure 3.1). The KBS sequences do not appear to share a common ancestor within the terrestrial cluster, but are distributed throughout this group. In addition, there appears to be no relationship between the history of treatment of a soil and the phylogenetic position of the sequences from that soil. The clones from the NC fields are as likely to be related to clones from the CT fields as they are to other clones from NC fields, and the opposite is true as well.

Using oligonucleotide probes specific for 16S rRNA, we determined the abundance of nonthermophilic crenarchaeotal 16S rRNA in the NC and CT soil samples. The concentration of rRNA in a cell generally increases

with growth rate, and so the abundance of rRNA in an environmental sample is a function of both the growth rate and the population size of the organism under consideration (39). The contribution of nonthermophilic crenarchaeotal 16S rRNA to total community 16S rRNA was lower in NC samples (0.37 ± 0.13%) than in CT samples (1.42 ± 0.59%) (Figure 3.4A). This observation could be explained by either by lower amounts of crenarchaeotal rRNA or by larger contributions of rRNA from bacterial populations in NC samples. When the percentage of nonthermophilic crenarchaeotal 16S rRNA was normalized to the total amount of rRNA per gram (dry weight) of soil, the actual sizes of the nonthermophilic crenarchaeotal 16S rRNA pools were similar in the NC and CT samples (Figure 3.4B). The differences in abundance were therefore due to increased contribution of 16S rRNA from organisms other than *Crenarchaeota*.

In samples from CT fields, archaeal 16S rRNA was found to account for 1.5 ± 0.59% of the total community rRNA. A previous study in which fluorescent in situ hybridization was used showed that *Archaea* account for 0.21 ± 0.65% of the microscopically detectable cells in a forest soil (40). These two values, determined by independent methods, confirm that *Archaea* represent a measurable component of soil microbial communities. The archaeal 16S rRNA abundance determined for CT fields was nearly equivalent to that of nonthermophilic *Crenarchaeota* in the same fields

(Figure 3.4A). The similarity in the abundance values for the *Archaea* and nonthermophilic *Crenarchaeota* in CT fields suggests that the nonthermophilic *Crenarchaeota* represent a majority of the archaea in the CT field soil samples.

Molecular approaches have demonstrated that nonthermophilic *Crenarchaeota* are found in diverse environments and are globally distributed; however, the physiological characteristics and ecological significance of these organisms remain unknown. The phylogeny presented in this paper suggests that the nonthermophilic *Crenarchaeota* may have a common ancestor and that there are several distinguishable groups within this lineage. We describe the use of a new probe that is specific for all of the currently identified members of the nonthermophilic *Crenarchaeota* and the presence and abundance of this group in soil samples from the KBS in Hickory Corners, Mich. There were no detectable differences in the diversity or abundance of *Crenarchaeota* in the fields sampled despite considerable differences in the disturbance history and plant community diversity associated with these plots. Further investigations are needed to characterize the distribution and abundance of the globally distributed nonthermophilic *Crenarchaeota*, to understand the ecological significance of these organsisms, and to help design strategies for their enrichment and isolation.

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CHAPTER 4

FACTORS INFLUENCING THE DISTRIBUTION OF VERRUCOMICROBIA IN SOIL

INTRODUCTION

Molecular phylogenetic studies continue to redefine our definitions of microbial diversity as microorganisms representing novel lines of bacterial descent are now recovered almost routinely from environmental samples. For each newly discovered phylotype, questions remain about their distribution and abundance in the environment as well as about the roles these organisms play in the ecosystem. The Verrucomicrobia constitute a newly identified bacterial division that appears to be widely distributed in both aquatic and terrestrial systems, but for which only a handful of organisms have been cultivated in isolation (12). Verrucomicrobia are present in 91% of the 16S rDNA clone libraries generated from soil microbial communities and on average represent 11 % ± 4 % (s.e.) of the 16S rDNA clones in these libraries as calculated from published reports (1, 2, 8, 14-16, 18, 25, 27). In addition, Verrucomicrobia are a numerically

abundant component of certain soil microbial communities as determined by RT-PCR analysis of rRNA (10) and by quantitative PCR analysis of rDNA (15). Despite their widespread distribution in the environment and evidence that they are a numerically abundant component of soil microbial communities nothing is known about the ecological significance of the *Verrucomicrobia.*

The distribution of an organism in relation to biotic and abiotic characteristics of its environment can provide important clues to its function within an ecosystem. Careful observation of the environmental distribution of an organism or group of organisms that have not yet been studied in cultivation can help us to improve our understanding of the nature of these organisms and can provide insights about the conditions required for their enrichment and isolation. We determined the abundance of the Verrucomicrobia in a series of replicated fields located at the W. K. Kellogg Biological Station Long Term Ecological Research site (KBS-LTER, Hickory Corners, MI) over a period of two years. An oligonucleotide probe was designed and tested that is specific for 16S rRNA from the Verrucomicrobia, this probe was used to determine the abundance of verrucomicrobial rRNA in soil RNA extracts. Soil sampling at the KBS-LTER site allowed us to describe the influence that management history,

time, soil depth, and soil moisture content have on the abundance of verrucomicrobial rRNA in the soil.

METHODS

Site description and soil sampling

Soil samples were taken from the Long Term Ecological Research (LTER) site located at the Michigan State University Kellogg Biological Station (KBS) in Hickory Corners, Michigan. The KBS-LTER site, established in 1989 to study ecological processes in agroecosystems, includes a large-scale replicated field experiment located on 48 hectares of land that had been uniformly farmed for over fifty years prior to its establishment. The KBS LTER also includes nearby successional fields that not been historically cultivated (21). Soils at the site are Typic Hapludalfs, sandy to silty clay loam and are of moderate fertility (21). The site has a mean annual air temperature of 9.4 °C and a mean annual rainfall of 860 mm (7). A total of 30 fields were sampled representing four treatments from the main experiment site, and two sets of successional fields that had never been historically cultivated but were located near the experimental site. The fields sampled from the main experimental site consisted of conventionally tilled (CT) agricultural fields managed under a corn/soybean/wheat rotation (in corn during 1996), fields planted with poplar trees (PL), historically

cultivated successional (HCS) fields that had been abandoned from cultivation in 1989, and subplots of the HCS fields that received annual tillage (HCST). The other fields sampled consisted of never cultivated successional (NCS) fields located several hundred meters from the main experimental site, and late successional (LS) fields that were located approximately one kilometer from the main experimental site. Plant communities in the NCS and LS fields were dominated by herbaceous forbs and were similar in species composition to the plant communities in HCS fields. Five Fields were sampled from each treatment by taking a single soil core (2.5 cm diameter, 10 cm depth) from each of five permanent sampling locations distributed across each field replicate. The soil cores from each field replicate were pooled, sieved (4 mm mesh), frozen in liquid nitrogen (generally within 10 minutes of sampling), and stored at -80°C.

Soil samples were taken at four times over a period of two years. On October 3, 1996 and May 23, 1997 soil was sampled from three field replicates of the CT, HCS, and NCS treatments. On June 6, 1998 soil was sampled from five field replicates of treatments CT, HCS, HCST, and NCS. June 1998 sampling included both 5 cm deep soil cores and 10 cm deep soil cores to assess potential differences in microbial community structure due to soil depth. On July 28, 1998 five field replicates were sampled from all of the described treatments (CT, PL, HCS, HCST, NCS, LS). Ten grams of soil

from each of the July 1998 samples were used to determine gravimetric soil moisture content.

Probe design

To determine the abundance of Verrucomicrobia in the soil an oligonucleotide probe that targets verrucomicrobial 16S rRNA was designed with the help of the ARB software package (24). The specificity of the probe was empirically tested against RNA extracted from *Verrucomicrobium spinosum* ATCC 43997 and from closely related non-target organisms consisting of *Ketogulonigenium vulgare* DSM4025, *Nitrosomonas europaea* ATCC 25978, *Planctomycetes limnophilus* ATCC43296, and *Acidobacterium capsulatum* ATCC 51196. RNA was extracted from bacterial cultures and 100 ng, 80 ng, 60 ng, 40 ng, and 20 ng of RNA were immobilized on nylon membranes for use in hybridization experiments. The wash temperature providing the appropriate probe specificity was determined empirically. Hybridization experiments were carried out as described below.

Nucleic acid extraction and hybridization

RNA for use in hybridization experiments was extracted from soil as previously described (5, 6). In brief, a 10 g portion of each frozen soil sample was suspended in a buffer suitable for sample homogenization and RNA stabilization. Microbial cells were lysed using beadmill homogenization with 10 g of 0.1 mm silica/zirconia beads in a 32 ml chamber for a duration of two minutes (Beadbeater, Biospec Products, Inc.). RNA from homogenized samples was concentrated by precipitation with polyethelene glycol and then purified using both hydroxyapetite and Sephadex G-75 columns. RNA samples were finally precipitated, resuspended in 200 µl of Rnase-free ddH_2O, and stored at –20°C.

Quantitative filter hybridization was performed as previously described (6, 22). Nucleic acids from soil samples and standards were denatured with 0.5% glutaraldehyde-50 mM Na_2HPO_4, serially diluted to provide a range of sample concentrations, blotted onto nylon membranes using a 96 well dot blot manifold, and immobilized by UV crosslinking. RNA isolated from the pure cultures mentioned above were included on all membranes as standards to control for differences in the specific activity of labeled probes and to account for the possibility of nonspecific probe binding. All membranes used for hybridization were prepared in duplicate for hybridization with either the probe Ver47 or the probe Univ1390.

Hybridization protocols for ^{32}P-5'-labeled oligonucleotide probes were previously described in detail (22). Hybridization between radio-labeled probes and RNA immobilized on filters proceeded at 45°C for at least 12 hours. Following probe hybridization, filters were washed twice for 30 minutes at 45°C. The specifically bound probe that remained on the membrane was visualized using a phosphorimaging system (Storm 860, Molecular Dynamics), signal intensity was quantified using Image Quant software v 5.0 (Molecular Dynamics).

Data analysis

The relative abundance of rRNA derived from a specific group was measured as the ratio of the signal derived from a group-specific probe to the signal derived from the universal probe. This approach for determining microbial rRNA abundance has been used previously to describe aspects of microbial community structure (22). Relating specific probe binding to universal probe binding controls for variability in the total amount of RNA recovered from each soil sample, and also controls for the presence of hybridization inhibitors that may co-purify with RNA from soil. Positive controls were included on each membrane to correct for variations in the labeling efficiency of different oligonucleotide probes while negative controls were used to correct for the possibility of non-specific probe

binding. Every RNA sample was represented by five aliquots in a dilution series to examine potential differences in signal intensity due to inhibition or membrane saturation. The ratio of signal intensities obtained for specific and universal probe binding to an RNA sample was defined as $R = \Sigma^{n}_{i=1}[G_i(U_i)^{-1}]n^{-1}$, where G_i and U_i represent, respectively, the corresponding signal intensities obtained for group specific and universal probe binding to each aliquot representing the sample, and n equals the total number of aliquots representing the RNA sample. The value R was calculated for each soil RNA sample (R_s), and a mean value of R was determined for all positive (R_p) and negative (R_n) controls present on each membrane. The relative abundance (expressed as a precentage) of rRNA from a specific microbial group was then defined as $(R_s - R_n)(R_p - R_n)^{-1} \times 100$.

Percent rRNA abundance data was arcsine transformed prior to statistical analyses to control for statistical artifacts that may result when the variance is proportional to the mean in a dataset, as is common for percentage data. A repeated measures ANOVA design was used to examine the main effects of treatment and sampling time, as well as the interaction of these effects on verrucomicrobial rRNA abundance in treatments CT, HCS, and NCS over all four sampling dates. A repeated measures ANOVA design was used to examine the main effects of soil depth and treatment on verrucomicrobial rRNA abundance in samples from June 1998, while

ANCOVA was used to examine the effects that field treatment and soil moisture content have on verrucomicrobial abundance in samples from July 1998. Significant ANOVA results were investigated using Fisher's Protected Least Significant Difference test to perform all pairwise comparisons. Statistical tests were performed using StatView v 5.0 (SAS Institute, Inc.).

RESULTS

Development of an oligonucleotide probe for the Verrucomicrobia

The probe Ver47 (Figure 4.1) is complementary to 100 % of the Verrucomicrobia 16S rRNA genes that have been sequenced in the probe target region and are present in public databases (29 sequences total). In addition, the probe has two or more base pair mismatchs with 99.9 % of the 16S rRNA sequences in the current release of the Ribosomal Database Project (> 22,000 sequences, (17)). In total there are only 24 gene sequences that have a single base pair mismatch to the probe. The specificity of the probe was determined empirically by hybridization against RNA from Verrucomicrobia and against RNA from microorganisms in closely related phylogenetic groups (Figure 4.2). A final wash temperature of 45°C was found to be sufficient to achieve probe specificitiy under the hybridization conditions described.

Ver47 Sequence	5' GAC	TTG	CAT	GTC	TTA	WC	3'
a) *V. spinosum*	3' CUG	AAC	GUA	CAG	AAU	AG	5'
Controls							
b) *K. vulgare*	3' • • •	• • •	• • •	• • C	• • •	CC	5'
c) *N. europaea*	3' • • •	• • •	• • •	• • C	• • •	• C	5'
d) *P. limnophilus*	3' • • •	• • •	• • •	• G •	• U •	• •	5'
e) *A. capsulatum*	3' • • •	• • •	• • •	• • C	• • •	CC	5'

Figure 4.1. Sequence of the Ver47 probe specific for rRNA from Verrucomicrobia. The Ver47 probe sequence is depicted along with the complimentary 16S rRNA sequence from the verrucomicrobial positive control and homologous sequences from species used as negative controls. The letter W indicates that either of the bases A or T can be present at this position.

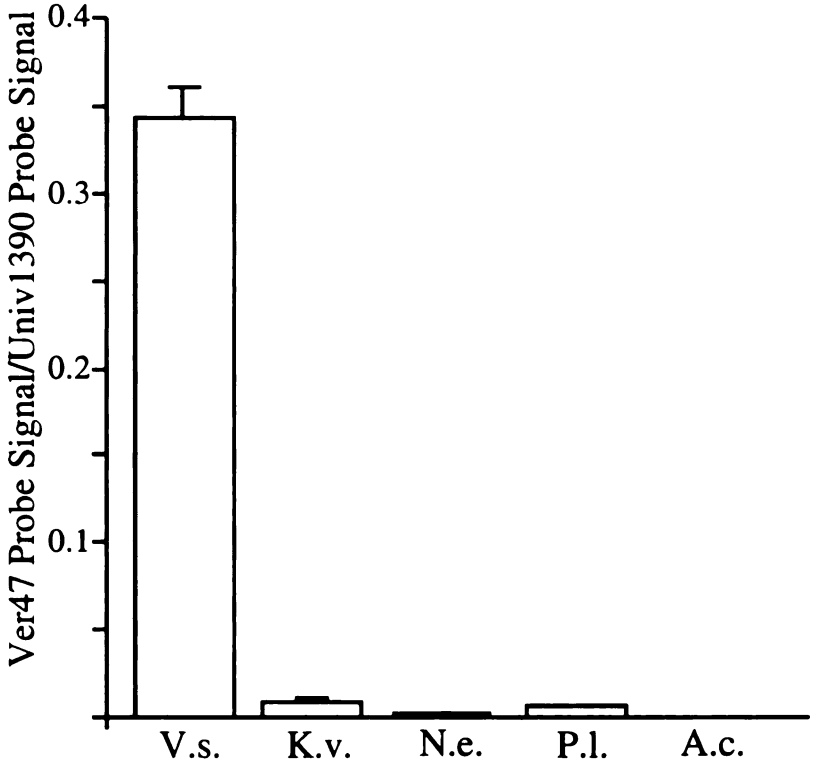

Figure 4.2. A summary of hybridization results with probe Ver47. Each bar represents the signal intensity for probe Ver47 bound to sample RNA divided by the signal intensity for probe Univ1390 bound to RNA from the same sample, thereby controlling for differences in the amount of 16S rRNA present in each sample. Values are expected to be less than unity as a result of differences in the labeling and hybridization efficiency of the two probes. Each bar represents the mean and standard error from 6 separate experiments each performed at 5 different RNA concentrations. The RNA samples used are *V. spinosum* (V.s.), *K. vulgare* (K.v.), *N. europaea* (N.e.), *P. limnophilus* (P.l.), and *A.. capsulatum* (A.c.).

Verrucomicrobial rRNA abundance

In the 30 fields examined over the two years, verrucomicrobial rRNA accounted for between 0 and 9.8 % of the rRNA present in the soil. The mean abundance of verrucomicrobial rRNA in KBS-LTER soil microbial communities was 1.9 % ± 0.2 % (standard error (s.e.), n = 85). Analysis of CT, HCS, and NCS fields at four times indicated slight differences in verrucomicrobial rRNA abundance between the treatments, but these differences were not significant (Figure 4.3A). Sampling time had a significant impact on the abundance of verrucomicrobial rRNA in the soil ($F_{3, 18} = 9.913$, $P = 0.004$). The highest abundance of verrucomicrobial rRNA occurred in May 1997 (2.66 ± 0.37 % (s.e.)) with significantly lower values occurring in June 1998 (1.4 ± 0.24 % (s.e.)), July 1998 (1.24 ± 0.39 % (s.e.), and October 1996 (0.72 ± 0.35 % (s.e.) (Figure 4.3B). The interaction between the main effects of treatment and sampling time was not significant.

