16s ribosomal RNA analysis to detect soil microbial community shifts in response to changing agricultural practices from row crops to short rotation woody crops

Julia O'Neal Stair

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I am submitting herewith a thesis written by Julia O'Neal Stair entitled "16s ribosomal RNA analysis to detect soil microbial community shifts in response to changing agricultural practices from row crops to short rotation woody crops." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Gary S. Sayler, Major Professor

We have read this thesis and recommend its acceptance:

Gary Stacey, Robert Moore

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
To the Graduate Council:

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And recommend its acceptance:

Robert N. Moore

Accepted for the Council:

[Signature]
Associate Vice Chancellor and
Dean of The Graduate School
16S Ribosomal RNA Analysis to Detect Soil Microbial Community Shifts in Response to Changing Agricultural Practices from Row Crops to Short Rotation Woody Crops

A Thesis Presented
For the
Master of Science Degree
The University of Tennessee, Knoxville

Julia O'Neal Stair
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ABSTRACT

To monitoring soil microbial community dynamics at an Ames Plantation study in which agricultural management practices were changed from growing row crops to short rotation woody biomass crops, 16s rRNA was directly extracted from soil and without further amplification the RNA was blotted onto a membrane and hybridized with a hierarchical array of rRNA probes. The RNA was quantified by comparison to standard concentrations of RNA. The relative proportions of RNA from the three major domains; Eubacteria, Eukaryotic and Archaeal were compared. The relative proportions of Proteobacteria (Alpha, Beta, Gamma, and Delta subgroups), High G+C and Low G+C content bacteria and the Cytophaga/Flavobacterium/Bacteriodes bacteria were also assessed. The microbial community composition in each test plot was compared using a Fisher's least significant difference test to see which components of the microbial community changed significantly with the changing agricultural practices.

The results showed that the techniques could be used to obtain quantifiable amounts of RNA and the hierarchical probe approach could be used to detect community shifts which occurred due to changing agricultural practices from row crop management to woody biomass production. Community shifts were detected between the test sites. Perhaps most interesting was the amount of unexplained diversity present at the sites that could be detected with the Universal probe, but was not detected using the individual group probes.
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1. INTRODUCTION

Identifying what microorganisms are present in an environmental sample can be a difficult task. Many methods have been used in the past to accomplish this feat. They include isolation of colonies on various agar media, enrichment cultures, biochemical tests such as Biolog, or phospholipid fatty acid analysis (PLFA). Unfortunately, there are problems associated with using these methods for characterizing microbial communities from environmental samples.

For one, it has been shown that some organisms that are known to be present in a sample are unculturable. These organisms have been seen under a microscope, but elude isolation. One example is *Epolopiscium fishelsoni*, a symbiont that inhabits the gut of the surgeonfish. *E. fishelsoni* is easily seen due to its large size 500 by 80μm, but has yet to be cultured (Fishelson et al 1985). Unculturability of an organism may be the result of unknown growth requirements that are not present in a medium or the lack of necessary interactions with other organisms (Pace 1996). It has been suggested that less than 1% of the microorganisms from an environmental sample can be cultured (Bakken 1985, Faegri et al 1977, Hopkins et al 1993, Richaume et al 1993, Torsvik et al 1990, Tsuji et al 1995).

Characterizing organisms on the basis of carbon source utilization or membrane lipids may not reveal true relationships between various organisms. Boivin-Jahns et al ( Boivin-Jahns et al 1995) compared the identification obtained by sequencing the 16s rDNA gene and the identification obtained by conventional phenotypic typing of 74 environmental isolates. They described 100 phenotypic characteristics such as cellular...
morphology, Gram staining, enzyme production, and ability to use various sole carbon sources. They found 42 discrepancies between phenotypic and sequence identifications during the first round of testing. They repeated the identifications and found that the 16SrDNA analysis results were identical to the first results. For 26 strains, a second phenotypic analysis resulted in a completely different genus determination from the first analysis. Furthermore, Boivin-Jahns et al observed differences in the phenotypic characteristics and the ability to utilize various carbon sources for freshly isolated organism and for those that had been stored in culture collections. They attributed the differences to potential loss of plasmids, which may contain the genes necessary for alternate carbon utilization and may be lost during storage. They also suggested that the changes might be due to a long-term adaptation response.

Biochemical pathways may change when microorganisms are faced with varying conditions, which can problematic when using the biochemical pathways for identification. For example, certain fatty acids are specific to certain bacteria and different groups of bacteria may have different fatty acid compositions (Goodfellow and Minnikin 1985, Lechevalier, 1977). These specific fatty acids are known as "signature lipids" and have been used to determine microbial community structure through the use of fatty acid profiles (Bobbie and White 1980, Gillan et al 1983, Dowling et al 1986, Edlund et al 1985, Mancusco et al 1986, Nichols et al 1985).

It has been shown that Bacillus species can be identified by their "signature lipids" using standardized growth conditions. Without careful control, fatty acid
profiles can vary substantially. For example, the membrane lipids present in fifteen *Bacillus* species have been extensively surveyed by Kaneda et al according to Nes (Nes and Nes 1980). Kaneda found that the *Bacillus subtilis* species could be defined as principally having the anteiso form of a C$_{15}$ lipid, whereas the *Bacillus cereus* species possessed the iso form of the C$_{15}$ lipid. Both *Bacillus* species possess some quantities of each isomeric form of the C$_{15}$ lipid. It was noted that when amino acids were added to the growth medium of the *B. subtilis* species, it began producing iso C$_{15}$ as its predominant lipid. Under these conditions, the *B. subtilis* would look more like a *B. cereus* when using the isomeric form of the C$_{15}$ lipid to identify an unknown species. These results show that one must use caution when using “signature lipids” to identify organisms in their environment, as lipid biomarkers described from pure culture studies may not be relevant to environmental organisms.

It is clear that phenotypic traits alone cannot be used to correctly identify and determine relationships between bacterial species. Traits found in two seemingly related organisms may have arisen from lateral gene transfer and there may not be a true evolutionary relationship between the two organisms. Alternatively, a gene for a trait may have been lost during evolution (Stahl 1997). Leading one to ask; is there a more constant biochemical component of a cell that could be used to group and identify microorganisms?

Researchers are looking to ribosomal ribonucleic acid (rRNA) as a way to group and identify organisms. The RNA molecule contains regions of highly conserved and highly variable sequences. Oligonucleotide probes can be designed that are
complementary to the nucleotide sequences in these regions. Probes have been designed which hybridize to the highly conserved regions and can be used to detect RNA in all types of organisms and are known as Universal probes (Amann et al 1990b). Probes have been developed that differentiate between the three domains of life, Archaea, Eubacteria and Eukarya (Stahl and Amann 1991, Amann et al 1990b). More specific probes have been designed which use the most variable regions to identify classes and subclasses of organisms. By using the most highly variable sequences it may be possible to differentiate between species (Manz et al 1992). The probes can be labeled with either a radioactive or fluorescent label. The probes can be used to detect RNA that has been extracted from an organism or they can be used to detect RNA within a cell.

By using an array of probes, one can determine the community composition of a sample. One such analysis is the Nested Probe or Hierarchical Probe Analysis. As the name implies, the Hierarchical Probe Analysis uses an array of probes ranging from the most general probes to increasingly more specific probes. A Universal probe is used to determine the total amount of RNA present in a sample. Domain level probes assess the proportion of the three domains and the specific group probes detect organisms within each domain. Probes can also be produced and used to detect differences on the species level. One advantage of the hierarchical probing approach to community analysis is the ability to validate ones observations since the amount of RNA detected by the probes should be additive. The amount of RNA detected using the three domain probes should equal the amount of RNA detected using the
Universal probe. Likewise, the total amount of RNA detected for the various group probes should be equal to the amount of RNA detected by the domain probe. Using the probes, an estimation of the microbial community composition can be assessed.

Why do we need to know about microbial community structure? Norman Pace has said “Although the chemical balance of the biosphere depends upon microorganisms, we have little understanding of the makeup and dynamics of the microbial ecosystems responsible for maintaining this balance” (Pace 1996). David Stahl suggests that because our current understanding of the microbial world is based mainly upon pure culture isolation and phenotypic characterization, we do not know whether pure culture organisms are representative of natural populations and what their exact roles are in a specific natural process. We need to know what is out there to better understand how microbial physiology largely defines and sustains the biosphere (Stahl 1993).