Verrucomicrobial abundance and soil depth

In July 1998 the effect of soil depth on verrucomicrobial abundance was investigated in four fields (CT, HCST, HCS, NCS) by comparing either 5 cm deep soil cores or 10 cm deep soil cores. Consistent with previous

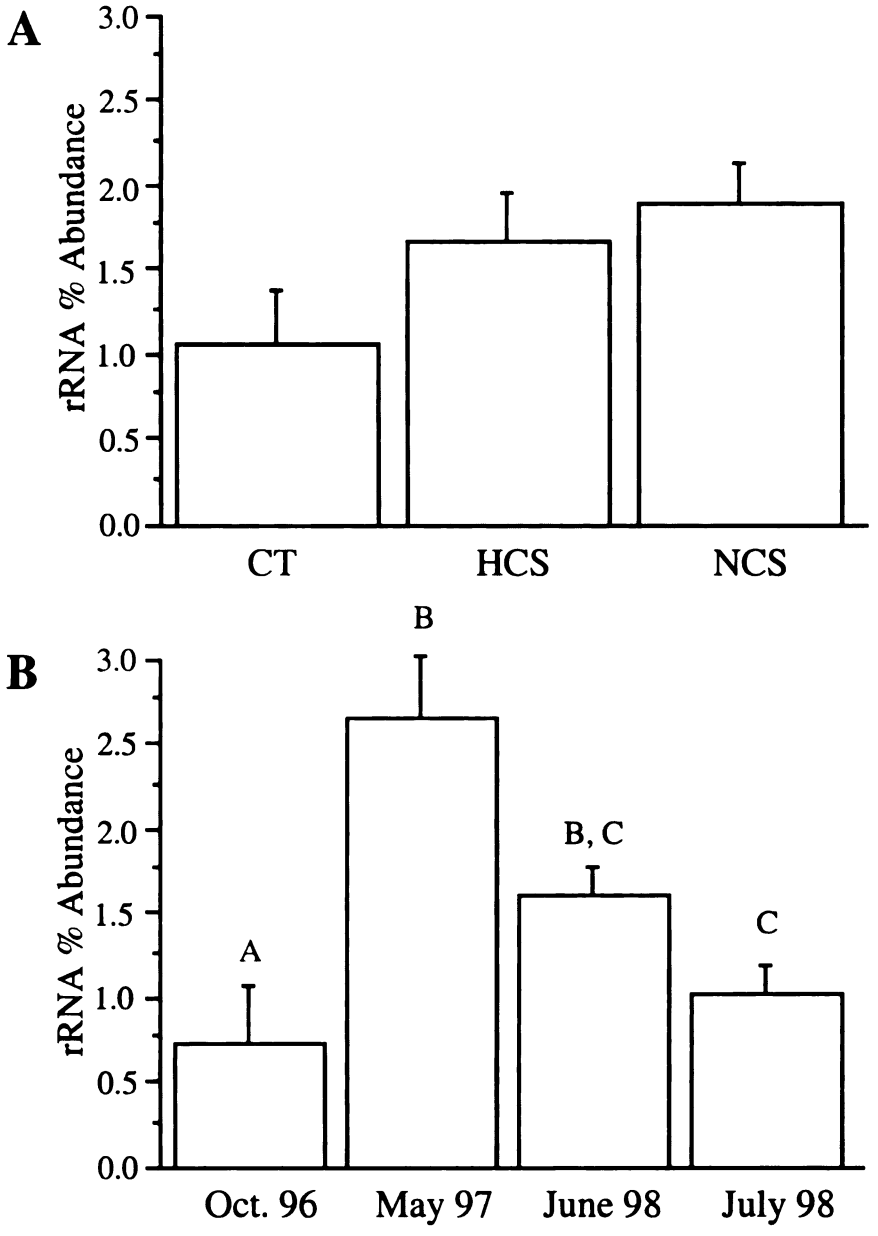

Figure 4.3. Abundance of verrucomicrobial rRNA in fields from the KBS-LTER site at different times. Average abundance of verrucomicrobial rRNA in fields that have either been cultivated (CT), abandoned from cultivation (HCS), or never cultivated (NCS) for samples from October 1996, May 1997, June 1998, and July 1998 (Panel A). Abundance of verrucomicrobial rRNA in October 1996, May 1997, June 1998, and July 1998 averaged over all fields examined (Panel B). Bars with different letters are significantly different from each other (Fishers PLSD, $P < 0.05$). Bars and whiskers represent the mean and standard error.

analyses, differences in verrucomicrobial abundance between the different treatments were not significant. Verrucomicrobial rRNA abundance, however, did vary significantly with depth ($F_{1, 15} = 23.159$, $P = 0.0002$) (Figure 4.4). Verrucomicrobial rRNA was more than twice as abundant in the 5 cm deep soil cores (3.87 % ± 0.53 %) than in the 10 cm deep soil cores (1.59 % ± 0.17 %). The interaction of the main effects of treatment and depth was not significant indicating that treatment effects do not influence differences due to depth.

Verrucomicrobial abundance and soil moisture

For the July 1998 samples verrucomicrobial rRNA was positively correlated with soil moisture content ($r = 0.510$, $P = 0.0041$; Figure 4.5A). ANCOVA was used to determine if the variation in the abundance of the verrucomicrobial rRNA across treatment was due to variation in soil moisture content. A test of the homogeneity of slopes revealed that the effect of moisture on verrucomicrobial abundance did not differ significantly between the treatments examined, so interaction effects were excluded from subsequent analyses. There were significant differences in verrucomicrobial rRNA abundance among the treatments examined in July 1998 ($F_{5, 22} = 4.500$, $P = 0.0056$). Verrucomicrobial rRNA abundance tended to be significantly lower in fields with a history of cultivation (CT, PL, HCST,

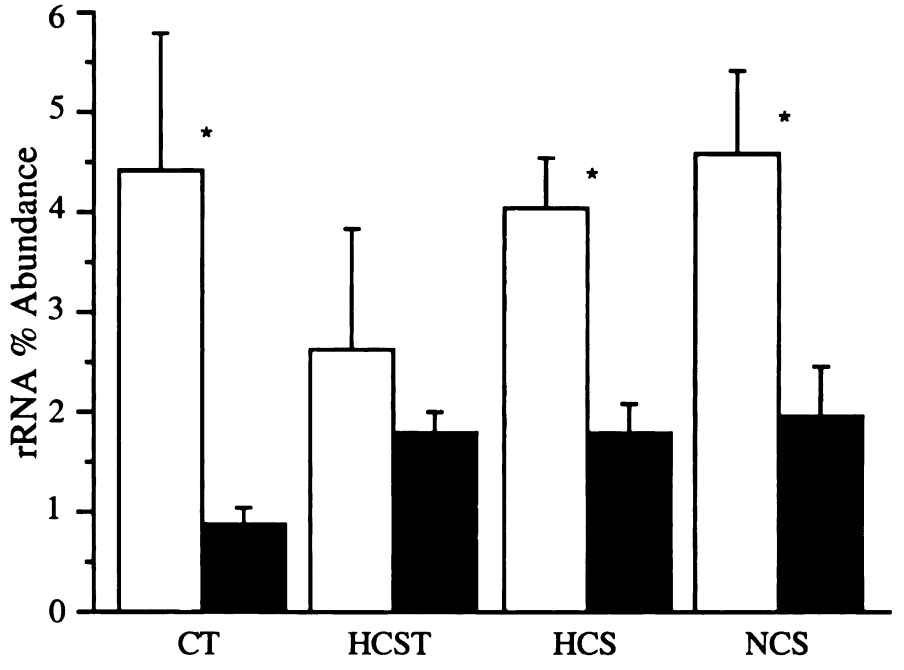

Figure 4.4. The effect of depth on verrucomicrobial rRNA abundance. Abundance of verrucomicrobial rRNA in either 5 cm deep soil cores (open bars) or 10 cm deep soil cores (black bars) in fields that have either been cultivated (CT), abandoned from cultivation (HCS), abandoned from cultivation but tilled annually (HCST), or never cultivated (NCS). Asterisks indicate significant differences between 5 cm and 10 cm cores that were detected using unpaired T-tests ($P < 0.01$). Bars and whiskers represent the mean and standard error respectively.

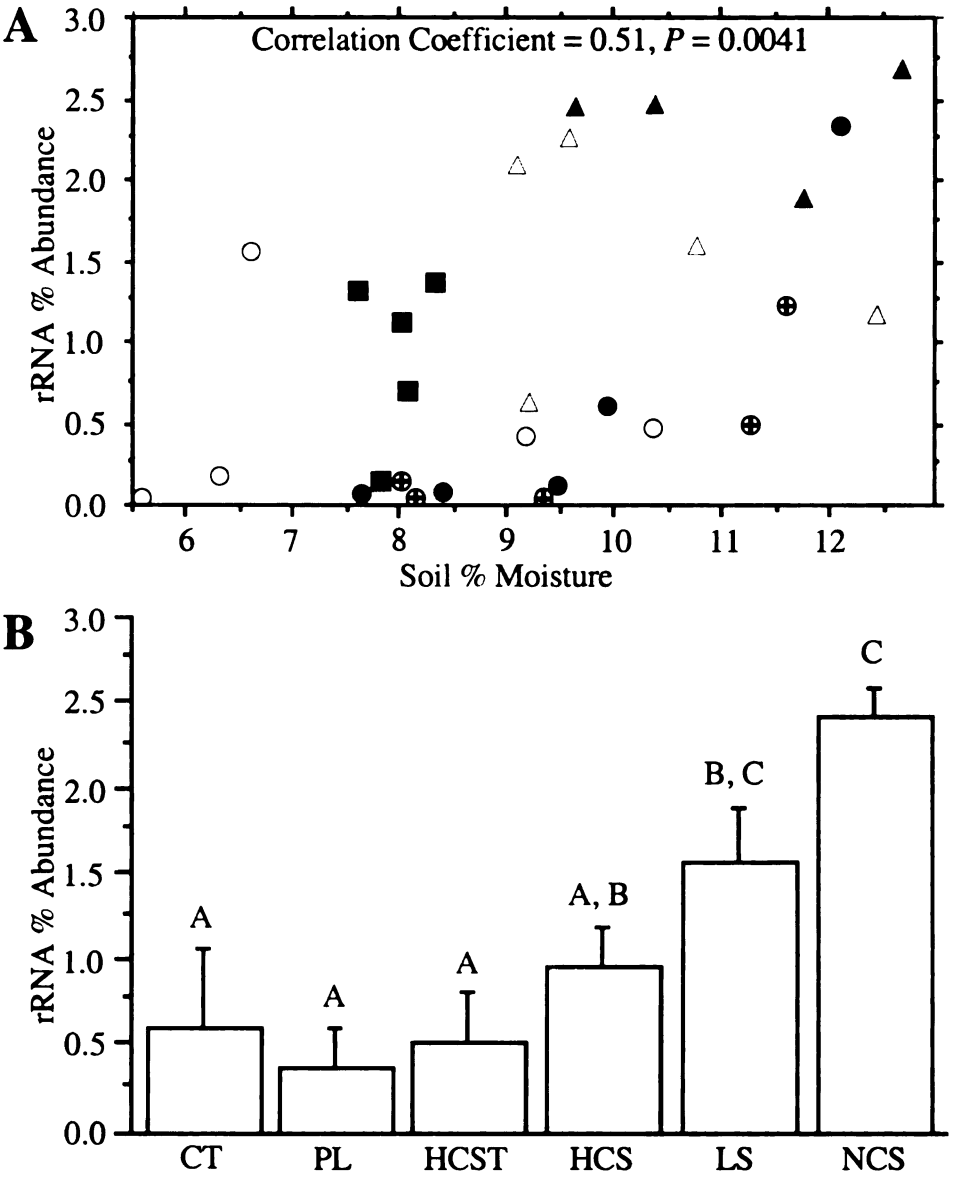

Figure 4.5. Abundance of verrucomicrobial rRNA in KBS-LTER fields in July 1998 with respect to soil moisture and management. Verrucomicrobial rRNA abundance in soil samples (n = 29) from July 1998 are plotted against soil moisture content (Panel A). Soil samples are from fields that have either been cultivated (CT, ●), planted with poplar trees (PL, ≈), abandoned from cultivation but tilled annually (HCST, ○), abandoned from cultivation (HCS, ■), or never cultivated (LS, Δ; NCS, ▲). The average verrucomicrobial rRNA abundance for fields belonging to each treatment are also shown (Panel B). Bars with different letters are significantly different from each other (Fishers PLSD, $P < 0.05$). Bars and whiskers represent the mean and standard error.

HCS) than in fields that had never been cultivated (LS, NCS) (Figure 4.5B). In addition, there was evidence that there is a statistically significant relationship between soil moisture content and the abundance of verrucomicrobial rRNA ($F_{1, 22} = 8.182$, $P = 0.0091$) indicating that soil moisture may be a useful variable for predicting differences in verrucomicrobial abundance in soil.

DISCUSSION

The oligonucleotide probe Ver47 was effective in determining the abundance of verrucomicrobial rRNA in soil microbial communities. This new probe provides the first measurements of verrucomicrobial abundance in the soil by a technique that does not require PCR amplification of nucleic acids from the microbial community. The mean verrucomicrobial rRNA abundance found at four different times in fields from the KBS-LTER site was 1.9 % ± 0.2 % (s.e.). Sampling at the KBS-LTER site was designed to address the relative importance of plant community composition, soil management history, soil depth and soil moisture on the abundance of verrucomicrobial rRNA in the soil. In addition, samples were taken at different times to allow for an assessment of the temporal variability in verrucomicrobial abundance.

The HCS and NCS fields both support diverse plant communities that are similar in species composition but very different from the much less diverse plant community present on the CT fields. Prior to their abandonment in 1989, the HCS fields were treated identically to the CT fields and as a result had total soil carbon and nitrogen concentrations that at the time of sampling were similar to those found in CT fields and significantly lower than those found in the NCS fields (7, 19). If plant community composition is a major influence on the abundance of Verrucomicrobia in soil than verrucomicrobial abundance should be similar in the HCS and NCS fields. Alternatively, if soil characteristics relating to cultivation history are a major influence on the abundance of Verrucomicrobia than verrucomicrobial rRNA abundance should be similar in the CT and HCS fields. Observations of verrucomicrobial abundance in the CT, HCS, and NCS fields at four times do not provide convincing evidence that either plant community composition or soil management history have a significant influence on the abundance of Verrucomicrobia in the soil. As this analysis was restricted to three replicate fields from each treatment it is possible that subtle differences in verrucomicrobial abundance may occur between the treatments at different times and that these differences were not detected due to the magnitude of temporal variability. For example, when the numbers of treatments sampled and the numbers of

fields sampled per treatment were increased and the analyses were limited to the July 1998 samples it was possible to detect differences in verrucomicrobial rRNA abundance between fields that had differences in past management history (Figure 4.5B). This result most likely indicates that the abundance of the Verrucomicrobia is influenced by changes in the soil caused by past management history, but that these differences in abundance may be small in comparison to those caused by temporal variability or only apparent at certain times of the year. In contrast, similarities observed in verrucomicrobial rRNA abundance between the CT, HCS, and HCST fields provides no evidence to indicate that plant community composition has an influence on the abundance of verrucomicrobial rRNA in the soil.

Though we found no evidence that verrucomicrobial rRNA abundance varies between the treatments examined there is evidence of significant temporal variability in verrucomicobial rRNA abundance in the soil (Figure 4.3B). Changes in verrucomicrobial abundance could result from repeatable seasonal variations or from isolated meteorological phenomena that occurred prior to the time of sampling. The data are sufficient to show that verrucomicrobial rRNA abundance can change significantly at time scales relevant to seasonal or metorological events though they are not sufficient to distinguish the specific causes of the temporal variability.

Soil moisture content varies considerably with time and has been observed to influence both the activity of soil microorganisms (9, 20, 23), and the structure of soil microbial communities (3, 4). To examine whether soil moisture has an influence on the abundance of the Verrucomicrobia in soil, the moisture content was determined for soil samples from July 1998. Though only a weak positive correlation was observed between the abundance of verrucomicrobial rRNA and soil moisture content, the use of soil moisture as a covariate in ANCOVA revealed that a significant amount of the variability in verrucomicrobial rRNA abundance can be explained by soil moisture content. Clearly soil moisture content is not the only variable influencing the abundance of Verrucomicrobia in the soil, but it is interesting to note that the few species of Verrucomicrobia that have currently been isolated have originated from either aquatic environments or saturated soils (11, 13).