Microorganisms live and function as communities in nature. They are responsible for transforming, degrading and synthesizing many substances in the environment. In order to know whether or not a microbial transformation will occur and to quantitatively predict those transformations, one must first know what organisms are present and whether or not they are active. Furthermore, it is hypothesized that microbial communities contribute to fundamental soil structure and formation, soil quality, soil fertility and plant nutrition in agricultural soils (Bacon 1968). Microorganisms can control the flux of carbon, nitrogen, sulfur and phosphorus in agricultural systems. If these compounds are not available to growing plants, the soil
may need to be amended with fertilizer containing these chemicals. Excess fertilizer can disrupt the balance in an ecosystem. Microorganisms are also partly responsible for breaking down the detrital foliage into soil components. When changes in agricultural practices occur they are accompanied by changes in the microbial communities, which may enhance or be deleterious to the above processes (Smith et al 1993).

A study at Ames Plantation sought to monitor the microbial community changes, which would occur in changing from an annual row crop to a short rotation woody biomass crop. These fast-growing trees can be used for the production of woody biomass for energy production. Initially, they are capable of producing enough biomass for harvesting within ten years. Repropagation from the stump allows for production of a second harvest within 5-8 years. The Department of Energy has identified 156.8 million hectares of land suitable for short rotations woody crops. Approximately 92% of this land is currently being used for other agricultural purposes. Changing the crop management system will change the soil microclimate and effect the soil biota (Paul and Clark 1989). To understand the changes that might occur, four sites were chosen for preliminary studies. Annual row crops such as corn and winter wheat were replaced at the Ames Plantation Site, in 1985 and 1993, with a short rotation woody crop of Sycamore (*Platanus occidentalis* L.) trees. Various parameters of the row crop and tree crop were measured as the trees matured. The physical and chemical properties of the soil such as erosion rates, moisture content, pH, carbon, nitrogen and phosphorus content were measured. In addition, the
macrobiota and microbiota were monitored. As part of the microbiota analysis, researchers in the Department of Plant and Soil Sciences at the University of Tennessee performed DNA analysis, phospholipid fatty analysis, acridine orange direct counts and plate counts. In collaboration with this group, the 16s ribosomal RNA was analyzed to monitor microbial community shifts.

A goal of this research was to optimize a protocol to rapidly obtain from soil high yields of RNA suitable for membrane hybridizations with hierarchical probes. An objective of the research was to optimize hybridization and washing condition for the hierarchical probes. Another objective was to apply the above methodologies to the Ames Plantation research to test the following hypothesis: that changes occur in the microbial community structure that compensate for changing from annual row crops to short rotation woody biomass crops in order to maintain and complete biogeochemical cycles.
2. LITERATURE REVIEW

2.1 Classical Methods of Monitoring Microorganisms in the Environment

Traditional methods used to characterize microorganisms in various environmental samples included selective enrichment, pure culture isolation and most-probable number estimates. The microbes, once isolated, are characterized by phenotypic (non-evolutionary) relationships. Various properties are used to define each microbe such as morphology, physiological properties (for example utilizable nutrients) and cellular structure (such as membrane lipid composition) (Pace 1996). A classification system based on these characteristics is employed to group and classify microorganisms in a manner similar to those methods used for plants and animals. Microscopically, microbes, that appear spherical under the microscope, are separated from the rod-like microbes. Anaerobes are separated from the aerobes and so on. Unfortunately, analyzing the phenotypic traits often provides little information about the evolutionary relatedness of organisms and the natural structure of microbial communities completing biogeochemical cycles in ecosystems.

Differences in physiological characteristics can mask genetic similarities, alternatively, similar physiological and morphological properties may conceal genetic diversity (Stahl 1988). Schleifer and Ludwig noted that adaptive characteristics have been acquired independently by unrelated taxa either through convergence or lateral gene transfer (Schleifer and Ludwig 1994). In other cases, organisms can resemble one another merely by lacking a particular characteristic (Fox et al 1980).
example, Gibson et al noted that the fact that *Rhodomicrobium* divides by budding does not distinguish it strongly from nonbudding representatives of the purple nonsulfur bacteria (Gibson et al 1979). Another example is the ability for spore formation as a phylogenetic indicator. All spore formers are related. However, the lack of sporeforming capacity does not necessarily exclude an organism from phylogenetic groupings defined by sporeformers (e.g., *Eubacterium*, *Lactobacillus*, and *Streptococcus*). One particularly confounding group is the *Pseudomonads*, which have actually been determined to be a collection of at least five separate groups of bacteria (Fox et al 1982). Woese concluded that unlike animals and plants, which possess complex morphological traits, bacteria have simple morphologies and thus a phenotypically based characterization is not adequate for unambiguous classification (Woese 1987, 1994).

Another problem associated with the classical techniques is that cultivation is a prerequisite for examining these phenotypic properties and the microorganisms cannot be fully characterized in their natural settings. Norman Pace likened our understanding of microorganisms to an understanding of biology based solely on visits to zoos (Pace 1996). It has been suggested that the culture-dependent methods fail to adequately detect all members of an environmental microbial community (Giovannoni et al 1988, Amann et al 1990b, DeLong 1992).

### 2.2 The Great Plate Count Anomaly and Underestimating Diversity

Colony forming units (CFUs) or most-probable-number techniques (MPNs) are still reported in most literature as an means of enumerating microorganisms in a
sample. However, because these methods select for organisms that are capable of growing under stringent conditions, forcing rapid growth at high carbon source concentrations, these methods may not be accurate. It has been shown that for oligotrophic to mesotrophic aquatic habitats, direct microscopic counts exceed viable-cell counts by several orders of magnitude and the same can be said for sediment and sandy soils (Amann et al 1995). Staley and Konopka coined the phrase “great plate count anomaly” to describe this phenomenon (Staley and Konopka 1985).

Amann et al attributes the phenomenon to two different types of cells: (i) known species for which the cultivation conditions are not suitable or which have entered a non-culturable state and (ii) unknown species for which suitable cultivation methods have yet to be discovered (Amann et al 1995). It has been shown that some bacteria may enter a nonculturable state upon exposure to salt water, freshwater or low temperature (Roszak et al 1984, Colwell et al 1985, Button et al 1993, Oliver et al 1991).

About 5,000 species of bacteria have been described (Bull et al 1992) with the Approved List of Bacterial Names currently containing less than 3,000 entries. Furthermore, it is estimated that the pure culture collection represents only a small fraction of the true environmental diversity (Barns 1994 et al, Leisack and Stackebrandt 1992). The classic species definition for higher organisms only applies to organisms with sexual reproduction and is therefore difficult to apply to microorganisms. Bacterial taxonomists agreed to define a species on the basis of DNA-DNA similarity of more than 70% (Schleifer and Stackebrandt 1983, Wayne et
al 1987, Stackebrandt and Goebel 1994). Torsvik et al extracted DNA samples from a soil sample and suggested that perhaps 4,000 different standard sizes bacterial genomes existed in this sample (Torsvik et al 1990). They suggested that by using the 70% criterion, there might be as many as 13,000 species in this sample alone.

Another estimate of the potential of bacterial diversity comes from Dasch et al. They state that there are at least 800,000 insect species and each insect species harbors millions to billions of bacteria and at least 10% of insect species harbor obligate symbionts. Thus consideration of insect symbionts alone would increase the current number of bacterial species by several orders of magnitude (Dasch et al 1984). What can we do to better assess microbial species diversity in the environment?

2.3 Using Molecular Methods for Determining Phylogeny

With the advent of gene sequencing the world of microbial phylogeny was changed forever. In 1965, Zuckerkandl and Pauling laid down the conceptual foundation for molecular systematics by introducing the idea of using sequence information to determine evolutionary relatedness (Zuckerkandl and Pauling 1965). New methods allowed for evolutionary analyses based not on phenotypic characteristics but on phylogenetic relationships (Woese 1987).

With the advent of gene sequencing, comparative analysis of protein sequences for a variety of cellular components were reported. Sequence data from cytochrome c from purple photosynthetic bacteria (Gibson et al 1979), ferredoxin from Clostridium (Tanake et al 1971), heat-shock proteins and ATPases (Brown and Doolittle 1997), and proteins associated with transcription, translation, amino acid metabolism and
transport (Doolittle et al 1996 and Tatusov et al 1997) have all been used to determine relationships between bacterial groups and other domains.

Eventually nucleic acids sequences were obtained and compared. Initially, comparisons were made between the homology of different 5S rRNA gene sequences (Woese and Fox 1977), then by comparing the homology between different 16S rRNA gene sequences (Pace and Campbell 1971, Johnson and Francis 1975, DeSmedt and DeLey 1977, DeLey et al 1978). The phylogenetic results obtained by 5S rRNA and 16S rRNA gene sequences and protein sequences were in reasonable agreement with one another. The differences were minor and did not affect the major conclusions (Fox et al 1980). Jensen has issued a word of caution for the newly found classification system. He states, “Phylogenies derived from sequence analysis have to be accepted for what they minimally are: hypotheses to be tested and either strengthened or rejected on the basis of other kinds of data” (Jensen 1985). With sequence comparisons came the question: are the relationships obtained via sequence comparisons true evolutionary relationships?

2.4 Molecular Chronometers for Measuring Evolutionary Relationships

In his review of the conceptualization of the molecular evolutionary clock, Emile Zuckerkandl notes that early researchers of protein and nucleic acid sequences, Ernest Baldwin and Marcel Florkin, failed to perceive the unique value of protein and nucleic acid sequences for molecular evolution (Zuckerkandl 1987). According to Zuckerkandl, Christian Anfinsen pioneered the use of proteins and nucleic acid sequences to study molecular evolution in 1959 (Zuckerkandl 1987). Zuckerkandl
recalls that he himself first realized the concept of a molecular evolutionary clock in late 1960 or 1961 and later introduced the idea that evolutionary rates of amino acid replacements in hemoglobin chains were roughly proportional to time (Zuckerkandl 1987, Zuckerkandl and Pauling 1965).

Evolution occurs on the level of the genotype and of the phenotype. The changes in the deoxyribonucleic acid (DNA) sequences occur more or less continuously on an evolutionary time scale. According to the neutral theory proposed by Kimura, the majority of changes to DNA sequences occur more or less continuously on an evolutionary time scale and are either deleterious or selectively neutral. The changes, therefore, do not alter the phenotype (Kimura 1987). On the other hand, changes on the phenotypic level are relatively rare and sporadic (Schleifer and Ludwig 1994). An analogy presented by Carl Woese is a car and its motor. A car does not go unless the motor is running, but the motor can run without the car moving. In this analogy, the motor is the genotypic change and the car is the phenotypic change (Woese 1987).

A molecule that changes in time is a candidate for consideration as a molecular chronometer. To be a useful chronometer, a molecule must meet certain specifications. According to Woese, a molecular chronometer must (1) possess clocklike behavior in changing its sequence as randomly as possible, (2) span the range of evolutionary distances to be measured, and (3) be large enough a molecule to provide an adequate amount of information (Woese 1987).

Proteins used in phylogenetic studies have included ATPases, protein elongation factors and cytochromes (Goodfellow and O'Donnell 1993, Hennecke et al 1985).
However, some problems have arisen in using such proteins for determining evolutionary relationships. Gene transfer between species may cause misclassification of some organisms (Smets et al 1990). Discussed in detail by Michael Syvannen, cross-species gene transfer may involve not only entire genes, but short regions of coding sequences, perhaps tens of nucleotides long rather than entire gene (Syvannen 1987). He states the growing evidence that viruses can contribute to transferring genetic information from one host to another species. As an example, Syvannen states the first report of horizontal gene transfer was the probable transfer of a retroviral sequence from Old World monkeys to a recent ancestor of domestic cats. He also notes that other kinds of mobile elements also appear to have been transferred across species. An example is the P-element found in modern populations of *Drosophila melanogaster* that was presumably introduced from a different species. Syvannen concedes that these examples may represent rare occurrences and few viruses have the ability to infect highly unrelated species. However, he concludes these incidences may be frequent enough to be a major factor in speciation (Syvannen 1987).

Is there a more stable molecular chronometer that could be used to determine evolutionary relatedness among microorganisms? Comparative sequencing of the rRNAs has had the most far-reaching application and Woese has proclaimed it “the ultimate chronometer” (Woese 1982). RNAs can be sequenced directly and rapidly using the enzyme reverse transcriptase (Lane 1985a). The RNAs show a high degree of functional constancy, the molecules are large and they consist of many domains.
There are about fifty helical stalks in the 16S rRNA secondary structure and roughly twice that number in the 23S rRNA, which makes them accurate molecular chronometers. The 16S rRNA contains approximately 1,500 nucleotides, providing a large amount of information for phylogenetic inferences. The 23S rRNA contains approximately 3,000 nucleotides and should provide a greater accuracy in phylogenetic inference, but the smaller 16S molecule is much easier to sequence and has therefore become the established reference (Stahl 1997). The 23S rRNA should be used for resolving closely spaced evolutionary branching (Ludwig et al 1992).

There are now over 5,000 16s rRNA sequences available in the databases. RNAs have proved to be the most encompassing of the available molecular frameworks with which to explore the natural microbial diversity and phylogeny (Pace et al 1986, Stahl 1986, Ward et al 1992).

2.5 Phylogenetic Analysis Using 16S rRNA

In the paper, “Towards a Natural System of Organisms”, Woese et al detail how the current phylogenetic tree came into being (Woese et al 1990). In 1866, Haeckel challenged the opinion of the day that the living world was divided into two parts, the plant and animal divisions. Haeckel recognized that the single-celled forms, the protists, did not fit into either the plant or animal category and must have arisen separately from both. Haeckel therefore concluded that the tree of life must have three branches, Plantae, Animalia and Protista. Copeland later decided that the tree should consist of four branches with a new kingdom containing the bacteria and Whittaker proposed a fifth kingdom for the fungi. The five kingdom tree divided into Animalia,
Plantae, Fungi, Protista, and Monera is the most widely received view of the basic organization of life today. However, with the advent of 16S rRNA sequence comparison, Woese proposed that there are truly only three domains in life, Archaea, Bacteria and Eucarya (Woese et al 1990).

Woese proposed the three domains of life after extensively studying the 16S rRNA structure with Harry Noller, Robin Gutell and Bryn Weiser. The results of the study were presented in a 1985 Progress in Nucleic Acid Research and Molecular Biology article entitled “Comparative Anatomy of 16-S-like Ribosomal RNA” (Gutell 1985). Woese summarized the results in his paper “Bacterial Evolution” (Woese 1987). There are signature sequences in the rRNA molecule that are highly conserved or invariant in one kingdom, but possess a different (highly conserved) composition in one or both of the other kingdoms. These differences define the three kingdoms, Archaea, Bacteria (Eubacteria) and Eukarya.

Comparisons of the 16S rRNA molecule and other molecular sequences, such as cytochrome C, have totally redefined the microbial phylogenetic system. It has been discovered that some groupings based on cellular membrane composition were correct, while others were not. The current grouping of Gram positives is a phylogenetically correct grouping, but the Gram-negative grouping is not. Indeed, Woese suggests that the current Gram-negative grouping encompasses some 10 distinct groups, each the equivalent of the Gram-positive one (Woese 1987). Furthermore, it has been found that photosynthetic bacteria do not form a separate group from the nonphotosynthetic bacteria (Imhof et al 1984). Likewise, autotrophs
and heterotrophs are not phylogenetically separate but are instead mixed within the various eubacterial phyla (Fox et al 1980).

The Proteobacteria, formally known as the purple bacteria, contain most but not all of the traditional Gram-negative bacteria. Originally thought to include only the purple photosynthetic phenotype, the group was found to include many non-photosynthetic members as well. Since some members possess photosynthetic capacity and others do not it has been suggested that photosynthetic capacity may have been lost many times in this phylum (Woese 1987).

The Proteobacteria fall into four subgroups awaiting formal nomenclature: the alpha (α), beta (β), gamma (γ) and delta (δ) subgroups. Two helices in 16S rRNA, positions 184 to 193 and 198 to 219 (E.coli numbering) help to define and distinguish the four subdivisions (Woese et al 1983). The first of the two helices (positions 184 to 193) contains only three base pairs in α, β, and γ subdivision sequences, but about ten base pairs in the δ subdivision. The three base pair form of the helix is rare and has been found outside the proteobacteria group only in the cyanobacteria and the planctomyces. In all other cases, a much longer version of the helix occurs, which therefore is likely to be its ancestral form. The second helix (positions 198 to 219) contains approximately eight base pairs in all, except in the α-subgroup, where only two base pairs make up the stalk (Woese et al 1983). However, the short version is common among bacteria and even occurs in archaea. The longer version is rare among bacteria. The long version has a common structure in most sequences from the
β and γ subgroups, but the δ subgroup long version differs from the β and γ subgroup (Woese 1987).

The Alpha subgroup is a mixture of metabolic types. Aerobic metabolism appears to have arisen a number of times in this subgroup. There is a close association of microorganisms that can reduce and oxidize nitrogen compounds suggesting some sort of evolutionary connection between the two metabolic pathways. They also have a tendency to form intimate if not intracellular associations with eukaryotic cells. For example, rhizobacteria essential for nitrogen fixation in legumes and the agrobacterium, which are pathogenic for plants, are in this group (Woese 1987). Other members of the alpha subdivision include: Acetobacteraceae, the Beijerinckia group, the Bradyrhizobium group, Caulobacter, Hyphomicrobium, Rhizobiaceae, Xanthobacter and the Zymomonas group.