The most striking difference in verrucomicrobial rRNA abundance occurs with depth in the soil. Verrucomicrobial rRNA was significantly more abundant in the top 5 cm of soil than in cores taken deeper into the soil (0 - 10 cm). Because the 0 - 5 cm portion of the soil is included in the 0 - 10 cm soil cores our measurements actually underestimate the difference in verrucomicrobial abundance that occurs with depth. Soil characteristics such as total organic carbon, total nitrogen, and soil moisture all decrease with

depth in the soil (26). It is likely that verrucomicrobial rRNA abundance is high in the 5 cm soil cores relative to the 10 cm soil cores because the Verrucomicrobia respond favorably to growth conditions that are present near the surface of the soil.

Though rarely isolated from the soil, Verrucomicrobia are commonly detected in this environment through the use of cultivation-independent analyses of soil microbial communities (1, 2, 8, 10, 14-16, 18, 25). At the KBS-LTER site *Verrucomicrobia* were observed to account for as much as 9.8 % of the 16S rRNA present in the soil. The abundance of verrucomicrobial rRNA in soil was strongly influenced by sampling time and depth in the soil. In addition, there was evidence that verrucomicrobial abundance may be influenced by changes in the soil caused by past cultivation history. If the Verrucomicrobia respond to changes in the soil environment that are associated with changes in soil moisture content as suggested by data from July 1998 than this relationship may help to explain the variability in verrucomicrobial rRNA abundance observed in relation to sampling time, soil depth, and soil management history. To understand if there is a relationship between verrucomicrobial abundance and soil moisture content and how such a relationship could influence the behavior of Verrucomicrobia in the soil will require experimentation on verrucomicrobial isolates from the soil in addition to further observation of

environmental samples. The probe described in this study can be used to measure the abundance of Verrucomicrobia in environmental samples and can also be used to verify the successful enrichment and isolation of Verrucomicrobia from the soil.

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CHAPTER 5

THE STRUCTURE OF MICROBIAL COMMUNITIES IN SOIL: PATTERNS OF MICROBIAL DISTRIBUTION, THEIR DYNAMIC NATURE AND THE LASTING IMPACT OF CULTIVATION

INTRODUCTION

Soil microbial communities are integrally involved in biogeochemical cycles, and their activities are crucial to the productivity of terrestrial ecosystems. Despite the importance of these communities, little is known about the distribution of specific bacterial groups in the soil or the manner in which these organisms respond to environmental stimuli. The dearth of information about soil microbial communities is a consequence of their enormous complexity and genetic diversity, and the fact that the characterization of microbial community structure requires sophisticated techniques that cannot provide real time measurements in the field (45). Furthermore, the microorganisms that can be isolated from soil and studied in pure culture in the laboratory represent only a small portion of the

microbial groups present *in situ* (18). As a result, much of the research on soil microbial communities has focused on community-level properties that are more easily measured, such as microbial biomass or microbial respiration rates. While these approaches simplify measurements by treating the soil microbial community as a homogeneous group, they do not provide information about the internal dynamics of microbial communities or the relationships between community structure and biogeochemical processes in the environment. Soil microorganisms do not behave as a homogeneous trophic level (29), and the species composition of a soil microbial community can influence specific microbial processes in the soil both qualitatively and quantitatively (14). To understand the factors that influence the composition of microbial communities in the soil and the impact that microbial community composition may have on terrestrial ecosystem function it is important to investigate the distribution of microorganisms in relation to spatial and temporal characteristics of their environments.

The two most common approaches for studying microbial community structure rely on analyses of either microbial fatty acids or ribosomal RNA (rRNA) genes. The composition of fatty acids extracted from the cell membranes of microorganisms can be used to compare microbial communities from different soil samples (49, 54). Soil microbial communities exhibit changes in whole-community fatty acid profiles in

response to changing environmental conditions (4-6, 13, 26, 34, 36). However, a difference between whole-community fatty acid profiles can indicate either a change in the species composition of the communities or a difference in the physiological status of the cells within the communities. Thus, while it is a relatively easy to detect differences between communities, it is difficult to attribute those differences to changes in species composition based on whole-community fatty acid profiles (54).

Ribosomal RNA gene sequences are conserved in all living organisms and are commonly used to determine the phylogenetic relationships between organisms (50). The recovery and analysis of rRNA genes has proven to be a useful tool in revealing the general taxonomic composition of soil microbial communities (24). While analyses of rRNA genes reveal a tremendous amount of species richness within soil microbial communities, a large fraction of the rRNA gene sequences recovered fall into one of several broad groups of organisms. Examination of the rRNA genes recovered from soil microbial communities at diverse sites reveal that eight bacterial groups are present in a majority of soil microbial communities (10). These groups are the Alpha, Beta, and Gamma groups of the Proteobacteria, the Actinobacteria (Gram positive bacteria with high mol % G + C genome content) the Cytophagales, the Acidobacteria, the Planctomycetes, and the Verrucomicrobia. In addition to these bacterial groups, eukaryotic

microorganisms such as fungi and protozoa are commonly found in soil. The relative abundance of microbial group rRNA in soil microbial communities was determined by extracting RNA from soil samples and using radio-labeled oligonucleotide probes that specifically bind to the rRNA molecules from the microbial groups mentioned above.

There is currently a fundamental lack of information about the distribution of microorganisms in terrestrial ecosystems and the basic structure of microbial communities within the soil. The objective of this research was to determine if cultivation influences the distribution of microbial groups in the soil resulting in differences in microbial community structure between fields with different management histories. Microbial community structure in the soil was assessed over a period of two years to determine whether patterns of community structure are discernable at the scale of treatments composed of several one hectare field replicates and to determine if either plant community composition or past management history impacts microbial community structure in the soil. This survey allowed an assessment of the temporal variability of microbial community structure and a crude examination of the time scale at which such variability is evident. In addition, microbial community structure was examined at different soil depths to see if patterns of microbial distribution change with depth in the soil. This research addresses basic questions about the structure

of microbial communities, the distribution of microorganisms in the soil and the response of these microorganisms to environmental change. By observing the distribution of microbial groups in relation to environmental stimuli we can begin to generate and test hypotheses regarding the rules which govern the organization and distribution of microorganisms in terrestrial ecosystems.

METHODS

Site description and soil sampling

Soil samples were taken from the Long Term Ecological Research (LTER) site located at the Michigan State University W. K. Kellogg Biological Station (KBS) in Hickory Corners, Michigan. The KBS-LTER site, established in 1989 to study ecological processes in agroecosystems, includes a large-scale replicated field experiment with seven treatments representing different cropping systems and types of management, as well as several nearby successional sites (For detailed agronomic protocols see http://lter.kbs.msu.edu). The main field experiment is located on 48 hectares of land that had been uniformly farmed for over fifty years prior to establishment of the LTER site (39). Soils are typic hapludalfs, sandy to silty clay loam and are of moderate fertility (39). The site has a mean annual air temperature of 9.4 °C and a mean annual rainfall of 860 mm (14). Three to

five fields were sampled to represent each treatment. Fields were sampled by taking a single soil core (2.5 cm diameter, 10 cm depth) from each of five permanent sampling locations distributed across each field replicate. The soil cores from each field were pooled, sieved (4 mm mesh), frozen in liquid nitrogen (generally within 10 minutes of sampling), and stored at -80°C.

Soil samples were obtained at four times over a period of two years. On October 3, 1996 and May 23, 1997 soil was sampled from three field replicates of two of the historically cultivated treatments (the Cultivated Tilled (CT) and Historically Cultivated Sucessional (HCS) fields) and from a site that had never been cultivated, that was adjacent to the LTER experimental site (the Never Cultivated Successional (NCS) fields) (Table 5.1). At the time of sampling in October 1996 corn was present on CT fields. For a month prior to sampling in October 1996 the KBS-LTER site had experienced a mean temperature of 16.2 °C and a total rainfall of 73.3 mm. In the two weeks prior to sampling in May 1997 the CT fields had been fertilized and tilled and soybeans had been planted, but had not yet begun to sprout. The mean temperature and rainfall at the KBS-LTER site for a month prior to sampling in May 1997 were, respectively, 10.2 °C and 97.8 mm.

On June 6, 1998 soil was sampled from five field replicates of the CT, HCS, and NCS fields and from tilled subplots of the HCS fields (HCST). In addition, June 1998 sampling included both 0 - 5 cm deep soil cores and

Table 5.1. Codes and descriptions of experimental treatments and reference communities on the KBS-LTER site.

Symbol	Description	Till	Management History	Plant Community
CT	cultivated field	yes	historically cultivated	Annual rotation, corn/soybean/ wheat
PL	poplar plantation	no	historically cultivated, *Populus* clones planted in 1989	ground cover dominated by perennial grasses
HCS	historically cultivated successional	no	historically cultivated, abandoned in 1989	herbaceous perennials
HCST	historically cultivated successional, tilled subplot	yes	annually tilled HCS subplots	dominated by annual grasses
LS	late successional	no	historically cultivated, abandoned in 1951	herbaceous perennials
NCS	never cultivated late successional	no	never cultivated	herbaceous perennials

0 - 10 cm deep soil cores to assess potential differences in microbial community structure due to soil depth. The mean temperature for one month prior to sampling in June was 17.9 °C with a total rainfall of 34.7 mm. Wheat had been planted on CT fields at the end of 1997 and was present on CT fields in June and July; these fields received fertilization but no tillage in the Spring of 1998. On July 28, 1998 five field replicates were sampled from all of the treatments mentioned above (CT, HCS, HCST, and NCS), from a treatment consisting of poplar plantations grown on historically cultivated fields (PL), and from a treatment consisting of late successional fields (LS) that had been historically cultivated prior to abandonment in 1951 (Table 5.1). For a month prior to sampling in July the KBS-LTER site experienced a mean temperature of 21.6 °C and 113.4 mm of rainfall.

In 1994 there were no significant differences between the historically cultivated fields (CT, PL, HCS, HCST) in either soil organic carbon or total nitrogen content (35). In contrast, both soil organic carbon and total nitrogen content were significantly lower in the historically cultivated fields (HCS) than in the NCS fields (35).

The successional fields sampled (HCS, NCS, LS) were all dominated by herbaceous forbs (12, 21, 23). Following abandonment from cultivation in 1989 the plant communities in the historically cultivated successional

fields (HCS) had progressed from initial dominance by annual species to be dominated by herbaceous perennial forbs (23).

Sample processing

RNA extraction.

RNA for use in hybridization experiments was extracted as previously described (8, 11). In brief, a 10 g portion of each frozen soil sample was suspended in a buffer suitable for sample homogenization and RNA stabilization. Microbial cells were lysed using beadmill homogenization with 10 g of 0.1 mm silica/zirconia beads in a 32 ml chamber for a duration of two minutes (Beadbeater, Biospec Products, Inc.). RNA from homogenized samples was concentrated by precipitation with polyethelene glycol and then purified using both hydroxyapatite and Sephadex G-75 columns. RNA samples were finally precipitated, resuspended in 200 μl of Rnase-free ddH_2O, and stored at –20°C.

RNA hybridization.

Quantitative filter hybridization was performed as previously described (11, 43). Nucleic acids from soil samples and standards were denatured with 0.5% glutaraldehyde-50 mM Na_2HPO_4, serially diluted to provide a range of sample concentrations, blotted onto nylon membranes

using a 96 well dot blot manifold, and immobilized by UV crosslinking. RNA isolated from pure cultures (*Ketogulonogenium vulgare* DSM 4025, *Nitrosomonas europaea* ATCC 25978, *Cytophaga johnsonae* ATCC 17061, *Arthrobacter globiformis* ATCC 8010, *Verrucomicrobium spinosum* ATCC 43997, *Planctomyces limnophilus* ATCC 43296, *Acidobacterium capsulatum* ATCC 51196, and *Saccharomyces cerevisiae* American Ale Yeast 1056 (Wyeast Labs, INCS.)) were included on all membranes as standards to control for differences in the specific activity of labeled probes and to account for the possibility of nonspecific probe binding. Hybridization protocols for ^{32}P-5'-labeled oligonucleotide probes were previously described in detail (43). Radio-labeled oligonucleotide probes that bind to the rRNA molecules from specific microbial groups were then used to determine the relative abundance of microbial group rRNA. Oligonucleotide probes specific for bacteria from the alpha subclass of the Proteobacteria (Alf1b), the beta subclass of the Proteobacteria (Bet42a), the Actinobacteria (HGC69a), the Planctomycetes (Pla46R), the Verrucomicrobia (Ver47), and the Cytophaga-Flavobacterium cluster of the Cytophagales (CF319a) have all been previously described (3, 9, 32). The probe specific for the Acidobacteria (Acd31, 5'-GATTCTGAGCCAGGATC –3') was designed (modified from Barns et al., 1999) and verified empirically as specifically recognizing acidobacterial 16S

rRNA under the hybridization conditions indicated below. In addition, the probe specific for all of the Eukarya (Euk1195), and the probe universal to all 16S rRNA (Univ1390) have also been described previously (3). Hybridization between labeled probes and RNA immobilized on filters proceeded at 45°C for at least 12 hours. Following probe hybridization, filters were washed for 30 minutes at 45°C, and then washed for an additional 30 minutes at a higher temperature to provide stringency (45 °C for Univ1390, Euk1195, and Ver47; 50 °C for HGC69a; 53 °C for Acd31; 55 °C for Alf1b and CF319a; and 62 °C for Bet42a). The specifically bound probe that remained on the membrane was visualized using a phosphorimaging system (Storm 860, Molecular Dynamics), signal intensity was quantified using Image Quant software v 5.0 (Molecular Dynamics).

Determination of rRNA abundance

Within a soil sample, the relative abundance of rRNA derived from a specific group was measured as the ratio of the signal derived from a group-specific probe to the signal derived from the universal probe. This approach for determining microbial rRNA abundance has been used previously to describe aspects of microbial community structure (43). Relating specific probe binding to universal probe binding controls for variability in the total amount of RNA recovered from each soil sample, and also controls for the

presence of hybridization inhibitors that may co-purify with RNA from soil. Positive controls were included on each membrane to correct for variations in the labeling efficiency of different oligonucleotide probes while negative controls were used to correct for the possibility of non-specific probe binding. Every RNA sample was represented by five aliquots in a dilution series to examine potential differences in signal intensity due to inhibition or membrane saturation. The ratio of signal intensities obtained for specific and universal probe binding to an RNA sample was defined as $R = \Sigma^{n}_{i=1}[G_i(U_i)^{-1}]n^{-1}$, where G_i and U_i represent, respectively, the corresponding signal intensities obtained for group specific and universal probe binding to each aliquot representing the sample, and n equals the total number of aliquots representing the RNA sample. The value R was calculated for each soil RNA sample (R_S), and a mean value of R was determined for all positive (R_P) and negative (R_N) controls present on each membrane. The relative abundance of rRNA from a specific microbial group was then defined as $(R_S - R_N)(R_P - R_N)^{-1} \times 100$.

Data analysis

Percent rRNA abundance data was arcsine transformed prior to statistical analyses to compensate for relationships observed between the mean and variance of samples. After transformation certain data suffered

from both departures from normalty and heteroscedasticity, as a result all parametric tests were verified through comparable nonparametric tests (44). Except where explicitly stated to the contrary, all conclusions drawn from parametric statistical tests were supported by nonparametric analyses as well. In addition, abundance values for the Cytophaga-Flavobacteria group and the Acidobacteria frequently fell below detection limits (approximately 0.5 % rRNA abundance). A large number of zero values may bias statistical tests, so where noted these groups have been omitted from community-level analyses. Statistical tests were performed using StatView v 5.0 (SAS Institute, Inc.), and SAS v 7.0 (SAS Institute, Inc.).

Repeated measure MANOVA (RMANOVA) was used to examine the main effects of treatment and sampling time, and their interaction for all microbial groups simultaneously in treatments CT, HCS, and NCS over all four sampling dates (with three field replicates for each treatment at each time). Significant MANOVA results were investigated by using Hoetelling's T^2 test to perform pairwise comparisons, while ANOVA was used to examine the effects within each individual microbial group. Correspondence analysis was used to graphically represent relationships between the fields sampled at different times and the microbial groups from those fields. The above series of analyses were used to examine most data with the following exceptions. A 2 X 4 repeated measures design was used for the MANOVA

and ANOVA that examined the effects of treatment, sampling time, and their interaction on fields sampled in June and July 1998, while one way MANOVA and ANOVA were used to examine the treatment effects in all fields sampled in July 1998 (with five field replicates representing each treatment in June and July 1998).

To determine if soil depth has an effect on microbial community structure in samples from June 1998, community structure in 0 - 5 cm cores was compared to community structure in 0- 10 cm cores using Hoetelling's T^2 test. The effect of the treatments CT, HCS, HCST, and NCS on changes in community structure with depth were investigated by subtracting microbial group abundance in 5 cm cores from that in 10 cm cores from the same field replicate. Depth differences due to treatment were examined using one way MANOVA and ANOVA.

In addition to multivariate analyses of community structure, analyses were made of community variability in the treatments sampled. Variability in community structure was measured by first determining the coefficient of variation (CV) of rRNA relative abundance for each microbial group in each treatment. Variability in overall community structure in each treatment was then assessed as the mean of the CVs of relative abundance for all of the microbial groups examined in each treatment. ANOVA was used to examine treatment effects on microbial community variability.

RESULTS

Overall microbial community structure

Microbial community structure was assessed by determining the abundance of rRNA from eight microbial groups in relation to the total amount of rRNA present in the soil. Though there was considerable variation in the relative abundance of microbial groups in the 89 soil samples analyzed, a general profile for the structure of the microbial communities was apparent in these soils (Figure 5.1). The mean and standard deviation of rRNA relative abundance was determined for each microbial group across all of the samples analyzed. The dominant microbial group observed was the Alpha Proteobacteria (24.7% ± 13.2%), followed in abundance by the Actinobacteria (11.1% ± 7.6%), the Eukarya (9.7% ± 5.7%), the Planctomycetes (7.2% ± 4.7%), the Acidobacteria (3.5% ± 5.7%), the Beta Proteobacteria (2.3% ± 1.9%), Verrucomicrobia (1.9% ± 1.9%), and the Cytophaga-Flavobacteria (0.4% ± 0.9%).

In the overall data set, the coefficient of variation (CV) for microbial rRNA relative abundance increases as the relative abundance of a microbial group in the community decreases. We examined this phenomenon further using a subset of the rRNA relative abundance data in fields from the three treatments (CT, HCS, and NCS) that were sampled on all four sampling

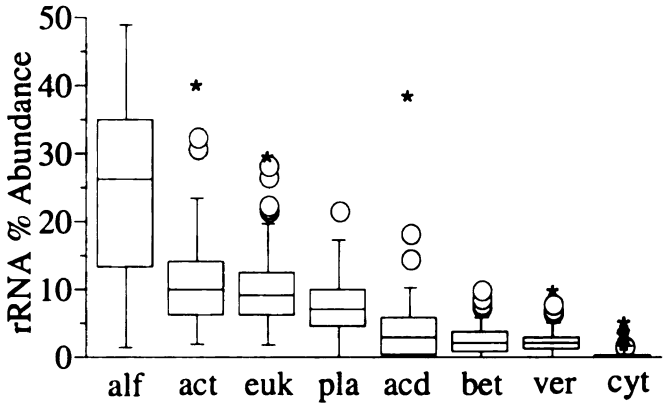

Figure 5.1. Summary of values for rRNA abundance from Alpha Proteobacteria (alf), Actinobacteria (act), Eukarya (euk), Planctomycetes (pla), Acidobacteria (acd), Beta Proteobacteria (bet), Verrucomicrobia (ver), and Cytophagales (cyt) as measured in all samples analyzed in this study (n=89). For each set of observations the median is shown as a horizontal line while each box extends from the first to the third quartile of observations (IQR), whiskers represent data points within 1.5 IQR of each edge of the box, mild outliers are indicated by open circles while extreme outliers are indicated by asterisks.

dates. We were concerned that experimental error or statistical artifacts associated with the use of relative abundance data could inflate the CV of microbial groups with low relative abundance. However, when the CV is determined for microbial groups within each treatment at a given time (with the fields from each treatment distributed randomly across the landscape) there is no relationship between CV and abundance (Figure 5.2A). Any analytical or statistical artifacts that result from calculating the CV of rRNA relative abundance would be apparent regardless of the organization of data by fields or treatments. In contrast, calculation of the CV for microbial group abundance from all fields sampled within a single time (regardless of treatment) reveals that variation increases as the abundance of a group decreases (Figure 5.2B), and the relationship exists at all four sampling times. The same relationship is observed when the CV is calculated for the abundance of each microbial group over the four sampling times in each individual field (Figure 5.2C). That variability in microbial group abundance increases with decreasing microbial group abundance in fields across different treatments and across time, but not in field replicates of the same treatment suggests that this pattern of variation occurs as a result of spatial and temporal variability in the soil environment.

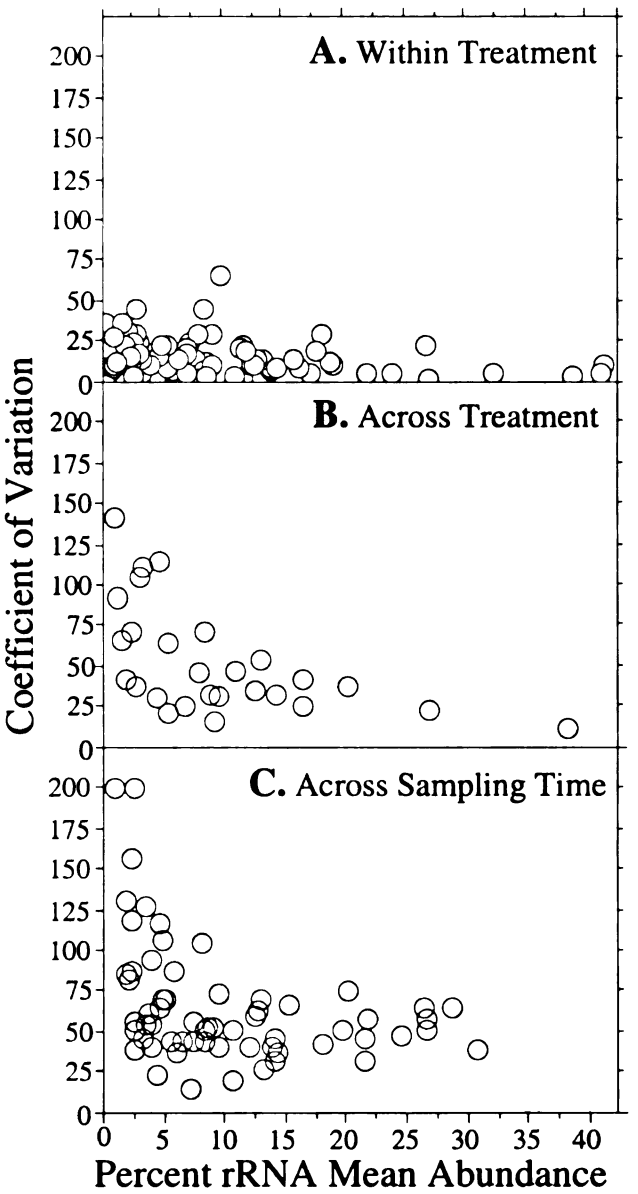

Figure 5.2. The mean and coefficient of variation (CV) in microbial group rRNA relative abundance were calculated for different sets of fields to demonstrate the effect that treatment and time have on the relationship between abundance and variability in abundance. Data points represent the mean and CV of microbial group abundance in each treatment at a single time (panel A), across all treatments at a single time (panel B), and in individual fields across the four sampling times (panel C).

Effect of cultivation

Microbial communities in fields from CT, HCS, and NCS were examined at four different sampling times to observe whether characteristics of these fields such as plant community composition, current cultivation status, or cultivation history impact variability in the structure of microbial communities in soil (Figure 5.3). The failure to detect any significant variability in microbial community structure between these fields would support previous observations that microbial community composition is largely homogeneous across continuous landscapes (19). Data from the Acidobacteria and the Cytophaga-Flavobacteria were not used in these analyses because a large proportion of zero abundance values rendered these data invalid for the statistical tests performed. RMANOVA of microbial group rRNA abundance revealed that the main effects of treatment and sampling time are significant (Table 5.2). There is also evidence for a significant interaction between the effects of treatment and sampling time, indicating that while there is temporal variation in microbial community structure this variation is influenced by treatment effects (Table 5.2). Subsequent pairwise tests provided no evidence for differences in microbial community structure between CT and HCS while indicating that the microbial communities in both of these treatments differ significantly from the NCS fields (Table 5.2). Correspondence analysis was used to visualize

Figure 5.3. Microbial group rRNA abundance in samples from October 1996 (Oct 96), May 1997, June 1998, and July 1998 (mean ± s.e., n = 9). F statistics are shown for the effects of sampling time (T) where significant. Bars that have different letters were revealed to be significantly different by the Scheffe test ($P < 0.05$).

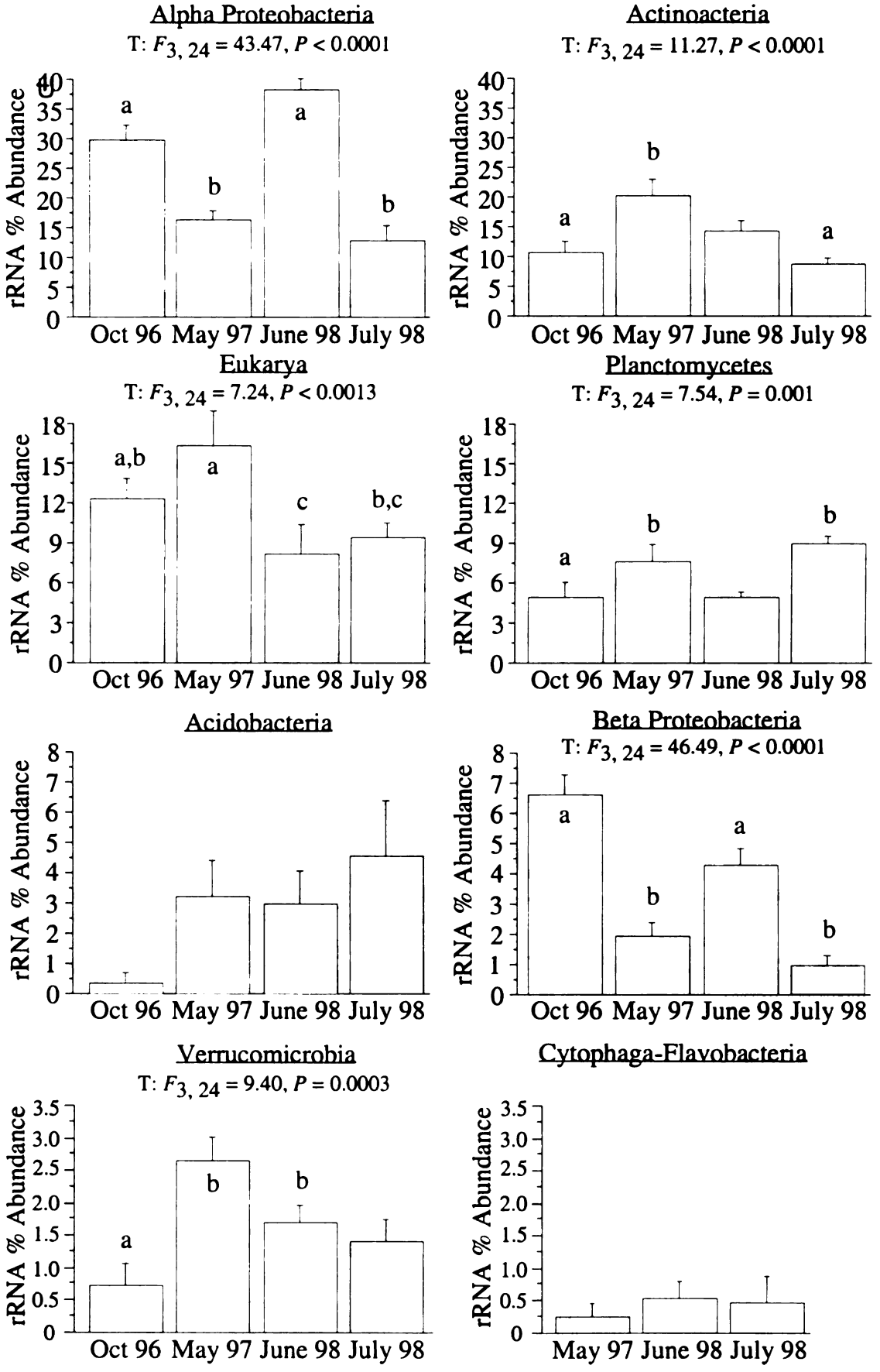
Alpha Proteobacteria
T: $F_{3, 24} = 43.47$, $P < 0.0001$
rRNA % Abundance
a
b
a
b
Oct 96 May 97 June 98 July 98
Actinoacteria
T: $F_{3, 24} = 11.27$, $P < 0.0001$
rRNA % Abundance
a
b
a
Oct 96 May 97 June 98 July 98
Eukarya
T: $F_{3, 24} = 7.24$, $P < 0.0013$
rRNA % Abundance
a,b
a
c
b,c
Oct 96 May 97 June 98 July 98
Planctomycetes
T: $F_{3, 24} = 7.54$, $P = 0.001$
rRNA % Abundance
a
b
b
Oct 96 May 97 June 98 July 98
Acidobacteria
rRNA % Abundance
Oct 96 May 97 June 98 July 98
Beta Proteobacteria
T: $F_{3, 24} = 46.49$, $P < 0.0001$
rRNA % Abundance
a
b
a
b
Oct 96 May 97 June 98 July 98
Verrucomicrobia
T: $F_{3, 24} = 9.40$, $P = 0.0003$
rRNA % Abundance
a
b
b
Oct 96 May 97 June 98 July 98
Cytophaga-Flavobacteria
rRNA % Abundance
May 97 June 98 July 98

Table 5.2. Summary of parametric and nonparametric MANOVA examining the effects of treatment (CT, HCS, or NCS) and sampling time (October 1996, May 1997, June 1998, July 1998) on microbial community composition as measured by the rRNA abundance of the Alpha Proteobacteria, Beta Proteobacteria, Actinobacteria, Verrucomicrobia, Planctomycetes and the Eukarya.

	RMANOVA			RMANOVA by Ranks		
Effects	Pillai's Trace	df	F value [a]	Pillai's Trace	df	L value [a]
Treatment	1.307	12	6.28 ***	1.296	12	45.360 ***
Time	2.467	18	16.20 ***	2.461	18	86.135 ***
Treatment x Time	2.208	36	2.32 ***	2.176	36	76.160 ***
Pairwise Tests						
CT v HCS	0.638	6	4.107	0.544	6	12.512
CT v NCS	0.866	6	15.124 ***	0.827	6	19.021 ***
HCS v NCS	0.740	6	6.627 ***	0.647	6	14.881 *

[a] The symbols (*, **, and ***) indicate $P < 0.05$, 0.01, and 0.005 respectively.

differences in community structure due to the effects of treatment and sampling time (Figure 5.4). Correspondence analysis reveals similarities in community structure between samples from October 1996 and June 1998, and between May 1997 and July 1998 (Figure 5.4A). Careful observation reveals that the microbial communities in CT and HCS are most distinct from those in NCS in both June 1998 and May 1997, though such differences are not as apparent in October 1996 and July 1998.

By restricting the analysis to fields sampled in both June and July 1998 an additional tilled treatment (HCST) can be added to the analysis and the number of field replicates representing each treatment can be increased from three to five. MANOVA used to analyze these data from June and July 1998 continues to indicate that the main effects of treatment (Pillai's Trace: $F_{18,81} = 4.878$, $P < 0.0001$) and sampling time (Pillai's Trace: $F_{6,25} = 50.857$, $P < 0.0001$) are significant while also providing evidence for a significant interaction between the effects of treatment and sampling time (Pillai's Trace: $F_{18,81} = 3.526$, $P < 0.0001$). Additional pairwise tests confirm that the structures of the microbial communities in the NCS fields are significantly different from those shared in the historically cultivated fields (Hoetellings T^2 test: $F_{6,29} = 11.88$, $P < 0.0001$). Neither the current tillage regime or plant community composition of the fields have any significant effect on the structure of microbial communities in soil. These results indicate that in

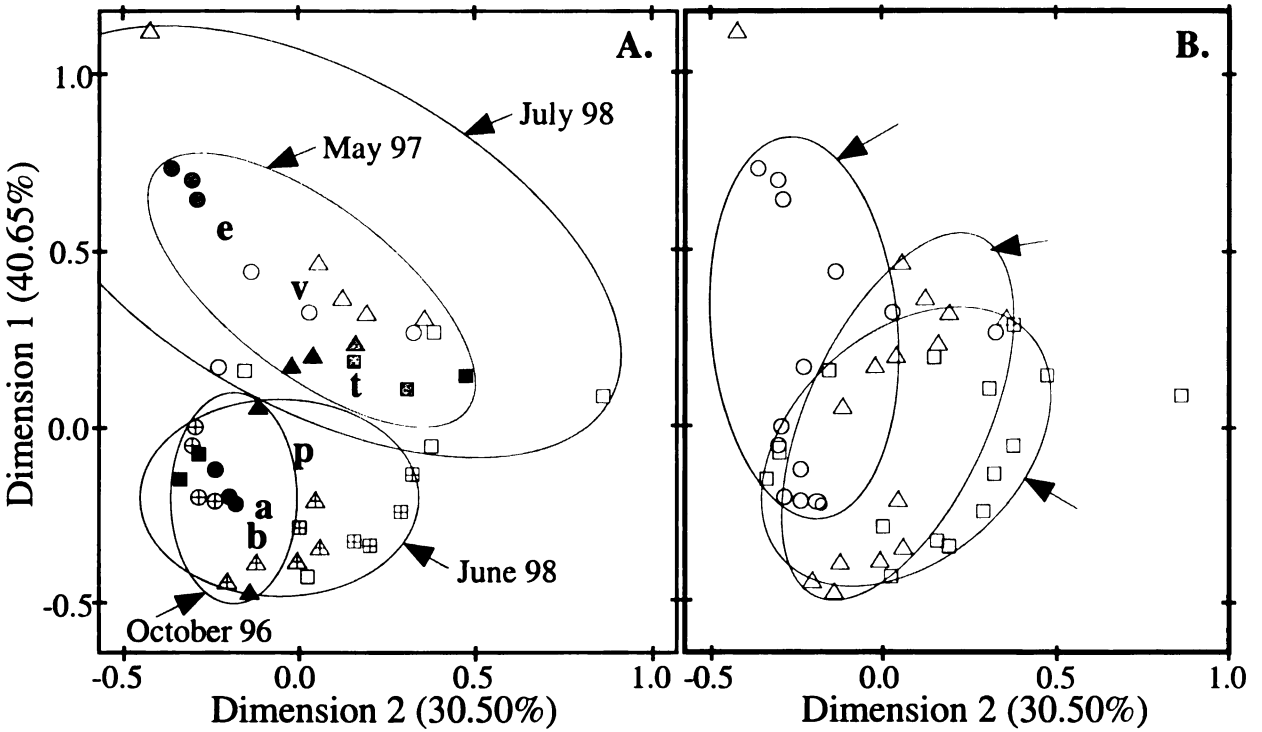

Figure 5.4. Correspondence analysis of community structure in treatments CT (squares), HCS (triangles), and NCS (circles) at four sampling times. The same data is presented in both panels, ellipses and color-coding are used to help visualize differences in microbial community structure in relation to either sampling time (Panel A) or treatment (Panel B). In Panel A symbols are filled to indicate sampling time for October 1996 (black fill), May 1997 (grey fill), June 1998, (crosses), and July 1998 (no fill). Community structure is represented by the rRNA abundance of alpha Proteobacteria (a), beta Proteobacteria (b), Actinobacteria (t), Verrucomicrobia (v), Planctomycetes (p) and the Eukarya (e).

these fields the cultivation history of soil has more of an impact on microbial community composition than does current management or plant community composition.