Beta species differ from their alpha counterparts in their rRNA sequences and in their cytochrome c type. Beta cytochromes are of the small-subunit type, while cytochromes from the alpha subdivision are of the medium or large type (Dickerson 1980). Furthermore, the photosynthetic beta species possess photoreaction centers that differ in structure from the photosynthetic alpha species (Woese 1987). Some members of the beta subdivision are Acidovorax, Alcaligenaceae, Ammonia-oxidizing bacteria, Burkholderia, Gallionella, Janthinobacterium, Thiobacillus and Zoogloea.

The gamma subdivision is a mixture of phenotypes. There are photosynthetic and non-photosynthetic, aerobic and anaerobic, heterotrophic and chemolithotrophic}
microorganisms. The beta group, as previously mentioned, is very closely related to this group and may eventually become a deep-branching subgroup of the gamma group (Woese 1987). Some members of the gamma subdivision are *Alteromonas*, the *Azotobacteraceae*, the *Enterobacteriaceae*, *Pseudomonas*, *Shewanella* and *Thiomicrospira*.

The delta subdivision is composed of very different phenotypes. The sulfur and sulfate-reducing bacteria, the *Myxobacteria* and relatives, the *Bdellovibrios* and *Geobacter*. This subdivision may be important in soil communities for several reasons. The delta subdivision plays an important role in global sulfur cycling. In agricultural soils, the sulfur and sulfate-reducing bacteria may play less of a role since they are strictly anaerobic. *Geobacter* may play a role in iron cycling. *Bdellovibrios* are important in their role as predators of other gram-negative microorganisms. The myxobacteria are chemoorganotrophs that are capable of decomposing many different macromolecules (Bergeys 1993).

The Gram positives or Firmicutes are divided into several subdivisions, those with a relatively high G+C DNA content (>55%), those with a low G+C DNA content (<50%), a third photosynthetic subdivision-*Helibacterium chlorum*, and a fourth group, comprised of *Megasphaera*, *Selenomonas* and *Sporomusa*. The third and fourth groups do not possess Gram-positive cell walls (Woese 1987). The 16S rRNA of Gram-positives has two characteristic adjacent A-G pairs, positions 1425-6 to 1474-5, that occur in the penultimate helix of almost all Gram-positive 16S rRNAs
sequenced to date (Woese 1987). This arrangement has yet to be seen outside of this
group.

The High G+C subdivision is composed of the *Actinomyces*, which includes the
*Arthrobacter* genus and the *Brevibacterium* genus. This subdivision also includes the
*Bifidobacterium, Propionibacterium, Streptomycyes* and *Mycobacterium* (Woese
1987).

The Low G+C subdivision usually expresses a clostridial phenotype. They tend to
be anaerobic, rod shaped and endosporeforming, although several members have lost
one or more of these characteristics (Woese 1987). Low G+C has several groups of
interest: *Bacillus* (aerobic), *Lactobacillus, Streptococcus*, the mycoplasmas and
*Clostridium* (Fox et al 1980 and Woese et al 1980b). Because the other members in
this group are predominantly anaerobic it has been suggested that *Bacillus* may have
came aerobic during the course of evolution (Woese 1987).

Another disparate grouping is the *Cytophaga, Flavobacterium* and *Bacteriodes*
(CFB) Group. This group is defined by their commonality in their 16S rRNA
sequences in the following respects. A uracil residue at position 570 is found in all
members of this group and in the planctomyces phylum, but nowhere else among
bacteria. An adenosine residue at position 995 is usually a cytosine in other bacterial
groups, except in the green sulfur bacteria. Finally, there is an adenosine at position
1532 which is exclusive to this group of bacteria and separates it from all other
bacteria, archaea and eukarya (Balch et al 1979, Paster et al 1985 and Woese et al
The anaerobic Bacteriodes are separate from the aerobic Cytophaga and Flavobacterium.

The Cyanobacteria group is composed of the classical blue-green algae. Cyanobacteria are photosynthetic bacteria that possess chlorophyll a. Cyanobacteria also possess either a uracil or adenosine residue at position 799 in their 16S rRNA. This residue is typically a guanine in all but 1 to 2% of other bacterial 16S rRNA (Woese 1987).

Several other bacterial groups exist that have not been fully characterized by their 16S rRNA sequences. One of the few groups correctly identified by classical morphological criteria is the photosynthetic Green Sulfur Bacteria. Included in this group are Chloroflexus, Herpetosiphon and Thermomicrobium. Another group the Planctomyces and their relatives, Planctomyces, Pasteuria and Pirella (Stackebrandt et al 1984) and a hot spring organism Isocystis pallida, are all noted for the fact that their cell walls contain no peptidoglycan (Bonen and Doolittle1976). It was previously thought that the Planctomyces inhabited only aquatic systems. However, new evidence obtained by fluorescent in situ hybridization suggests that Planctomyces may be an important component in terrestrial ecosystems as well (Zarda et al 1997).

The domain Archaea is another collection of disparate phenotypes, the methanogens, the extreme halophiles, and the extremely thermophilic sulfur-metabolizing species (Woese 1982, Woese and Wolfe 1985b). The domain possesses a unique 16S rRNA structure and all archaea have branched-chain, ether-linked lipids
The methanogens are divided into three main groups *Methanobacteriales, Methanococcales, and Methanomicrobiales*. The extreme halophiles are capable of living in very high salt concentrations and are the only photosynthetic archaea. The extremely thermophilic sulfur-metabolizing Archaea grow at very high temperatures, some near the boiling point of water (Woese 1987).

For the most up to date taxonomic listing of bacteria, one can consult the National Center for Biotechnology Information (NCBI) website at http://www.ncbi.nlm.nih.gov. The NCBI taxonomy browser can be used to access nucleotide or protein sequences from GenBank for each taxonomic level. The data is listed by the superkingdoms (i.e., domains), Archaea, Eubacteria and Eukaryota. It also lists information for viroids, viruses and unclassified organisms. Family, group and genus information is detailed below each superkingdom.

### 2.6 Molecular Analyses for Determining Microbial Community Structure

Many of the organisms listed in the NCBI Taxonomy Database are listed as uncultivated, unidentified clones from various environmental sources such as soil, activated sludge or hydrothermal vents. If these organisms defy classic means of isolation, how can they be detected in the environment? Furthermore, how can one determine the composition of an entire microbial community in an environmental sample? There have been many advances in this area of research during the last decade with several new methodologies emerging, such as, PCR Amplification, Cloning and Sequencing, Denaturing Gradient Gel Electrophoresis, Restriction Endonuclease Analyses (RFLP and ARDRA), In-situ hybridization and membrane
hybridization. Each of these methods will be discussed below for their advantages and their disadvantages.

2.6a PCR Amplification, Cloning and Sequencing

The first attempts to characterize microbiota in an environmental sample using rRNA began in the mid-1980s. The 5S rRNA was extracted from samples and the separated via electrophoresis. The sequences of the different 5S rRNA bands obtained from the gel were used for phylogenetically comparisons. This method was used to characterize bacterial symbionts of “chemoautotrophic invertebrates” living around deep-sea hydrothermal vents (Stahl et al 1984), bacteria from a hot-spring (Stahl et al 1985) and bacteria from a pond containing copper leachate (Lane et al 1985b). Since the 5S rRNA molecule is only about 120 nucleotides in length, limited inferences can be made to microbial communities that are very diverse.

Norman Pace proposed the use of larger (16S and 23S) rRNA molecules to gather more information for community comparisons (Olsen et al 1986). The following steps would be used to obtain sequence information for phylogenetic comparisons.

First, the total community DNA would be extracted from an environmental sample. Next a shot-gun DNA library would be prepared using bacteriophage lambda. The library would be screened by hybridization with a probe specific for 16S rDNA. The clones containing 16S rDNA would be sequenced and compared.

This method was first employed to analyze the microbial community in a marine picoplankton sample (Schmidt et al 1991). Schmidt et al determined that screening the shotgun library was very tedious since only 0.125 to 0.3 percent of the clones
contained all or part of the RNA gene. Another problem arose in sequencing redundant clones, which wasted time and money. It was found that a second screening of the clone library using species or group specific probes could help prevent sequencing redundant clones (Olsen et al 1986, Ward et al 1992). There is still a consideration of time and cost accrued by this second screening of the shotgun clone library. Advances in sequencing technologies have greatly improved the ease of sequencing rDNA and at the same time greatly reduced the time and money invested.