Effects on microbial groups

ANOVA was used to examine both treatment and sampling time effects for each microbial group individually (Figure 5.3). The main effect of sampling time was significant in five of the six microbial groups examined, while the treatment effect was only significant for the Eukarya. This result is in contrast with the MANOVA of all groups simultaneously which showed treatment, time, and treatment X sampling time effects to all be significant. Differences in eukaryal rRNA abundance were not solely responsible for the detection of significant treatment effects by MANOVA, as these effects were still significant when eukaryal data were excluded from analyses (Pillai's Trace: $F_{10, 42} = 4.266$, $P = 0.0004$). The fact that a treatment effect was only observed for one microbial group, and that no interaction effects were observed, are likely due to the decreased power of ANOVA relative to MANOVA. Correspondence analysis revealed that certain microbial groups have similar patterns of abundance at different sampling times (Figure 5.4A). The rRNA abundance of the Eukarya, Verrucomicrobia, and Actinobacteria were all maximal in May 1997 relative to the other sampling

times while the Alpha Proteobacteria, the Beta Proteobacteria, and the Planctomycetes all had abundance maxima in October 1996 and June1998 (Figure 5.3, and Figure 5.4).

Supplementary observations from July 1998

In July 1998 microbial community structure as defined by rRNA abundance was assessed for five replicates from each of the treatments CT, PL, HCS, HCST, LS, and NCS (Figure 5.5). Treatment PL was included to disentangle the effects of current plant community composition and historical cultivation on microbial communities. Plant communities in NCS fields have a larger proportion of perennial grasses than HCS or CT fields (K. L. Gross, pers. Com.) so it is possible that the presence of perennial grasses is responsible for the difference in microbial community structure between the NCS fields and the CT and HCS fields. If so, then microbial communities in the PL treatment, which also has a high proportion of perennial grasses, would be most similar to the NCS treatment. However, if historical effects are more influential, than the structure of the microbial communities in PL fields should be most similar to those in CT and HCS fields. Additionally, the LS field was sampled to investigate the length of time that is required for the microbial communities in historically cultivated fields to resemble those in fields that have not been cultivated. The LS field

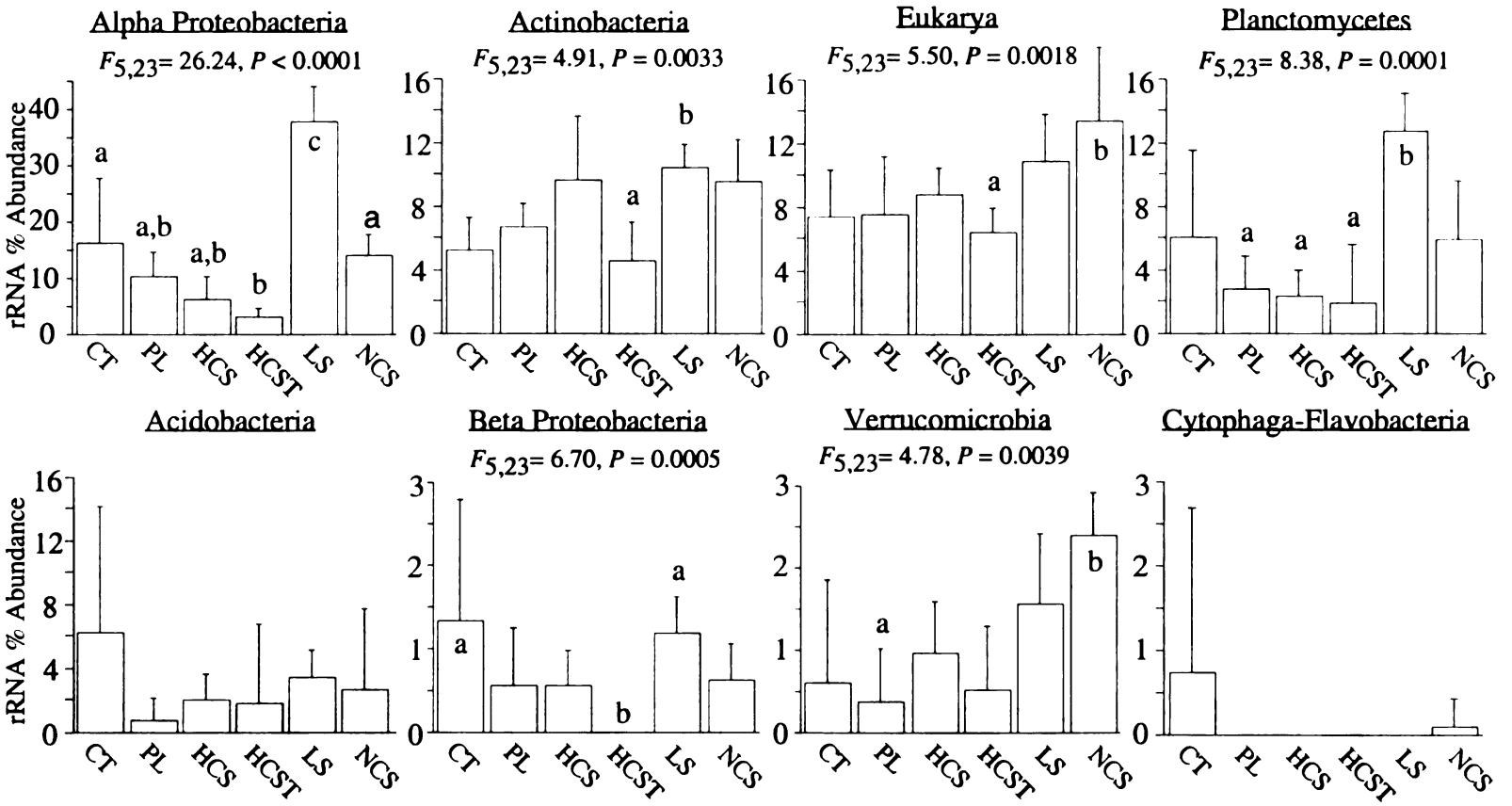

Figure 5.5. Microbial rRNA abundance (mean ± s.e., n = 5) in all treatments sampled in July 1998. F statistics are shown, where significant after Bonferroni correction, for treatment differences in microbial rRNA abundance. Values that were found to be significantly different ($P < 0.05$) by the Scheffe test are indicated by different letters.

was abandoned from cultivation prior to 1951 and is located approximately one kilometer from the main KBS-LTER experimental site.

The MANOVA for microbial group rRNA abundance determined in July 1998 provides strong evidence for a significant difference in community structure due to treatment (Table 5.3). Individual ANOVA reveal significant treatment effects for six of the seven microbial groups analyzed (Figure 5.5). Pairwise tests indicate that the microbial communities in the historically cultivated treatments (CT, PL, HCS, HCST) differed significantly from those in both the NCS and LS treatments (Figure 5.3). There was no detectable difference in the microbial communities of the NCS and LS treatments (Table 5.3).

Depth distribution of microbial groups

We examined the effect of soil depth on microbial community composition by comparing the microbial communities in surface surface (0 - 5 cm) and deeper (0 - 10 cm) soil cores sampled from treatments CT, HCS, HCST, and NCS in June 1998. We observed that microbial community structure differs significantly with soil depth (Pillai's Trace: $F_{7,23} = 8.647$, $P < 0.0001$). There was no evidence for any interaction between field treatment and depth on microbial community structure, indicating that

Table 5.3. Results of both parametric and nonparametric one-way MANOVA used to examine treatment effects on microbial community composition in July 1998 samples (CT, PL, HCS, HCST, LS, NCS). HC includes all of the historically cultivated fields on the main experimental site (CT, PL, HCS, HCST).

	MANOVA			MANOVA by Ranks		
Effects	Pillai's Trace	df	F value [a]	Pillai's Trace	df	L value [a]
All Treatments	2.538	35	3.091 ***	2.286	35	64.008 ***
Pairwise Tests						
HC v NCS	0.805	7	9.432 ***	0.716	7	16.468 *
HC v LS	0.873	7	16.743 ***	0.707	7	16.968 *
NCS v LS	0.985	7	9.249	0.993	7	8.937

[a] The symbols (*, **, and ***) indicate $P < 0.05$, 0.01, and 0.005 respectively.

neither tillage nor the historical effects of cultivation influenced differences seen in community structure between 5 cm and 10 cm cores. To examine the effect of soil depth on the relative abundance of individual microbial groups, the difference in rRNA abundance between 0 - 5 cm and 0 - 10 cm soil cores was calculated separately for each field replicate (Figure 5.6). ANOVA of the depth difference in rRNA abundance for each microbial group revealed that three microbial groups differed significantly with depth. The Alpha Proteobacteria (mean difference = –6.26%, P = 0.0105) and Beta Proteobacteria (mean difference = -2.12%, $P < 0.0001$) were respectively 18% and 61% lower in rRNA abundance in surface cores than in deeper cores, while Verrucomicrobia rRNA was 135% higher in surface cores than in deeper cores (mean difference = 2.27%, $P = 0.0003$).

Microbial community variability

Variability in microbial community structure due to treatment and sampling time was examined by determining the coefficient of variation (CV) for the rRNA abundance of each microbial group surveyed across the field replicates for each treatment. A 3 X 4 repeated measure ANOVA was used to examine the effects of treatment and sampling time on variability. Microbial community variability was influenced by the main effects of treatment ($F_{2, 54}$= 6.91, $P = 0.0059$), sampling time ($F_{3, 54}$= 3.08, $P = 0.0348$),

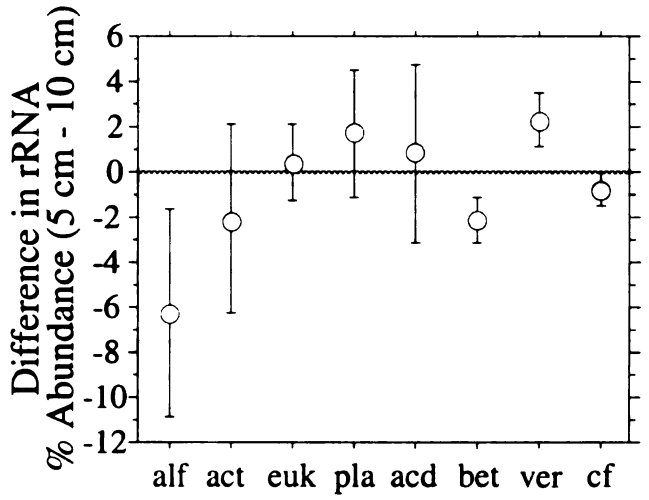

Figure 5.6. Mean difference in rRNA abundance between 0 - 5 cm soil cores and 0 - 10 cm soil cores. Values are estimated from all fields sampled in June 1998 (n = 19). Microbial groups depicted are the Alpha Proteobacteria (alf), Actinobacteria (act), Eukarya (euk), Planctomycetes (pla), Acidobacteria (acd), Beta Proteobacteria (bet), Verrucomicrobia (ver), and Cytophaga-Flavobacteria (cf). Means are shown with 95 % confidence intervals.

and by the interaction of these effects ($F_{6, 54}$= 4.33, P = 0.0012). Interestingly, variability in community structure was highest among HCS fields (20.34 ± 2.48) and lower in the CT (14.44 ± 2.03) and NCS fields (10.87 ± 1.78). Microbial community variability due to sampling time was highest in July 1998 (20.44 ± 2.52) relative to October 1996 (14.28 ± 3.63), May 1997 (11.58% ± 1.72%), and June 1998 (14.58 ± 1.62). The high variability for July 1998 samples relative to other sampling times is evident in Figure 5.4A by the wide distribution of points representing these samples compared to the smaller distributions seen for the other sampling times.

DISCUSSION

The microbial groups we examined accounted for between 11 % and 100 % (mean 59 % ± 23 % s.d.) of the total rRNA present in the soil microbial communities examined at the KBS-LTER site. Intracellular concentrations of rRNA increase with growth rate and change in response to alterations in the nutrient status of a cell (47). Thus, high relative abundance of rRNA for a microbial group in a community may indicate either that the group is numerically dominant or that it is growing rapidly within that community. Analysis of rRNA in relation to soil treatment and sampling time provides valuable insight into the responses of particular groups of microorganisms and of soil microbial communities in general. Each of the

microbial groups we examined encompasses a considerable amount of taxonomic and functional diversity. Although this approach sacrifices information about individual microbial species, by focusing on the distribution and abundance of broad groups we have obtained an unprecedented view of soil microbial community structure.

Our analyses show that microbial community composition did not differ significantly between conventionally managed agricultural fields (CT) and fields that had been abandoned from cultivation for ten years (HCS) (Table 5.2). In contrast, the microbial communities in both of these treatments differed significantly from those in nearby fields that had never been cultivated (NCS) (Table 5.2). The composition of microbial communities in another set of successional fields that had not been cultivated for > 45 years (LS) also differed significantly from those communities in fields having a history of agriculture, but were similar to the microbial communities in the NCS fields (Table 5.3). These results provide further support for previous observations indicating that the long-term effects of cultivation influence community structure in the soil at the KBS-LTER site (11, 26). Cavigelli and Robertson (2000) have shown differences between denitrifier community composition in CT and NCS fields, while Bruns et al. (1999) have shown that autotrophic ammonia oxidizer communities also differ between these fields. Furthermore, rates of methane

consumption by microbes in the never cultivated fields are much greater than methane consumption in either CT or HCS fields (40). These observations demonstrate that soil microorganisms are influenced by historical characteristics of the soil that are detectable long after changes of management practice.

Although microbial community composition did not differ in the CT and HCS fields, there is some evidence that after ten years community-level differences may be emerging in the historically cultivated fields. For example, microbial community variability as assessed by the mean CV of microbial rRNA abundance is significantly higher in the HCS fields than in either the CT or NCS fields. Such variability could result from successional processes occurring as HCS recovers from the effects of agriculture and may be related to plant community composition (21). There is growing evidence that plants influence microbial community structure in soil immediately adjacent to plant roots (17, 25, 33, 34, 51), but there is still conflicting evidence as to whether plant communities influence microbial distribution across individual fields (7, 13, 19, 36).

This study does not provide convincing evidence that plant community composition is influencing soil microbial community structure at the KBS-LTER site. There are several possible explanations for why we may have failed to detect a relationship between plant community

composition and microbial community composition if such a relationship actually exists. Firstly, it is possible that the effect of plant community composition on microbial communities in these fields is masked by the overwhelming influence of past agricultural practices that cause changes in soil structure and depletion of soil carbon and nitrogen levels (16). Secondly, microbial community structure in the historically cultivated treatments could differ due to plant community composition for scales below the resolution of the analyses presented in this study. A final possibility is that differences in microbial community structure that occur as a result of plant community composition occur at taxonomic levels that cannot be resolved by determining the abundance of entire microbial groups. Genetic differences in specific bacterial populations have been observed to coincide with differences in plant community composition (2, 20, 30), these differences in population structure are likely due to non-specific changes in local soil characteristics caused by the long-term deposition of plant materials (15, 21, 48).

Temporal variability in the composition of soil microbial communities may mask patterns of microbial abundance that occur in fields. Studies of whole-community phospholipid fatty acid profiles have shown that physiological changes can occur in soil microbial communities in response to seasonal cues (5, 6). Our results show that soil microbial

community structure exhibits significant temporal variability in the treatments examined (Figure 5.3 and Table 5.2). Indeed, microbial community structure changed considerably even during the seven weeks separating the sampling times in June and July 1998 (Figure 5.3 and Figure 5.4A). It is apparent from our data that microbial community composition in the soil can change dramatically at temporal scales relevant to seasonal or perhaps even meteorological events.

Correspondence analysis revealed that differences in community structure due to sampling time are largely driven by changes in the abundance of two sets of microbial groups (Figure 5.4). The Eukarya, Verrucomicrobia and Actinobacteria each achieve their highest rRNA relative abundance in May 1997 with lower abundance values seen in the other sampling times (Figure 5.3, Figure 5.4A). In contrast, the alpha Proteobacteria, beta Proteobacteria, and the Planctomycetes all have peaks of abundance in both October 1996 and June 1998 with lower abundance values seen in May 1997 and July 1998 (Figure 5.3, Figure 5.4A). Though we could not identify specific environmental parameters that influence the abundance of individual microbial groups, it is clear that microbial community composition varies over time.

An additional trend observed in these data was that the relative variation in microbial rRNA abundance in fields from different treatments

and times increased as their abundance in the community decreased (Figure 5.2B and 5.2C). In contrast, a relationship between microbial rRNA abundance and its coefficient of variation was not observed in fields from a single treatment at a single sampling time (Figure 5.2A). That the CV of microbial rRNA abundance increases across treatments and times is expected from significant MANOVA results for these effects (Table 5.2). However, more surprising is that scarce microbial groups tend to have increased variation relative to abundant groups as a result of differences they experience with treatment and time. This relationship could be a result of dispersal with the most abundant microbial groups dispersing more evenly across the landscape while scarce microbial groups have barriers to dispersal, though it seems unlikely that microbial community structure is significantly influenced by dispersal across a landscape over the time frame observed (28). Alternatively, this relationship could result if abundant microbial groups are more adaptable to variations experienced in the soil environment than are the scarce microbial groups. The adaptability of microbial groups may be related to differences in the ecological strategies of its members, e.g. generalists or specialists, or it may be related to the amount of genetic diversity present within the group allowing for adaptation through selection.

To determine whether microbial groups are homogeneously distributed with depth in the soil, we examined community structure in both 0 - 5 cm and 0 - 10 cm deep soil cores. We observed significant differences in microbial community structure due to depth. Both the Alpha and Beta Proteobacteria were significantly more abundant in deeper soil cores, while the Verrucomicrobia were significantly more abundant in surface soil (Figure 5.6). Soil depth has previously been shown to influence the community composition of nitrogen fixing bacteria in the soil (31, 42). None of the treatments (CT, HCST, HCS, NCS) influenced the depth distribution of microbial groups within the soil. It is interesting that microbial community structure varied with depth even in soils exposed to the homogenizing effects of tillage. Soil parameters such as total organic carbon, total nitrogen, and soil moisture have been observed to decrease with depth in agricultural fields with no significant change due to increases in tillage intensity (46). It is likely that variation in microbial group abundance with depth results as the organisms respond to soil characteristics that also vary with depth.

A possible explanation of the observation that microbial communities in fields abandoned from cultivation for ten years continue to resemble those in currently cultivated fields is that soil microbial communities respond to soil characteristics that require long periods of time to change from

disturbance. Long-term continuous agricultural management can cause soil carbon and nitrogen pools to be depleted by as much as 89% and 75%, respectively (27). The total carbon and nitrogen content of soil is significantly lower in the historically cultivated fields at the KBS-LTER site than in the NCS fields (14, 35). Recovery of the soil nitrogen and carbon pools to pre-agricultural levels may require decades or even centuries following abandonment (16, 27). While studies of spatial variability in soil resources indicate that the distribution of soil nutrients in post-agricultural fields can require decades to recover from the homogenizing effects of tillage (37, 38).

Relationships have been observed between microbial respiration and nitrogen content in the soil (1), and between carbon availability and microbial biomass in the soil (53). We hypothesize that changes in the composition of microbial communities are strongly influenced by soil characteristics such as soil carbon and nitrogen content that are slow to recover from the influence of cultivation. This hypothesis is supported by recent observations that soils with similar carbon and nitrogen contents have similar microbial communities as determined by both PLFA profiles (48, 52) and catabolic diversity (15). These data suggest that historical properties of the soil are more likely to influence microbial community composition than are contemporary land-use or plant community composition (48).

This research shows that soil microbial communities are heterogeneous entities with distinct components that are each capable of responding differently to environmental characteristics. Microbial community composition was shown to change with depth in the soil and with sampling time. Temporal changes in microbial community composition were observed to occur at scales that are relevant to seasonal events. In addition, it was demonstrated that cultivation has a significant impact on the composition of soil microbial communities and that the effects of cultivation on these communities are long lasting. As processes mediated by microorganisms in the soil are affected by the taxonomic composition of soil microbial communities (14, 22, 41), determining the impact that microbial community dynamics have on terrestrial ecosystems will require additional studies of microbial community composition in relation to soil characteristics and soil processes.

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CHAPTER 6

SUMMARY

1) Microbial community structure was determined in soil at the KBS-LTER site. Across all of the soil samples examined ten microbial groups were determined to account for 65.6 % of the rRNA present in KBS-LTER soils, these groups were: the Alpha Proteobacteria (24.7 %), the Actinobacteria (11.1 %), the Eukarya (9.7 %), the Planctomycetes (7.2 %), the Acidobacteria (3.5 %), the Gamma Proteobacteria (3.3 %), the Beta Proteobacteria (2.3 %), the Verrucomicrobia (1.9 %), the Archaea (1.5 %), and the Cytophagales (0.4 %).

2) The probe Cren745R was an effective tool for determining the abundance of Crenarchaeotal rRNA in soil. Crenarchaeotal rRNA composed 1.4 % of the rRNA present in the soil at the KBS-LTER site, a majority of the Archaeal rRNA present in the soil. The abundance and diversity of Crenarchaeota in soil was unaffected by the disturbance history or plant community composition of fields.

3) The probe Verr49R was an effective tool for determining the abundance of Verrucomicrobial rRNA in soil. Variation in Verrucomicrobial rRNA abundance in fields at the KBS-LTER site was correlated with variation in soil moisture content across treatments.

4) The structure of soil microbial communities as detected by rRNA probing varied with depth in the soil. Specifically, the Verrucomicrobia were more abundant at the surface of the soil (0 – 5 cm), while the Alpha and Beta Proteobacteria were both more abundant deeper in the soil (5 – 10 cm).

5) Soil microbial community differed across sampling time and across treatments. Sampling time was shown to have a significant effect on microbial community structure in soil with microbial community structure observed to change significantly over an interval of six weeks or less indicating that changes in community structure occur at time scales consistent with seasonal or meteorological changes in the environment.

6) Historical land-use was the primary field level characteristic influencing microbial community structure in the soil, indicating that microbial communities are sensitive to soil characteristics that changed by the effects of cultivation.

7) The microbial communities in fields abandoned from cultivation for nearly a decade were more similar to those in actively cultivated fields than to the communities in fields that have not been historically cultivated.

Microbial communities in the soil are dynamic, capable of significant change at temporal scales relevant to seasonal events. However, despite temporal change in the microbial community structure, the abundance of particular microbial groups responds to changes in the status of the environment that are induced by factors such as soil history, soil depth, soil moisture such that recognizable patterns of community structure exist in relation to field management. These data also indicate that cultivation significantly affects soil microbial community structure and that these effects are evident in fields abandoned from cultivation for as long as a decade.

APPENDIX A

OLIGONUCLEOTIDE PROBE HYBRIDIZATION METHOD TO DETERMINE rRNA RELATIVE ABUNDANCE IN SOIL

UTILITY OF RNA HYBRIDIZATION STUDIES

Ribosomal RNA (rRNA) molecules are highly conserved in all forms of life on Earth and as a result can be used to identify and classify microorganisms. The study of rRNA gene sequences recovered from environmental systems has vastly increased our understanding of microbial diversity. In soils, the study of PCR amplified 16S rRNA genes has revealed extraordinary diversity, including the discovery of entire groups of organisms not previously known to exist in the soil (4). PCR-based studies of microorganisms (cloning, DGGE, T-RFLP) can reveal the taxonomic composition of microbial communities, but these methods are not well suited to determine the abundance of microbial groups as the relative proportion of rRNA genes can change considerably after PCR amplification (2, 3, 8, 11).

Ribosomal RNA hybridization is a method that is useful for determining the activity and abundance of microorganisms in the environment (1, 7, 10). The sequence conservation of rRNA genes makes it possible to design oligonucleotide probes that target the rRNA molecules from specific groups of organisms. These probes can be radioactively labeled and then used to quantitatively measure the rRNA abundance of specific microbial groups in a larger microbial community. Once an oligonucleotide probe for a specific microbial group has been designed and empirically tested, rRNA abundance can be determined by radioactively labeling the probe, allowing the probe to hybridize to an RNA sample, and then measuring the amount of radioactivity associated with the RNA sample. The methods required for determining rRNA abundance through rRNA hybridization have been discussed in detail elsewhere (see Chapters 2, 3, and 4). This appendix will consider the use of rRNA hybrization for determining rRNA abundance in soil samples, the possible sources of experimental error encountered when determining rRNA abundance in soil samples, and how these sources of error can be eliminated or controlled.

The determination of rRNA abundance for a specific microbial group in a soil sample requires both the extraction of RNA from the sample and then the hybridization of a group-specific radio-labeled probe to that RNA

sample. These two steps, RNA extraction and hybridization, each have their own limitations and so each will discussed below.

ASSESSMENT OF RNA EXTRACTION TECHNIQUE

The extraction of RNA from soil has four primary considerations: cell lysis, RNA stabilization, yield, and purity. Relative to techniques depending on DNA and RNA amplification, RNA hybridization requires a large amount of nucleic acid (up to 1 μg per hybridization) and so the extraction protocol I designed needed to provide large quantities of RNA. In addition, humic acids in the soil tend to co-purify with nucleic acids. These contaminating humic acids can inhibit nucleic acid hybridization (9). The soil RNA extraction protocol I used was designed to yield RNA of sufficient quantity and quality to allow multiple RNA hybridization experiments (for details on extraction protocol see Chapter 3).

Cell lysis

Obviously cell lysis effects RNA yield, but more importantly the cell lysis procedure must be designed to minimize the possibility of differential cell lysis. If certain microbial groups are less susceptible to lysis than others and the lysis technique used is not sufficiently rigorous then certain microbial groups may be consistently underrepresented in extracted RNA.

To ensure that the RNA present in extracts is representative of the indigenous microbial community, a high cell disruption efficiency must be achieved. Methods that rely of mechanical lysis through bead mill homogenization provide higher rates of cell disruption than all other methods tested (5). The extraction protocol I designed relies on bead mill homoginization in the presence of a strong chaotrophic agent (Chapter 3). By making microscopic cell counts of DTAF-stained cells (Chapter 3) I was able to determine the total number of cells present before and after homogenization. I observed that my lysis protocol disrupted 97.3 % ± 1.6 % (s.e.) of cells in the soil. The observed lysis efficiency is consistent with previous measurements of lysis efficiency obtained for bead mill homogenization of soil samples (5). Thus while the lysis protocol employed does not provide complete cell lysis, the disruption rate is high enough to ensure that any bias caused by differential cell lysis will be kept below a few percent of the total rRNA abundance.

RNA degradation

RNA is very labile and the isolation of RNA requires special techniques to prevent RNA degredation (6). In addition, extraction artifacts could result if rRNA from different microbial groups degrades at different rates. Though there are no studies indicating that the rRNA from different

microbial groups may be more or less susceptible to degradation in cell extracts, the possibility for differential degradation exists. The use of guanidium isothiocyanate in the homogenization buffer along with the use of techniques that eliminate the presence and activity of RNA degrading enzymes prevents RNA degradation and therefore prevent any possible bias due to differential RNA degradation (6).

RNA yield and purity

The removal of humic acids from RNA extracts required extensive purification steps including nucleic acid precipitation with polyethelene glycol and purification on both hydroxyapetite and sephadex G-75 columns. Assessment of humic acid contamination of RNA extracts requires measuring sample light absorbance at 230 nm and 260 nm. As humics absorb light strongly at 230 nm and nucleic acid absorbs light at 260 nm, increasing A_{260}/A_{230} ratios tend to indicate increasing sample purity (12). The average 260/230 ratio for RNA extracted from pure cultures was 2.12 ± 0.13 while the average value obtained for purified soil RNA extracts was 1.8 ± 0.09. The 260/230 values obtained for soil RNA extracts indicates that these RNA samples are not as clean as the RNA extracted from pure cultures, though these A_{260}/A_{230} values are higher than those values reported for other soil nucleic acid extracts (12). In addition, the purity of RNA extracts varied

between soil samples from different agricultural treatments as the 260/230 ratio for RNA extracts from the conventionally tilled fields (1.89 ± 0.05) was consistently higher than that of RNA extracts from the never cultivated fields (1.71 ± 0.04) (Chapter 3).

The extensive purification steps required for removing humic acids from soil RNA extracts resulted in a low efficiency of RNA recovery. The total extraction efficiency was determined by amending soil samples with *E. coli* rRNA prior to RNA extraction. The total efficiency of the extraction was determined by using the orcinol reaction to measure the average RNA yield from amended soil samples and from identical unamended soil samples (Chapter 3). The determined efficiency for the RNA extraction protocol was 19 % ± 5.3 %. The amount of RNA recovered per gram of soil was higher on average in soil samples from never cultivated fields (3.00 ± 0.62) than from conventionally tilled fields (0.96 ± 0.06) (Chapter 3).

ASSESSMENT OF RNA HYBRIDIZATION TECHNIQUE

The A_{260}/A_{230} values of soil RNA extracts obtained by using my extraction method are high relative to existing soil RNA extraction techniques, but are still low compared to the A_{260}/A_{230} values of RNA samples extracted from pure cultures. This observation indicates that the soil RNA extracts may contain some residual humic acid contamination. Since

humic acids have been observed to inhibit hybridization experiments (9), and I observed that the purity of RNA extracts differs systematically among the fields that I sampled, there is the potential for a systematic bias in hybridization experiments caused by differing amounts of hybridization inhibition by humic compounds.

Humic acid inhibition of hybridization

An experiment was designed to assess whether or not impurities in my RNA extracts influence the results obtained from RNA hybridization experiments. The experiment consisted of preparing a dilution series of soil RNA extracts and spiking each diluted sample with 60 ng of RNA from Geobacter GS-15, an organism not suspected to be abundant in soil. Soil RNA extracts that had or had not been spiked with the indicator RNA were denatured and immobilized on a nylon membrane along with Gb RNA samples used as a positive control. For this experiment the probe Geo880 which hybridizes exclusively to Geobacter 16S rRNA was used to determine the abundance of Gb RNA (Bonnie Bratina personal communication). The probe was radio-labeled, hybridization was carried out, and the radioactivity associated with each RNA sample was determined as previously described. The amount of radioactive probe hybridized to each RNA sample was determined by counting radioactive decay events. The Counts Per Minute (CPM) for a 60

ng sample of Gb RNA was 60.9. Measured CPM for soil RNA extracts not receiving Gb RNA were at or below background levels (1 - 3 CPM) (Figure A.1). If compounds that inhibit hybridization are absent from soil RNA extracts than regardless of soil extract concentration the radioactive signal associated with all samples receiving a 60 ng spike of Gb RNA should be approximately 60.9 CPM. However, the soil RNA extracts tested clearly inhibit RNA hybridization as increasing concentrations of soil extract cause a logrithmic loss of radioactive signal (Figure A.1).