The introduction of using a thermostable DNA polymerase in polymerase chain reactions (PCR) made the production of gene libraries much easier (Saiki et al 1988). With the advent of PCR, it was possible to directly amplify 16S rDNA from mixed DNA. In theory, gene libraries made from PCR products should contain only the DNA sequence fragments which were specified for during the PCR process. These fragments can be rapidly sequenced using known priming sites. This method was first used by Giovannoni et al to determine the microbial composition of Sargasso Sea picoplankton (Giovannoni 1990a). Since the length of the amplification product is known, the clone library can be quickly screened for inserts of the proper length, thus identifying clones which may contain the desired 16S rDNA sequence.

Clone libraries can also be generated from 16S rRNA by using the enzyme reverse transcriptase to create rDNA from rRNA extracted from samples (Weller and Ward 1989). This method was first used to study the microbial community of a
cyanobacterial mat growing at 55°C by Ward et al (Ward et al 1990). They were able to distinguish eight different sequence types using this method.

The number of environmental microbial communities characterized via PCR amplification/cloning and sequencing has been tremendous and too numerous to mention completely. The technique has been used to characterize uncultivated hot spring microbial mats (Weller et al 1991). Other phylogenetic studies have been done using the technique and include analysis of dissimilatory iron reducing bacteria, propionate-oxidizing bacteria, sulfur and iron oxidizing bacteria and an obligate intracellular parasite of bovine erythrocytes (Lonergan et al 1996, Harmsen et al 1995, Lane et al 1992, Weisburg et al 1991). The method has been used to characterize organisms capable of degrading various compounds such as toluene and 3-chloro-4-hydroxybenzoate (Zhou 1997, Sanford 1996). Marine systems have particularly benefited by the ability to amplify low-biomass samples. The PCR technique has been used to study a Carolina Bay, an Adirondack mountain lake, a hypersaline environment and ocean ecosystems (Wise et al 1997, Hiorns et al 1997, Benlloch et al 1995, Rappe et al 1998, DeLong 1992 and DeLong et al 1993, Giovannoni et al 1996, Suzuki et al 1997). The method has been used to characterize microbial communities in sediments and soils (Thornhill et al 1995, Jurgens et al 1997, Kuske et al 1997).

Unfortunately, there are some problems associated with using the PCR method to create clone libraries for use in determining the microbial community composition from an environmental sample. It has been shown that some 16S rDNA may be
preferentially amplified from mixed DNA and, in the worst case, some rDNA may not be amplified at all. When a mixture of DNA containing yeast rDNA and archaeal rDNA was amplified using PCR, the yeast rDNA was preferentially amplified. The archaeal rDNA could be amplified only after addition of 5% acetamide to the PCR mixture. This apparently minimized nonspecific annealing of primers to the yeast rDNA (Reysenbach et al 1992). One reason for unequal amplification may be due to the inability of the primers to bind to the 16S rDNA due to higher-order structural elements. For example, it was found that high G+C content templates were discriminated against due to lower efficiency of strand separation during the denaturation step of the PCR reaction (Reysenbach et al 1992). In another study, it was found that when rRNA operons were clustered on the genome, rather than evenly distributed, the clustered genes dominated the PCR products (Farrelly et al 1995).

The number of rRNA gene operons present in a bacterial chromosome can range from 1 copy for *Mycoplasma* (Amikam et al 1984) to 10 copies for *Bacillus* (Jarvis et al 1988). This will influence the enumeration of certain populations when using the relative abundance of certain rDNA clones to estimate these populations.

The initial primers used in the PCR reactions will also effect the end result. Some primers pairs were shown to produce PCR products that tended toward a 1:1 ratio independent of the starting ratios of genes in a study by Suzuki and Giovannoni (Suzuki and Giovannoni 1996). In another study of clone libraries generated using different PCR priming pairs, the libraries were found to differ significantly in their compositions (Rainey et al 1994). They suggested that the fidelity of the PCR
amplification varies, depending on the DNA polymerase used because a polymerase may have misincorporation rates ranging from $2.5 \times 10^{-6}$ to 1.3 percent depending on the manufacturer. If the wrong bases are added in the first few cycles of amplification, the PCR product may have a large amount of incorrect sequences. The incorrect sequence may be erroneously concluded to be a new novel taxa.

Another way in which the wrong sequence may be construed to be a new taxa is by the formation of chimeric products. Chimeras may be formed during reannealing when segments of DNA from two different sources come together to form a new sequence of DNA that is a composite. During the next round of amplification, the composite DNA is amplified. This problem was observed by Liesack et al when they sequenced five clones from a pure culture of a strictly barophilic, psychrophilic bacterium and discovered the presence of two different 16S rDNA types (A and B) with 95% similarity and a sixth clone which contained a chimeric rDNA sequence. This clone contained about 1,000 nucleotides identical to type A at the 5' end and 500 nucleotides identical to type B at the 3' end (Leisack et al 1991). Leisack et al suggested two main factors in chimeric formation. For one, the availability of partial-length rDNA fragments present in low-molecular-weight genomic DNA preparations or generated by premature termination of elongation during PCR. Secondly, the percentage of highly conserved stretches along the primary structure of rDNA types, where after denaturation single strands originating from different rDNAs, can anneal in highly complementary regions (Leisack et al 1991). Once the chimeras are formed they will be amplified at the same frequency as
non-chimeric sequences. To check for chimeric sequences in PCR products, one should perform a comparative sequence analyses of different sections of the rDNA amplification products or look for complementary helical regions (Leisack et al 1991).

In their review on rRNA analysis of uncultivated microorganisms, Head, Saunders and Pickup, suggest caution when using rRNA sequence analysis to determine phylogenetic relationships. They stress the importance in correctly aligning rRNA sequences. This can be a rather easy exercise for highly conserved regions, but one that can be extremely difficult for highly variable regions. A comparison with the secondary structure model of rRNA can be used to resolve these difficulties, but they suggest that regions that cannot be unambiguously aligned should not be included in phylogenetic analyses (Head et al 1997).

Perhaps one of the most important biases incurred during PCR based analyses is that due to contaminating DNA in the PCR reaction. David Stahl suggests that persistent “inactive” DNA from either the environment or trapped within dead cells may be a factor in using PCR generated sequences in determining microbial phylogeny in a sample (Stahl 1997). Another source of contaminating rDNA may be from reagents used in preparing genomic DNA. A recent study showed a clone library generated in the absence of added template DNA yielded 16S rDNA sequences closely related to Duganella (formerly Zoogloea), Acinetobacter, Stenotrophomonas, Escherichia, Leptothrix and Herbaspirillum. These sequences have been found in low-biomass habitats and may not truly reflect an environmental
origin, but actual reagent contamination (Tanner et al 1998). They suggest the best way to check that a species is present in a sample is to use the obtained sequence data to create probes for in-situ hybridization studies.

Amann, in his review on phylogenetic identification of microbial cells without cultivation, discusses the advantages and disadvantages of using PCR to generate clone libraries for determining what organisms are present in an environmental sample. He suggests using caution in regarding the sequences from these collections as unbiased reflections of the microbial communities living in the natural ecosystem due to the number of steps involved in the retrieval of the rDNA sequences (Amann 1995). Head, Saunders and Pickup also conclude that one can never confidently extrapolate from sequence composition in a clone library to a quantitative population composition in an environmental sample due to issues of PCR and cloning efficiency and other aforementioned biases (Head et al 1997).

2.6b Denaturing Gradient Gel Electrophoresis (DGGE)

Fisher and Lerman developed denaturing gradient gel electrophoresis (DGGE) in 1979 (Fisher and Lerman 1979). DGGE is a method in which fragments of DNA with identical lengths but different sequence composition can be separated based on the changes in electrophoretic mobility of the DNA fragments migrating in a gel containing a linearly increasing gradient of DNA denaturants (urea and formamide). The temperature of the gel is held constant. The changes in fragment mobility are associated with partial melting of the double-stranded DNA in the melting domains. The melting temperature for each domain varies according to the concentration of the
denaturant in the gel, which changes according to the position in the gel. When the DNA enters a region of the gel containing sufficient concentrations of denaturants, the DNA becomes a partially melted molecule and migration is severely retarded. The sequence composition within the melting domains varies between the amplification products even if the products are the same lengths. Therefore, the sequence composition determines the position at which the DNA will stop migrating in the gel (Lerman et al 1984).

By using the method as first developed, approximately fifty percent of sequence variants studied could be detected in DNA fragments up to 1,000 bp in length. By adding a GC-rich sequence to the DNA fragment either by cloning (Myers et al 1987) or by PCR performed with a primer containing a 40bp GC-rich sequence (Sheffield et al 1989), one could resolve virtually all of the sequence variants. The method was first used to detect single-base changes in genes for diagnosis of human genetic diseases (Myers et al 1987). The method was first applied to the analysis of 16S rRNA genes from microbial mats and a biofilm by Muyzer et al in 1993 (Muyzer et al 1993). More specific information can be obtained by excising individual bands and re-amplifying and sequencing them (Ferris et al 1996) or by transferring the DNA to nylon membranes and hybridizing with an oligonucleotide probe (Muyzer et al 1996). Recent studies have used DGGE to study microbial mats (Bateson et al 1995, Ferris et al 1997a), a stratified marine water column (Teske et al 1996) and actinomycete communities (Heuer et al 1997).
After determining appropriate running conditions, DGGE is relatively rapid to perform and many samples can be run simultaneously. This method is particularly useful for examining a time series and population dynamics. Once the identity of an organism associated with a particular band is known, fluctuations in individual components of a microbial population in response to environmental changes can be assessed. However, assigning particular bands to a specific organism can be difficult especially in regions where multiple bands exist. Furthermore, separation of many fragments amplified from a highly diverse bacterial community may not be possible (Stahl 1997). This limits the assay to less diverse communities. Also, phylogenetic information obtained from the sequence of excised bands is limited due to their size (~500 base pairs). Another consideration is the rRNA operons of an individual organism can very significantly so individual organisms can potentially contribute to multiple bands on a DGGE gel (Stahl 1997). Perhaps the biggest concern with using DGGE is that it is a PCR based method and therefore is subject to all the potential biases introduced by PCR amplification (see section 2.6a).

2.6c Restriction Endonuclease Assays (RFLP and ARDRA)

There are two commonly used restriction endonuclease assays currently in use to determine phylogenetic relationships, restriction fragment length polymorphism (RFLP) and amplified ribosomal DNA and restriction endonuclease analysis (ARDRA). DNA is extracted from an environmental sample and cut into fragments using a restriction endonuclease specific for certain sequences. The restricted DNA is then run on a gel that separates the DNA based on the fragment length. The patterns
the bands create due to the differing fragment lengths are known as restriction length fragment polymorphisms (RFLP). The resulting population of different sized DNA fragments is used to infer a relationship between individual isolates or to resolve different environmental populations. The sequences must differ in sequences at the sites of DNA restriction or differ in length of DNA flanked by common restriction sites. For this reason, more than one restriction enzyme is generally used for restriction enzyme analysis.

The DNA may then be transferred to a membrane support for hybridization with a radiolabeled probe or with nonradioactive detection agents. The method of transferring DNA to a membrane was first described by Southern and is referred to as Southern blotting (Southern 1975). The rRNA gene is an optimal target for hybridization (Grimont and Grimont 1991), but other conserved elements (for example, nitrate reductase and formyltetrahydrofolate synthetase) have also been used for comparing microbial communities (Lovell 1991, Smith and Tiedje 1992).

Initial screening of a cDNA library by RFLP analysis of purified plasmid DNA or insert DNA obtained by colony PCR can greatly reduce the number of clones that require complete sequencing (Ward et al 1990). RFLP of the 16S rRNA gene has been used to characterize microbial communities from activated sludge, aquifer sand and termite guts (Liu et al 1997). RFLP has been used to determine differences in species and subspecies of bacterial isolates (Lee et al 1997).

Amplified ribosomal DNA and restriction endonuclease analysis (ARDRA) was originally described by Vaneechoutte et al in 1992 (Vaneechoutte et al 1992). The
amplified 16S rDNA gene is subjected to restriction endonuclease digestion and the resulting restriction fragment pattern is used to identify bacterial genomes on the basis that the restriction sites on the RNA operon are conserved according to phylogenetic patterns. The patterns of pure cultures can be used for a quick presumptive identification if they match band patterns of taxonomically described strains. This method can also be used to study microbial communities by creating a series of distinct band patterns resulting from the different microbial population members (Massol-Deya et al 1995). This method was used to define microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethene cometabolism (Fries et al 1997). This method was useful for monitoring whether the communities were changing in time, but did not define exactly what the changes were once they occurred.

The most common problem cited for restriction endonuclease analysis is due to the limited ability of a gel to resolve fragments of different lengths. For highly complex communities, the band patterns generated may be too complex (Massol-Deya et al 1995). Another problem is encountered when PCR is used to generate amplified products as this may create many biases in the PCR products as discussed in section 2.6a.

2.6d Fluorescent In-Situ Hybridization (FISH)

Rudolf Amann reviews fluorescent in-situ hybridization in the paper “Fluorescently labeled, rRNA-targeted oligonucleotide probes in the study of microbial ecology” (Amann 1995). Amann discusses the use of fluorescent probes to
detect microorganisms from different environmental matrices and problems associated with the technique. He begins with a description of in-situ hybridization or whole-cell hybridization as a technique whereby probes complementary to rRNA are incorporated into a morphologically intact cell, where upon the probe binds to rRNA within the cell. The probes can be detected either by radiography or by viewing with an epi-fluorescence microscope. In this manner, the phylogeny of an uncultured microorganism can be determined simultaneously with its morphology, abundance and spatial distribution. Olsen et al originally proposed using in-situ hybridization for enumeration and identification of organisms in 1986 (Olsen et al 1986). The microscopic identification of microbial cells using radioactively labeled rRNA targeted probes was first performed by Giovannoni et al (Giovannoni et al 1990a). Unfortunately, this method used microautoradiography and did not yield good in-situ results.

An immunofluorescence approach to labeling probes was tried by Bohool and Schmidt in 1980 (Bohool and Schmidt 1980). Later, fluorescently monolabeled, rRNA-targeted oligonucleotide probes were shown to detect individual cells by DeLong et al in 1989 (DeLong et al 1989). It was shown that the fluorescent probes yielded excellent spatial resolution and could be instantaneously detected by epifluorescence microscopy. This innovation made determinative studies possible in environmental samples (Amann et al 1990b). Later advances combined whole-cell hybridization with flow cytometry for a high-resolution automated analysis of mixed microbial populations (Amann et al 1990a).
As previously suggested, the probe signal may give an indication of the in situ growth rates of individual cells since the number of ribosomes present in a cell correlate to the growth rate (Schaechter et al 1958, Poulsen et al 1993, Cary and Giovannoni 1993, DeLong et al 1989). One consequence of this is that slowly growing cells are difficult to detect because of their low cellular rRNA content. Amann suggests caution when using rRNA content to determine growth rate as it is unclear whether the correlation observed in cell suspension holds true for attached growth in a biofilm or for carbon-starved cells. For example, some cells of *Vibrio* sp. strain S14 were starved of exogenous carbon for several days and were not growing, yet, they retained large numbers of ribosomes (Flardh et al 1992). Apparently the protein synthesis capacity far exceeded the apparent demand for translation and may be essential for the ability of this *Vibrio* strain to immediately regain high activity as soon as starvation is terminated by addition of substrates. Other studies have found that there is a correlation between rRNA/rDNA ratios (Kemp et al 1993, Kerkhof and Ward 1993).

Inaccessibility of the target rRNA molecule can also cause detection problems in whole-cell hybridization. Gram positive cell walls prevent the entry of probes into the cells unless they are pretreated with paraformaldehyde or lysozyme to increase cell permeability (Hahn et al 1992, Salama et al 1991, Schleifer et al 1991). Less predictable is the accessibility of the target sequence to the probe once the probe is within the cell. Some cells hybridize well with the 16S Universal probe and not at all with more specific probes. Some suggested reasons for this phenomenon are
noncomplementation of the probe to the target, ineffective probe labeling or non-optimal hybridization conditions. The hybridization may also be influenced by RNA-protein interactions or by the tertiary structure obscuring binding sites. These higher-order structural limitations may sometimes be overcome with the addition of formamide to the hybridization buffer. This weakens the hydrogen bonds, and thereby, softens the hindrance of higher-order structures (Amann et al 1992c).

Another limitation is the difficulty encountered when trying to bring a cell into the examining field of the microscope. Amann states that for a 20μl sample with a cellular concentration of $10^7$ cells/ml only approximately 40 cells will be in the viewing field at any time. For a $10^3$ cells/ml sample, several viewing fields may need to be examined in order to view one cell. Therefore, for low-biomass samples, the target cells must be concentrated through a filter, thus removing the spatial relationships. For sediment or soil samples in which non-cellular particles must be removed prior to concentration, some bacteria strongly adhered to the soil particles may be removed (Amann 1995).

Problems may stem from high background either from another bacterial population or from autofluorescence of the matrix. This is particularly problematic for soil samples. Initial attempts to detect bacterial cells in soil samples by Hahn et al failed for several reasons (Hahn et al 1992). Hahn et al found that the autofluorescence of organic particles was quite strong. Also, the cells were irregularly distributed over and attached to a very heterogeneous matrix. Finally, of the cells that overcame these first problems, only 1% of them showed detectable hybridization.
signals. It was unclear whether they were poorly permeabilized or had low cellular rRNA contents. Some of the problems associated with autofluorescence have been overcome by using a confocal laser scanning microscope to selectively visualize the probe-conferred fluorochromes (Assmus et al 1995).