Possible inhibition mechanisms

Though it is extremely difficult to completely remove humic acids from soil nucleic acid samples it may be possible to overcome the effects of humic compounds on RNA hybridization experiments if we can understand these effects. Humic acids are a heterogeneous group of carbon compounds that can be very large and contain numerous negatively charged side chains (9). There are two likely explanations for the inhibition of hybridization signals caused by humic acids. Firstly, during the immobilization of RNA on a membrane it is possible that humic acids may overlay RNA molecules and physically occlude probe-binding sites. Secondly, humic acids may prevent RNA from binding to membranes. The membranes used in hybridization experiments are positively charged in order to bind negatively charged

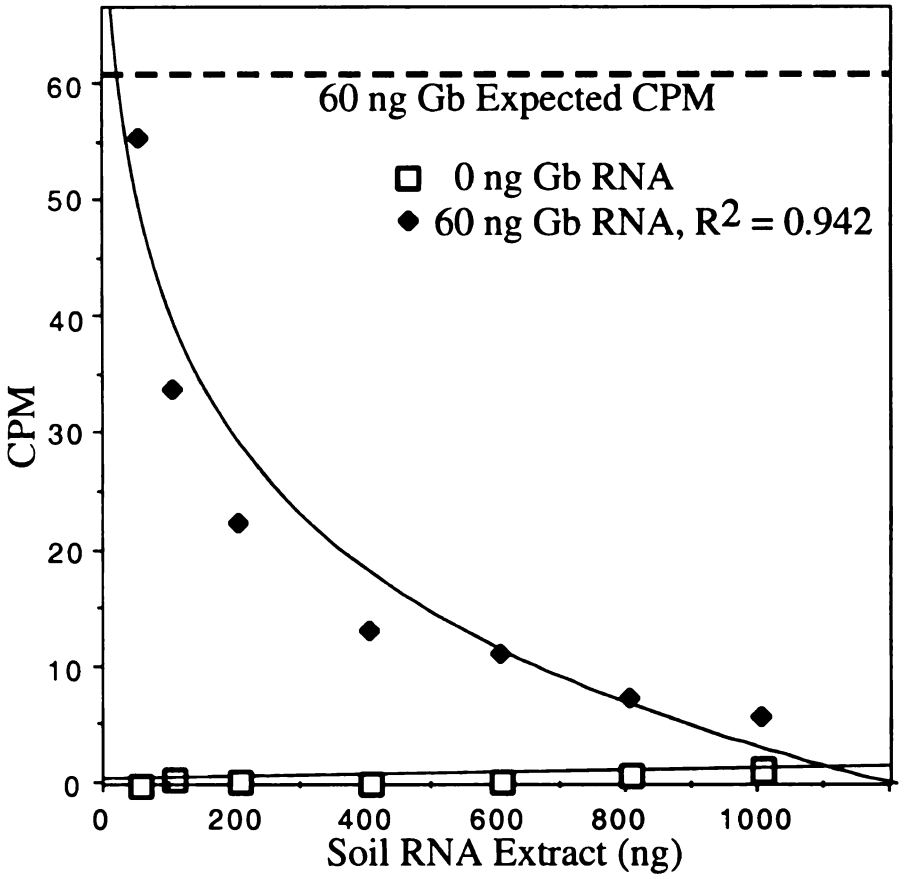

Figure A.1. A constant amount of Gb RNA (60 ng) was added to increasing amounts of soil RNA extract. After hybridization with the radio-labeled probe Geo880 the hybridization signal (as measured by CPM) of soil RNA extracts decreased logrithmically with increasing amounts of soil extract. The hybridization signals of soil RNA extracts devoid of Gb RNA addition were not distinguishable from background levels of radiation on the hybridization membrane (1 – 3 CPM), while the hybridization signal of 60 ng Gb RNA absent soil RNA extract was 60.9 CPM.

nucleic acid molecules. Since humic compounds are also negatively charged these compounds may saturate the charge on hybridization membranes and thereby prevent nucleic acid molecules from being bound. Any nucleic acid molecules that fail to bind to the hybridization membrane cannot be detected by subsequent analysis. Prior to any further experimental analysis the second model seems the more likely explanation as the inhibition of hybridization by a physical occlusion model would be expected to provide a linear decrease in hybridization signal with increasing humic acid concentration. The competition for membrane occupancy between RNA and humic acids predicted in the second model would be expected to obey second order kinetics resulting in a logrithmic decline of hybridization signal with increasing soil extract concentration as observed in my initial experiment (Figure A.1).

Experimental analyses

An experiment was designed to determine which of the above models is correct. Once again different concentrations of soil RNA extract were spiked with a constant amount of an indicator and immobilized on a nylon hybridization membrane. However, in this experiment the indicator was not Gb RNA but rather radio-labeled Geo880 probe. The probe is composed of single stranded DNA and like RNA binds to the membrane on the basis of

charge. By using radio-labeled probe instead of RNA the experiment tests directly for humic acid effects on nucleic acid binding to membranes. The amount of radioactive probe bound to membranes is then measured by placing the membrane in scintillation fluid (Bio-Safe II, Research Products International Corp.) and measuring radioactive decay events in a liquid scintillation counter (Minaxiβ Tri-Carb 4000 Series, Packard). Liquid scintillation counting of 2.5 ng of radio-labeled probe immobilized on a membrane recorded 4846 CPM. When this exact quantity of probe was added to membranes along with various concentrations of soil RNA extract the radioactive signal was observed to decrease logrithmically in response to increasing soil RNA concentrations (Figure A.2). The logrithmic decline of probe retention to hybridization membranes, independent of probe-RNA hybridization, provides strong evidence that the inhibition of hybridization by humic acids is caused by competition for charge on hybridization membranes. In addition, when increasing amounts of indicator nucleic acids are added to soil RNA extracts the relative proportion of signal inhibition increases (Figure A.3). This result indicates that humic acids preferentially occupy charged sites on hybridization membranes. There are a limited number of binding sites available on a membrane and once these sites have been occupied by humic acids any increase in the amount of nucleic acids in a sample will result in increased proportions of sample loss.

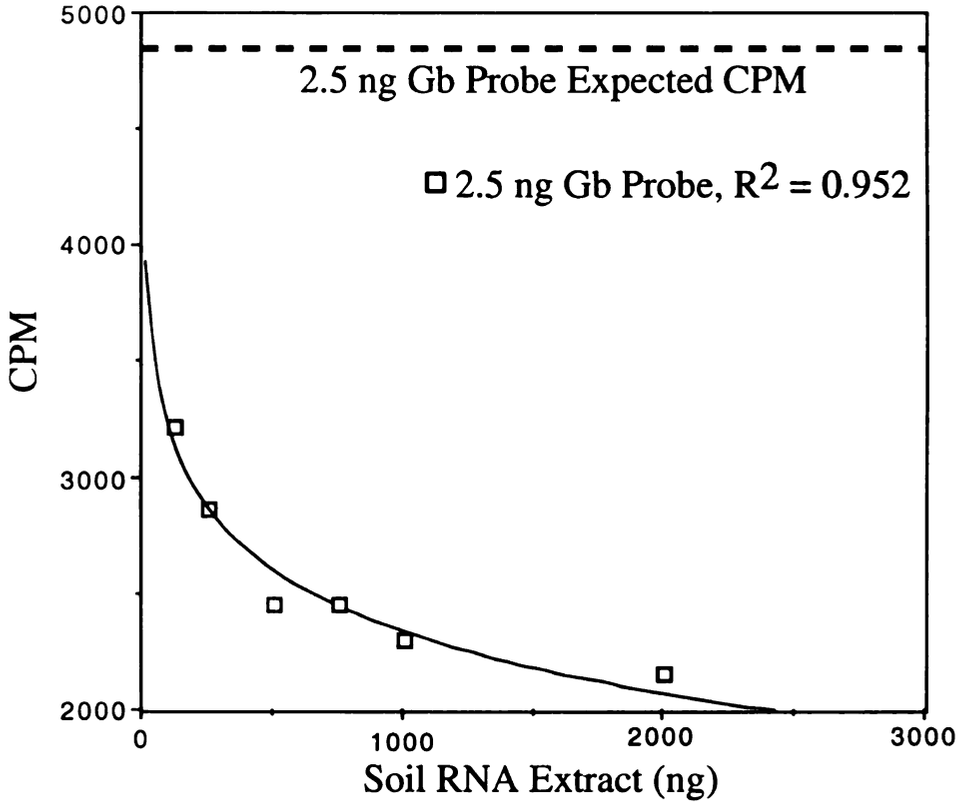

Figure A.2. A constant amount of radio-labeled oligonucleotide probe Geo880 (2.5 ng) was added to hybridization membranes along with varying amounts of soil RNA extract. Hybridization was not carried out and the amount of probe retained by the hybridization membranes was measured as the CPM associated with the membrane. Increasing the amount of soil RNA extract caused a logrithmic decline in the amount of Geo880 probe bound to the hybridization membrane.

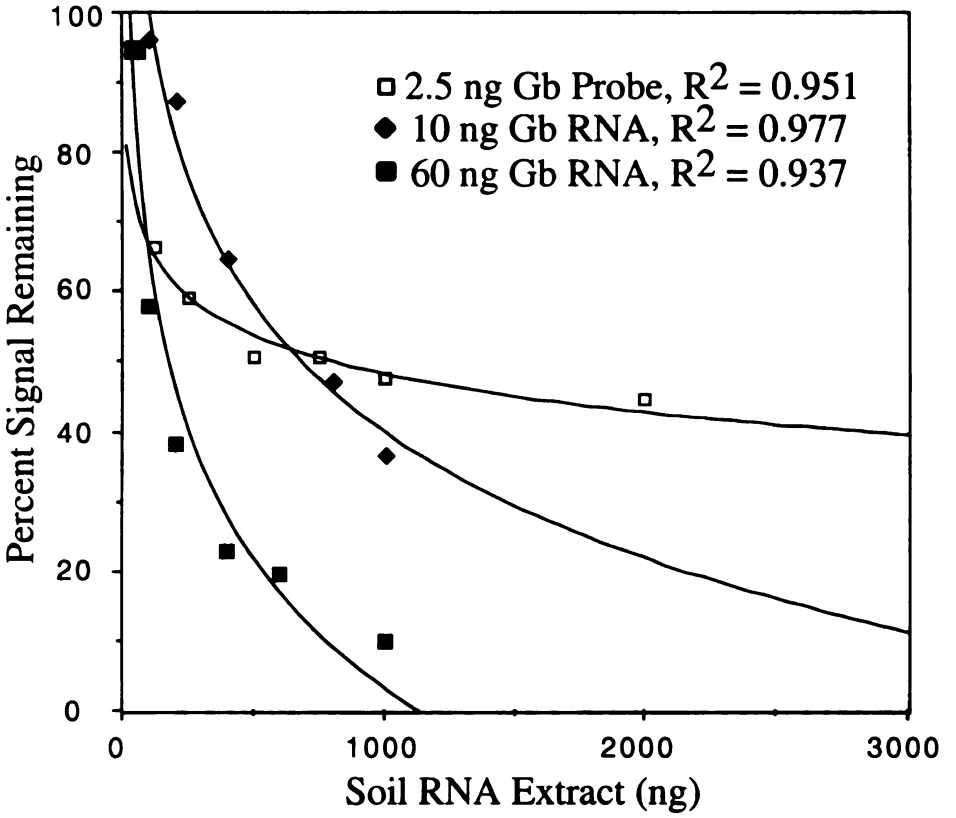

Figure A.3. Data from both hybridization experiments and probe retention assays are plotted as percent signal remaining (measured CPM / expected CPM * 100). This data reveals that the amount of indicator nucleic acid added to a hybridization membrane will influence the amount of signal loss even in a constant background of inhibitory compounds from soil RNA extracts.

Potential consequences of humic acid inhibition

I have documented that the amount of RNA and its purity can vary in different soil samples and that soil management can influence such variation. Further, I have shown that inhibition of RNA hybridization is a function of both the purity of RNA extracts and the total amount of RNA present. As a consequence, if not accounted for humic acids can cause systematic errors in the measurement of rRNA abundance. Fortunately, it is possible to design hybridization experiments in a controlled manner that accounts for these potential sources of error.

CALCULATION OF RRNA RELATIVE ABUNDANCE

The calculation of rRNA relative abundance from RNA hybridization data involved four levels of control that account completely for any possible problems caused by impurities in soil RNA extracts and variability in soil sample RNA abundance. To control for differences in RNA quantity and quality all determinations of rRNA relative abundance are based on the ratio of the results from two hybridization experiments carried out with two distinct probes. While several different rRNA-targeted probes were used to determine the abundance of different microbial groups, the hybridization

results from each of these probes was related to the hybridization signal obtained with a probe that binds to nearly all known 16S rRNA molecules (Univ1390). In all hybridization experiments, every RNA sample was present in exactly the same set of concentrations on each membrane. As a result the same amount of nucleic acids and humic acids will be present in each corresponding sample on the blots used for specific probe hybridization and on the blots used for universal probe hybridization. Thus the amount of inhibition for a given sample will be the same on both blots and dividing the hybridization signal for the sample on a specific blot by its signal on a universal blot will cancel out any inhibition effects giving an accurate measure of rRNA relative abundance.

A series of dilutions of each RNA sample is placed on every hybridization membrane to provide a second level of control. If hybridization signals are influenced by the presence of humic acids then we would expect our results to vary with changes in the amount of soil RNA extract present. Since there was very little variation in the ratio of specific probe to universal probe hybridization signal over a five-fold range of sample dilution, I can conclude that the ratio is effectively controlling for any sample impurities.

RNA molecules extracted from pure cultures of microorganisms are placed on every hybridization membrane to provide the final two levels of

control. These RNA samples are from both organisms that are targeted by the rRNA probe being used and from several microorganisms that are closely related but outside of the target group. The positive controls are used to account for variations in the labeling efficiency of different oligonucleotide probes, while the negative controls were used to account for any hybridization signal that results from nonspecific interactions. Calculations of relative abundance are made by taking the ratio of signal intensities obtained for specific and universal probe binding to an RNA sample as $R = \Sigma^{n}_{i=1}[G_i(U_i)^{-1}]n^{-1}$, where G_i and U_i represent, respectively, the corresponding signal intensities obtained for group specific and universal probe binding to each aliquot representing the sample, and n equals the total number of aliquots representing the RNA sample. The value R was then calculated for each soil RNA sample (R_s), and a mean value of R was determined for all positive (R_p) and negative (R_n) controls present on each membrane. The relative abundance (expressed as a precentage) of rRNA from a specific microbial group was then defined as $(R_s - R_n)(R_p - R_n)^{-1}$ x 100.

METHOD REPRODUCIBILITY

Samples were taken from different treatments at the Kellogg Biological Station Long Term Ecological Research site in May 1997, as

previously described (Chapter 3). These samples were archived at –80°C. In March 1997 RNA was extracted from soil samples from three field replicates of both the conventionally tilled and never tilled fields. These RNA samples were used in hybridization experiments to determine the rRNA relative abundance of the alpha proteobacteria and the cytophaga-flavobacteria group (Table A.1). The purpose of these experiments was to test the reproducibility of rRNA hybridization experiments on RNA from soil samples. In April of 1999 RNA was once again extracted from these archived soil samples as part of a larger experiment to assess microbial community structure (Chapter 3). There are no significant differences (by t-test or Kruskal Wallis test) between the rRNA relative abundance values obtained in 1997 and those obtained for the same soil samples in 1999 (Table A.1).

It should be noted the system used to measure the amount of radio-labeled probe bound to RNA samples changed between 1997 and 1999. The measurements made in 1997 were performed using a radioanalytic imaging system (AMBIS, Inc.) that directly measures radioactive decay events. The measurements in 1999 were performed using a phosphorimaging system (Storm 860, Molecular Dynamics) that requires radioactive signals to be stored in phosphor storage plates and then measures the amount of phosphorescent light given off when the storage plate is excited by laser

Table A.1. Relative abundance of rRNA for two microbial groups in soil samples from May 1997 as determined for identical samples processed in both 1997 and 1999.

	1997 Extracts	1999 Extracts
Alpha Proteobacteria		
Conventionally Tilled	19.5 ± 2.7	18.8 ± 3.9
Never Cultivated	NA	11.4 ± 0.8
Cytophaga-Falvobacteria		
Conventionally Tilled	0.8 ± 0.4	0.7 ± 0.9
Never Cultivated	0.0 ± 0.0	0.0 ± 0.0

radiation. Despite the use of different RNA extracts, the use of independently prepared batches of probe, the preparation and hybridization of RNA samples at different times, and fundamental differences in the techniques used to measure the quantity of bound radio-labeled probe, the measurements of rRNA relative abundance remained consistent. The similarity between independently obtained values for rRNA relative abundance confirms the reproducibility of this hybridization method.

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APPENDIX B

DATA TABLES

APPENDIX B OVERVIEW

This appendix includes tables of data for all of the rRNA abundance values that I determined for microbial groups at the KBS-LTER as part of this dissertation. All percent rRNA abundance values are calculated relative to the probe Univ1390. Also included is a table that cross-references the treatment designations that I have used against their official designations used at the KBS-LTER site.

Table B.1 Cross reference for treatment names used in this dissertation and KBS-LTER treatment designations.