2.6e Quantitative Dot Blot Hybridization

In order to circumvent the biases associated with PCR amplification, cloning and sequencing and the problems associated with in-situ hybridization, one may use quantification of 16S rRNA using a directly isolated nucleic acid mixture bound to a membrane and hybridized with a rRNA-targeted probe. This method is commonly know as the dot-blot hybridization method because the RNA extract is applied to the membrane using a 96 well assembly as a template to create concentrated dots of RNA on the membrane. A standard curve of known concentration of extracted rRNA that is complementary to the probe being used is blotted onto the membrane with the unknown samples. The RNA in the samples can be quantified by comparing the probe signal from the sample to the probe signal from the known concentrations (Stahl 1997). The relative abundance of RNA is calculated by dividing the amount of specific probe bound of a given sample by the amount of RNA hybridized by the universal probe. This method was first used to monitor populations of microbial communities in the rumen of cattle (Stahl et al 1988b). When radioactively labeled oligonucleotide probes were used in this particular study, rRNA sequences with a relatively low abundance between 0.1 and 1% could be quantified.
Since cells of different species have different ribosome contents ranging from between $10^3$ to $10^5$ ribosomes per cell, a direct translation into cell number cannot be made from the relative rRNA abundance. However, the relative rRNA abundance should represent a reasonable measurement of the relative physiological activity of the respective population, since it is the product of the number of detected cells and the average rRNA content (Amann 1995). Amann cautions that this does not indicate any kind of specific activity.

One advantage of this technique is that the extraction technique used to isolate RNA can be more disruptive than the methods used for DNA because RNA is less sensitive to mechanical shearing than is DNA (Stahl 1997). Methods of RNA analysis can be combined with the most disruptive of extraction methods, for example, mechanical breakage by reciprocal shaking with zirconium beads (Stahl and Amann 1991, Stahl et al 1988b). Furthermore, this method is does not require PCR amplified products, although PCR products or cDNA may be used in the Dot-blot assay.

The method is, however, subject to problems associated with using RNA. For example, although rRNA is present in the cell in a higher copy number than is DNA, the RNA is more prone to degradation. Therefore, when extracting RNA from environmental sources some RNA may be lost due to degradation. Please see section 2.7e for a further discussion on RNA degradation.

When combined with a nested probe analysis (section 2.6f), membrane hybridization using directly extracted rRNA can be used to monitor community
changes in environmental samples. This technique has been used to track changes in municipal solid waste and activated sludge systems (Griffin et al. 1997 and Reyes et al. 1997). The method has also been used to monitor ruminal bacteria under different substrate conditions (Shi et al. 1997) and to monitor sulfate-reducing bacteria (Devereux et al. 1996). The method was used to quantify *Crenarchaeota* in Lake Michigan sediments (MacGregor et al. 1997) and microbial communities associated with deep aquifers (Fry et al. 1997).

### 2.6f Nested Probe Analysis

Either DNA or RNA can serve as a nucleic acid probe, however, because DNA is easy to synthesize and is more stable it is used more often. Probes are designed to complement the rRNAs and are typically 15 to 25 nucleotides in length (Amann 1995 and Stahl and Amann 1991). There are two basic categories of DNA probes: group specific (phylogenetic or taxonomic) and functional (Stahl 1997).

Functional probes target a specific trait and can relate community structure and function. Some examples of functional genes, which are phylogenetically conserved, are genes for nitrogen fixation (Henneke et al. 1985), NiFe hydrogenase (Wawer and Muyzer 1995) and cellulases within some cellulolytic assemblages (Lin and Stahl 1995).

Group-specific probes generally target conserved biopolymers that can be used to infer phylogenetic relationships with the most wildly used target being 16S rRNA (Amann 1995 and Stahl and Amann 1991). Probes complementary to the most highly conserved regions of the 16S rRNA molecule are known as Universal probes. An
example is the Univ 1390 probe, which hybridizes to the nucleotides in positions 1390-1408 (E.coli numbering) (Zheng et al 1996). Probes have been designed that are complementary to the sequences that define the three domains, *Archaea, Bacteria* and *Eucarya* (Amann et al 1990b, DeLong et al 1989 and Giovannoni et al 1988). Probes are also available for the intermediate levels, for example, the Gram-negative sulfate-reducing bacteria (Amann 1992c), the alpha, beta and gamma subdivisions of the Proteobacteria (Manz et al 1992) and the *Cytophaga/Flavobacterium/Bacteriodes* (CFB) clusters (Manz et al 1996). Probes have been produced to identify the two archaeal kingdoms, *Crenarchaeota* and *Euryarchaeota* (Bull and Hardman 1991). Some probes have been generated to detect the two orders of the methanogens, the *Methanobacteriales* and *Methanococcales* (Raskin et al 1994b). The number of probes made for the lower taxa (genus, species-, and subspecies) specific probes are growing rapidly (Amann et al 1992a, Devereux et al 1992, Goebel et al 1987, Salama et al 1991, Stahlb et al 1988, Wagner et al 1994).

When these probes are applied to parallel subsamples in an ordered top-to-bottom approach, beginning with the universal probe and the three domain probes followed by probes of increasing specificity, more and more information is obtained about a particular communities composition (Raskin et al 1994a). This approach known as the nested probe or hierarchical probe approach can be used very rapidly in both dot blot and whole cell hybridizations (Manz et al 1993, Wagner et al 1993).

It is not necessary to apply the entire suite of probes initially. The information gained from the higher-level probes can be used to dictate which lower-level probes
should be used to gain increasing amounts of information. For example, if the archaeal probe shows a significant Archaeal presence in a sample, the probes for the *Crenarchaeota* and *Euryarchaeota* should be applied next (Burggraf et al 1994) before probing for the major orders of the *Euryarchaeota* (Raskin et al 1994b). In this manner, probing efforts can have a more concerted effort.

Another advantage of using the nested probe approach to characterize an environmental sample is the ability to validate results. If the more specific probes fully represent the larger phylogenetic group, then the sum of the specific probe hybridization values should equal that obtained by using the more general probe (Raskin et al 1997). For example, the total amount or RNA quantified using the three domain probes should be equal to the total amount of RNA quantified using the Universal probe (Raskin et al 1994b).

One drawback of this method is the probes are designed almost completely on the basis of sequences from cultured organisms and their specificities are evaluated by hybridization against reference organisms (Devereux et al 1992, Manz et al 1992). Since the culture collection currently contains a minor fraction of the real diversity of organisms and sequences this may limit the ability of the nested probe analysis. However, as the rDNA sequence database grows, newer probes can be produced to accommodate greater diversity. The nested probe approach has been used to analyze communities of symbiotic and magnetotactic bacteria (Moench and Kontzka 1978), marine picoplankton (Giovannoni et al 1990b), colonized biofilms (Bohlool and Schmidt 1980) and activated sludge (Wagner et al 1993 and 1994).
2.6g Evaluation of RNA/DNA Duplex Stability

An important parameter in quantitative hybridizations is the melting temperature \( T_m \) of the rRNA targeted probes. The \( T_m \) is defined as the equilibrium temperature at which half of the oligomers are dissociated for an oligonucleotide (Stahl and Amann 1991). The \( T_m \) for a probe is concentration dependent since association and dissociation reactions are intermolecular, but time-independent since the \( T_m \) is defined for equilibrium (Raskin et al 1997).

The dissociation temperature \( T_d \) or the duplex retention temperature (\( T_{dr} \)) is defined as the temperature midpoint of the transition between the duplex structure and the dissociated single-stranded target. The \( T_d \) does not correspond to equilibrium conditions and may be different from the \( T_m \). The \( T_d \) is calculated as follows:

**Calculation of \( T_d \) (Stahl and Amann 1991)**

\[
T_d = 81.5 + 16.6 \log M + 0.41 \% (G+C) - 820/n
\]

Where \( n \) is the number of bases in the oligonucleotide probe. \( M \) is the molar concentration of the salts in the buffer and \( \% G+C \) is the percentage of guanine and cytosine bases in the oligonucleotide probe.