Treatment Designation	KBS-LTER Designation
CT	T1
NT	T2
NI	T4
PL	T5
AF	T6
HCS	T7
HCST	T7t
NCS	T8
LS	SF2

Table B.2 Percent rRNA abundance of Alpha Proteobacteria.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	29.7	29.7	8.2
1	2	-	19.7	45.8	45.8	30.2
1	3	26.5	14.6	34.7	34.7	16.7
1	4	31.0	22.2	42.8	42.8	7.1
1	5	-	-	31.9	31.9	18.8
2	2	27.7	-	-	-	-
2	3	31.9	-	-	-	-
2	4	32.8	-	-	-	-
4	2	27.5	-	-	-	-
4	3	26.9	-	-	-	-
4	4	24.0	-	-	-	-
5	1	-	-	-	-	12.1
5	2	-	-	-	-	12.8
5	3	-	-	-	-	4.6
5	4	-	-	-	-	13.0
5	5	-	-	-	-	9.2
6	2	27.8	-	-	-	-
6	3	29.8	-	-	-	-
6	4	21.3	-	-	-	-
7	1	-	-	30.1	33.9	1.3
7	2	27.7	24.1	31.7	35.0	8.1
7	3	-	16.0	37.4	34.2	6.7
7	4	25.4	15.9	33.2	27.2	6.6
7	5	-	-	-	33.2	9.3
7t	1	-	-	20.4	27.6	4.9
7t	2	-	-	40.0	48.6	2.1
7t	3	-	-	23.8	28.1	4.1
7t	4	-	-	21.7	20.0	2.8
7t	5	-	-	40.6	45.4	1.9
8	1	-	-	23.3	40.7	14.9
8	2	42.3	11.6	47.0	37.5	10.7
8	3	36.4	12.1	38.1	37.9	16.3
8	4	37.2	10.6	34.0	47.1	14.0
SF1	McKav	-	-	-	-	23.1
SF2	Upper	-	-	-	-	34.0
SF2	Lower 1	-	-	-	-	43.1
SF2	Lower 2	-	-	-	-	31.9
SF2	Lower 3	-	-	-	-	38.2
SF2	Lower 4	-	-	-	-	42.1
SF3	Pond lab orchard		-	-	-	32.5
SF3	Field Kav	-	-	-	-	39.2
Bailey	-	-	-	-	-	23.2

Table B.3 Percent rRNA abundance of Beta Proteobacteria.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	2.3	1.9	1.1
1	2	-	3.1	2.4	5.4	0.6
1	3	4.7	3.8	4.6	2.7	3.4
1	4	7.7	2.7	1.7	5.0	1.0
1	5	-	-	0.8	3.8	0.5
2	2	2.8	-	-	-	-
2	3	4.6	-	-	-	-
2	4	7.8	-	-	-	-
4	2	3.9	-	-	-	-
4	3	5.6	-	-	-	-
4	4	3.5	-	-	-	-
5	1	-	-	-	-	1.3
5	2	-	-	-	-	0.6
5	3	-	-	-	-	0.0
5	4	-	-	-	-	0.9
5	5	-	-	-	-	0.0
6	2	3.1	-	-	-	-
6	3	4.7	-	-	-	-
6	4	3.3	-	-	-	-
7	1	-	-	1.9	3.3	0.0
7	2	3.3	3.1	1.6	4.7	0.8
7	3	-	2.7	0.6	3.1	0.7
7	4	7.1	1.3	1.7	3.6	0.5
7	5	-	-	5.3	5.9	0.8
7t	1	-	-	0.6	3.4	0.0
7t	2	-	-	1.6	6.5	0.0
7t	3	-	-	1.1	1.4	0.0
7t	4	-	-	0.1	0.4	0.0
7t	5	-	-	0.2	4.8	0.0
8	1	-	-	0.3	2.0	1.0
8	2	7.6	0.3	2.7	2.5	0.4
8	3	9.4	0.3	1.3	4.5	0.5
8	4	8.5	0.1	1.1	7.2	0.6
SF1	McKav	-	-	-	-	0.8
SF2	Upper	-	-	-	-	1.1
SF2	Lower 1	-	-	-	-	0.8
SF2	Lower 2	-	-	-	-	1.7
SF2	Lower 3	-	-	-	-	1.2
SF2	Lower 4	-	-	-	-	1.1
SF3	Pond lab		-	-	-	2.1
SF3	Field Kav	-	-	-	-	2.7
Bailey	-	-	-	-	-	2.5

Table B.4 Percent rRNA abundance of Gamma Proteobacteria.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	-	-	-
1	2	-	-	-	-	-
1	3	3.8	-	-	-	-
1	4	3.7	-	-	-	-
1	5	-	-	-	-	-
2	2	2.6	-	-	-	-
2	3	2.9	-	-	-	-
2	4	4.4	-	-	-	-
4	2	3.3	-	-	-	-
4	3	3.6	-	-	-	-
4	4	3.1	-	-	-	-
5	1	-	-	-	-	-
5	2	-	-	-	-	-
5	3	-	-	-	-	-
5	4	-	-	-	-	-
5	5	-	-	-	-	-
6	2	3.3	-	-	-	-
6	3	3.3	-	-	-	-
6	4	2.3	-	-	-	-
7	1	-	-	-	-	-
7	2	3.1	-	-	-	-
7	3	-	-	-	-	-
7	4	3.6	-	-	-	-
7	5	-	-	-	-	-
7t	1	-	-	-	-	-
7t	2	-	-	-	-	-
7t	3	-	-	-	-	-
7t	4	-	-	-	-	-
7t	5	-	-	-	-	-
8	1	-	-	-	-	-
8	2	4.4	-	-	-	-
8	3	3.5	-	-	-	-
8	4	2.9	-	-	-	-
SF1	McKav	-	-	-	-	-
SF2	Upper	-	-	-	-	-
SF2	Lower 1	-	-	-	-	-
SF2	Lower 2	-	-	-	-	-
SF2	Lower 3	-	-	-	-	-
SF2	Lower 4	-	-	-	-	-
SF3	Pond lab		-	-	-	-
SF3	Field Kav	-	-	-	-	-
Bailey	-	-	-	-	-	-

Table B.5 Percent rRNA abundance of Acidobacteria.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	1.9	2.6	0.8
1	2	0.0	10.0	2.7	7.2	2.9
1	3	0.0	3.5	3.7	7.8	17.7
1	4	0.1	7.0	6.1	5.4	4.3
1	5	-	-	6.7	9.2	6.3
2	2	-	-	-	-	-
2	3	-	-	-	-	-
2	4	-	-	-	-	-
4	2	-	-	-	-	-
4	3	-	-	-	-	-
4	4	-	-	-	-	-
5	1	-	-	-	-	0.9
5	2	-	-	-	-	2.8
5	3	-	-	-	-	0.0
5	4	-	-	-	-	0.0
5	5	-	-	-	-	0.0
6	2	-	-	-	-	-
6	3	-	-	-	-	-
6	4	-	-	-	-	-
7	1	-	-	0.0	1.5	0.0
7	2	2.9	5.0	2.6	2.9	2.0
7	3	0.1	2.0	0.6	0.0	3.4
7	4	0.0	0.9	3.5	2.8	1.8
7	5	-	-	8.0	0.0	3.1
7t	1	-	-	6.1	14.0	0.0
7t	2	-	-	4.4	5.8	0.0
7t	3	-	-	38.3	7.1	9.3
7t	4	-	-	9.6	7.9	0.0
7t	5	-	-	0.0	6.9	0.0
8	1	-	-	0.0	0.0	3.1
8	2	0.0	0.0	2.6	0.0	0.8
8	3	0.0	0.0	0.0	0.0	7.2
8	4	0.0	0.0	0.0	0.0	0.0
SF1	McKav	-	-	-	-	2.2
SF2	Upper	-	-	-	-	4.9
SF2	Lower 1	-	-	-	-	4.1
SF2	Lower 2	-	-	-	-	1.1
SF2	Lower 3	-	-	-	-	3.8
SF2	Lower 4	-	-	-	-	3.8
SF3	Pond lab		-	-	-	2.7
SF3	Field Kav	-	-	-	-	8.6
Bailey	-	-	-	-	-	1.8

Table B.6 Percent rRNA abundance of Cytophaga-Flavobacteria.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	0.0	0.5	0.0
1	2	-	0.4	0.0	1.4	0.0
1	3	-	1.8	0.0	1.4	3.6
1	4	-	0.0	0.0	1.7	0.0
1	5	-	-	0.0	3.3	0.1
2	2	-	-	-	-	-
2	3	-	-	-	-	-
2	4	-	-	-	-	-
4	2	-	-	-	-	-
4	3	-	-	-	-	-
4	4	-	-	-	-	-
5	1	-	-	-	-	0.0
5	2	-	-	-	-	0.0
5	3	-	-	-	-	0.0
5	4	-	-	-	-	0.0
5	5	-	-	-	-	0.0
6	2	-	-	-	-	-
6	3	-	-	-	-	-
6	4	-	-	-	-	-
7	1	-	-	0.0	0.2	0.0
7	2	-	0.0	0.0	0.0	0.0
7	3	-	0.0	0.0	0.0	0.0
7	4	-	0.0	0.0	0.1	0.0
7	5	-	-	0.0	0.0	0.0
7t	1	-	-	0.0	3.6	0.0
7t	2	-	-	0.5	5.1	0.0
7t	3	-	-	2.6	1.0	0.0
7t	4	-	-	0.1	0.0	0.0
7t	5	-	-	0.0	0.6	0.0
8	1	-	-	0.0	0.0	0.0
8	2	-	0.0	0.3	0.0	0.0
8	3	-	0.0	0.0	0.0	0.4
8	4	-	0.0	0.0	0.0	0.0
SF1	McKav	-	-	-	-	0.0
SF2	Upper	-	-	-	-	0.0
SF2	Lower 1	-	-	-	-	0.0
SF2	Lower 2	-	-	-	-	0.0
SF2	Lower 3	-	-	-	-	0.0
SF2	Lower 4	-	-	-	-	0.0
SF3	Pond lab		-	-	-	0.0
SF3	Field Kav	-	-	-	-	0.0
Bailey	-	-	-	-	-	0.0

Table B.7 Percent rRNA abundance of Actinobacteria.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	23.5	9.3	4.1
1	2	-	19.0	21.3	19.7	3.8
1	3	5.7	22.8	30.3	10.3	8.4
1	4	6.0	23.3	22.7	22.2	6.2
1	5	-	-	6.6	10.6	3.8
2	2	3.9	-	-	-	-
2	3	5.6	-	-	-	-
2	4	22.3	-	-	-	-
4	2	5.3	-	-	-	-
4	3	5.7	-	-	-	-
4	4	9.3	-	-	-	-
5	1	-	-	-	-	6.6
5	2	-	-	-	-	6.5
5	3	-	-	-	-	4.7
5	4	-	-	-	-	8.3
5	5	-	-	-	-	7.7
6	2	5.3	-	-	-	-
6	3	6.1	-	-	-	-
6	4	9.2	-	-	-	-
7	1	-	-	8.0	13.5	3.8
7	2	5.2	39.6	10.3	17.1	15.2
7	3	-	20.5	5.3	13.1	9.7
7	4	12.7	19.1	6.7	11.4	9.1
7	5	-	-	13.2	9.5	10.2
7t	1	-	-	2.7	7.3	4.6
7t	2	-	-	10.5	32.1	8.0
7t	3	-	-	4.3	5.6	2.7
7t	4	-	-	2.3	2.2	1.8
7t	5	-	-	4.4	10.6	5.6
8	1	-	-	4.3	7.7	12.7
8	2	15.3	15.0	5.8	6.0	8.9
8	3	17.0	11.5	5.6	10.5	10.3
8	4	18.7	9.7	8.6	18.2	6.4
SF1	McKav	-	-	-	-	7.7
SF2	Upper	-	-	-	-	9.9
SF2	Lower 1	-	-	-	-	11.5
SF2	Lower 2	-	-	-	-	12.0
SF2	Lower 3	-	-	-	-	8.4
SF2	Lower 4	-	-	-	-	10.2
SF3	Pond lab		-	-	-	9.6
SF3	Field Kav	-	-	-	-	14.2
Bailey	-	-	-	-	-	13.6

Table B.8 Percent rRNA abundance of Planctomycetes.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	4.7	3.6	2.4
1	2	7.4	6.6	5.1	5.9	5.2
1	3	8.4	4.4	6.8	5.1	13.4
1	4	7.6	5.9	4.1	3.8	3.5
1	5	-	-	4.2	6.3	6.1
2	2	-	-	-	-	-
2	3	-	-	-	-	-
2	4	-	-	-	-	-
4	2	-	-	-	-	-
4	3	-	-	-	-	-
4	4	-	-	-	-	-
5	1	-	-	-	-	2.3
5	2	-	-	-	-	2.6
5	3	-	-	-	-	0.4
5	4	-	-	-	-	4.7
5	5	-	-	-	-	4.1
6	2	-	-	-	-	-
6	3	-	-	-	-	-
6	4	-	-	-	-	-
7	1	-	-	8.5	8.7	0.0
7	2	11.6	7.7	9.2	7.4	3.4
7	3	8.4	4.3	9.7	6.9	2.8
7	4	7.0	3.9	9.2	6.1	2.3
7	5	-	-	21.2	6.9	3.2
7t	1	-	-	5.1	7.6	1.2
7t	2	-	-	7.3	5.5	0.6
7t	3	-	-	16.4	4.9	7.2
7t	4	-	-	10.0	7.0	0.9
7t	5	-	-	12.5	9.5	0.0
8	1	-	-	13.5	9.8	8.4
8	2	10.7	4.6	13.1	6.2	4.4
8	3	11.9	4.2	7.7	11.4	7.5
8	4	10.0	4.1	3.9	17.2	3.5
SF1	McKav	-	-	-	-	7.7
SF2	Upper	-	-	-	-	13.2
SF2	Lower 1	-	-	-	-	10.0
SF2	Lower 2	-	-	-	-	11.9
SF2	Lower 3	-	-	-	-	15.2
SF2	Lower 4	-	-	-	-	13.4
SF3	Pond lab		-	-	-	10.7
SF3	Field Kav	-	-	-	-	11.9
Bailey	-	-	-	-	-	6.3

Table B.9 Percent rRNA abundance of Verrucomicrobia.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	3.3	0.3	0.0
1	2	0.0	4.5	1.9	0.9	0.0
1	3	0.0	2.1	2.7	0.9	2.3
1	4	0.0	2.8	4.4	1.0	0.1
1	5	-	-	9.8	1.4	0.6
2	2	-	-	-	-	-
2	3	-	-	-	-	-
2	4	-	-	-	-	-
4	2	-	-	-	-	-
4	3	-	-	-	-	-
4	4	-	-	-	-	-
5	1	-	-	-	-	0.5
5	2	-	-	-	-	0.1
5	3	-	-	-	-	0.0
5	4	-	-	-	-	0.0
5	5	-	-	-	-	1.2
6	2	-	-	-	-	-
6	3	-	-	-	-	-
6	4	-	-	-	-	-
7	1	-	-	4.0	2.4	0.2
7	2	3.3	4.2	3.1	1.5	1.4
7	3	0.8	2.6	3.8	2.2	1.1
7	4	0.0	1.5	3.2	2.2	0.7
7	5	-	-	6.1	0.8	1.4
7t	1	-	-	1.2	1.3	0.0
7t	2	-	-	1.9	2.3	0.4
7t	3	-	-	7.4	1.9	1.6
7t	4	-	-	1.8	2.2	0.2
7t	5	-	-	0.9	1.4	0.5
8	1	-	-	3.0	1.3	2.5
8	2	0.4	1.8	5.1	1.6	2.7
8	3	0.9	1.5	6.8	1.6	2.5
8	4	1.1	2.9	3.4	3.4	1.9
SF1	McKay	-	-	-	-	0.5
SF2	Upper	-	-	-	-	0.6
SF2	Lower 1	-	-	-	-	1.2
SF2	Lower 2	-	-	-	-	1.6
SF2	Lower 3	-	-	-	-	2.3
SF2	Lower 4	-	-	-	-	2.1
SF3	Pond lab		-	-	-	1.5
SF3	Field Kay	-	-	-	-	1.9
Bailey	-	-	-	-	-	0.8

Table B.10 Percent rRNA abundance of Eukarya.

Treatment	Replicate	10/3/96	5/23/97	6/6/98 5 cm	6/6/98 10 cm	7/28/98
1	1	-	-	5.2	5.9	6.7
1	2	-	10.9	2.8	4.9	4.1
1	3	12.6	11.0	3.1	6.9	10.6
1	4	20.1	10.6	2.7	4.1	7.9
1	5	-	-	5.3	10.1	7.4
2	2	24.8	-	-	-	-
2	3	7.1	-	-	-	-
2	4	5.5	-	-	-	-
4	2	19.9	-	-	-	-
4	3	10.2	-	-	-	-
4	4	15.1	-	-	-	-
5	1	-	-	-	-	9.3
5	2	-	-	-	-	11.3
5	3	-	-	-	-	7.7
5	4	-	-	-	-	4.3
5	5	-	-	-	-	5.2
6	2	16.0	-	-	-	-
6	3	9.8	-	-	-	-
6	4	6.3	-	-	-	-
7	1	-	-	6.0	1.9	9.5
7	2	23.0	16.8	4.2	2.9	9.0
7	3	4.6	11.1	4.5	2.6	6.4
7	4	0.0	9.4	5.8	6.0	9.6
7	5	-	-	8.5	4.5	9.1
7t	1	-	-	5.8	9.7	5.6
7t	2	-	-	4.2	7.2	6.7
7t	3	-	-	16.1	8.2	8.4
7t	4	-	-	6.5	6.4	5.6
7t	5	-	-	7.2	3.9	5.8
8	1	-	-	26.2	19.7	16.2
8	2	14.5	28.0	13.1	12.6	13.5
8	3	19.7	29.3	20.3	21.9	14.4
8	4	13.9	20.7	12.4	12.4	9.3
SF1	McKav	-	-	-	-	14.3
SF2	Upper	-	-	-	-	10.6
SF2	Lower 1	-	-	-	-	14.4
SF2	Lower 2	-	-	-	-	9.7
SF2	Lower 3	-	-	-	-	11.4
SF2	Lower 4	-	-	-	-	8.1
SF3	Pond lab		-	-	-	9.8
SF3	Field Kav	-	-	-	-	8.6
Bailey	-	-	-	-	-	1.6