**Calculation of \( M \) (Stahl and Amann 1991)**

1X SSC contains 0.15M sodium chloride and 0.015M trisodium citrate. Therefore, the molar concentration of monovalent cations in 2X SSC equals

\[
M = 2 \times [0.15 + (3 \times 0.015)] = 0.39
\]

By calculating \( 81.5 + 16.6 \log 0.39 \) and substituting into the \( T_d \) equation, the equation can be simplified for a 2X SSC solution to:

\[
T_d = 88.28 + 0.41 \% (G+C) - 820/n.
\]
The $T_d$ value is determined by the stability of the oligonucleotide probe-target duplex and consequently depends on probe length, nucleotide composition, higher-order structure in the region of the target sequence and hybridization conditions. The hybridization wash temperature is generally set equal to or slightly above $T_d$ as calculated in the above formulation by Amann and Stahl. Since the calculated $T_d$ does not incorporate all of the parameters associated with hybridization washing such as probe concentration and hybridization temperature, the hybridization and washing conditions for each new probe should be evaluated (Amann and Stahl 1991, Raskin et al 1994b). This is very important since probes may differ by only one nucleotide, as is the case for the Beta and Gamma group probes. The optimal hybridization conditions must be carefully assessed in order to obtain specificity when using these probes.

2.7 Considerations in the Isolation of rRNA from soil

The method variations for isolating RNA from environmental matrices are almost as numerous as are the investigators attempting the extractions. There are four basic aspects to most methods: (1) cell lysis, (2) extraction of the RNA from the matrix, (3) concentration of the RNA via precipitation and (4) final purification of the extract. Some of the considerations relevant to each of these steps are presented in the sections that follow.
2.7a Cell Lysis

Perhaps one of the most critical steps in retrieval of RNA from an environmental matrix is the efficiency of cell lysis. In an environmental sample of highly diverse types of bacterial cell types, some of the cells will lyse easily while other cell types will require harsh treatment in order to open the cells. For example, Gram-negative cells will lyse quite easily and may be over-represented if the extraction method is too gentle. Gram positive bacteria or chitin-walled fungi are more difficult to break and will require a more harsh treatment.

There have been many published methods for extracting DNA (Bruce et al 1992, Fuhrman et al 1988, Holben et al 1988, Tsai and Olsen 1991, Ogram et al 1987) and RNA (Ogram et al 1995, Borneman et al 1996, Felske et al 1996) from natural samples. But there have been only a few systematic studies on the efficiency of cell lysis in environmental matrices. It is possible that the same lysis techniques that are successful in pure culture may not be successful in water, sediment or soil (Head et al 1997).

Mechanical lysis protocols such as bead-beating procedures are believed to give relative uniform recovery of RNA and introduce the least bias caused from cell lysis (Stahl et al 1988b). However, these data were obtained from extractions using pure cultures and few environmental samples have been systematically evaluated. A study by Raskin et al on ruminal samples showed that the recovery of RNA did not change significantly when comparing bead-beating times and the amount of beads added to the extraction tubes (Raskin et al 1997a). However, large standard deviations
indicated that lysis by bead-beating may introduce significant variability into extraction recoveries of RNA.

It has been shown that a combination of physical and chemical treatments, such as freezing and thawing, lysis with detergents and bead beating lysed approximately 96% of cells in soil including bacterial endospores (Moré et al 1994). Moré noted that bacterial endospores and the smaller cells (0.3 to 1.2μm) were more resistant to lysis, but 94% of endospores and small cells were lysed by a combination of sodium dodecyl sulfate (SDS) detergent and bead beating. Cellular resistance to lysis may be important in oligotrophic samples were cells may be in a state of starvation and as a result may be very small or may form endospores (Head et al 1997). Other studies have found that even without harsh physical treatments such as bead beating, up to 99.8% lysis can be obtained using lysozyme and freeze-thaw cycles (Rochelle et al 1992). Tsai and Olson also found that lysing by SDS and freeze-thaw cycles was an efficient means of obtaining nucleic acids from sediment samples (Tsai and Olson 1991).

Jeffrey et al compared extraction recoveries of mRNA using the bead beating method and boiling with SDS. The boiling lysis method recovered significantly more mRNA than did the bead-beating method. The boiling lysis method also recovered more Gram-positive organisms than did the bead beating method (Jeffrey et al 1994). Benlloch et al found that boiling samples for 2-5 minutes in STE buffer and 0.1 volume 10% sodium dodecyl sulphate (SDS) lysed essentially all of the bacterial cells and did not significantly denature the DNA. Cell lysis was monitored by microscopic
observation (Benlloch et al 1995). The advantages of using the boiling lysis method are it does not require any special equipment and the RNA is less susceptible to RNase degradation because the tubes can be more easily cleaned and treated to remove RNases.

A new mechanical bead-beater is now available from Savant, the FastPrep System. This machine is capable of lysing bacteria in a 500mg sample of soil within 45 seconds. A method by Borneman et al using the FastPrep system and a modification of the Bio101 FastPrep RNA kit yielded highly-purified RNA that was amplifiable by RT-PCR (Borneman et al 1997). However, given the heterogeneous nature of soil, one must wonder if the bacterial sample is actually representative of the bacterial population when using such a small volume of soil.

2.7b Extraction of RNA

Once the RNA is release from the cell, the RNA is subject to degradation from RNases and may also adhere to matrix materials such as clays (Stahl 1997). The adsorption of nucleic acids to soils and sediments may be significant according to Ogram et al (Ogram et al 1994). Ogram et al's results showed that the Freundlich adsorption coefficient (K) was highly dependent upon soil type. Soils with higher clay and organic carbon content exhibited a higher K value than did soils with low clay and organic carbon content when binding a 4.19kb fragment of DNA. However, no direct correlation was found between K values and the organic carbon contents, clay contents, pHs or cation exchange capacities of the soils.
The lysis-denaturant solutions may not be efficient for removing nucleic acids from soils and sediments. Ogram et al found that alkaline extractants with at least 0.1M phosphate are the most efficient extractants, however most lysis-denaturant solutions are slightly acidic (pH 5.2) to prevent the hydrolysis of RNA under alkaline conditions and most contain very little phosphate (Ogram 1987).

2.7c Purification of RNA

The next step in most isolation procedures for soils and sediments is the purification of the RNA away from organic contaminants that co-extract with the nucleic acids. Phenol and chloroform are two reagents commonly used to remove proteins and other cellular constituents from the nucleic acid extracts. Both phenol and chloroform cause proteins to become denatured and more soluble in the organic phase or in the interphase, while nucleic acids remain in the aqueous phase. Phase separation is enhanced by the addition of chloroform and isoamyl alcohol, which aids in removal of the aqueous phase. After centrifugation the aqueous phase is re-extracted with an equal volume of chloroform: isoamyl alcohol. This reduces the loss of RNA due to the formation of insoluble protein: RNA complexes at the interphase (Ambion #158 1998). The loss of RNA and DNA may be significant during the purification step when the extracts contain significant amount of organic carbon and low biomass concentrations (Ogram et al 1995).
2.7d Precipitation of RNA

RNA is typically precipitated with alcohol (either ethanol or isopropanol) or lithium chloride. Precipitation with alcohol requires a minimum concentration of monovalent cations for example, 0.2M Na\(^+\), K\(^+\) or 0.5M NH\(_4\)^+ (Wallace 1987). Ethanol precipitation requires 2.5 volumes of ethanol and isopropanol precipitation requires an equal volume of isopropanol. Isopropanol is somewhat less efficient at precipitating RNA. However, isopropanol in the presence of NH\(_4\) is better than ethanol at keeping free nucleotides in solution. RNA precipitation is faster and more efficient at higher RNA concentrations. For solutions less than 10\(\mu\)g/ml, an overnight precipitation is recommended and a carrier nucleic acid, (e.g., glycogen or linear acrylimide) should be added to facilitate precipitation (Ambion #159 1998).

Lithium Chloride may also be used to precipitate RNA and has the advantage of not precipitating carbohydrates, proteins or DNA. However, some studies have shown lithium chloride precipitation may not precipitate lower molecular weight RNAs (Ambion #160 1998). No alcohol is needed for lithium chloride precipitation. A final concentration of lithium chloride of 2 to 3 M is needed to precipitate RNA. Lithium chloride can effectively precipitate RNA from more dilute solutions, but for best results, the RNA concentration should be greater than 200\(\mu\)g/ml (Ambion # 159 1998).

2.7e RNA Degradation

It is well known that RNA is easily subject to degradation perhaps due to its primary and tertiary structure. RNA has a ribose sugar backbone rather than a
deoxyribose sugar backbone, which is found in DNA. Ribose contains a 2' hydroxyl group; whereas, deoxyribose contains hydrogen at the 2’ position. This additional hydroxyl group may provide a site for enzymatic attack. Furthermore, rRNA is typically double stranded in the helical regions, but remains single stranded in the loop regions of the molecule and this may make it more susceptible to degradation (Gutell et al 1985, Noller 1984).

RNases are ubiquitously distributed in nature and care must be taken when working with RNA to avoid contamination with ribonucleases (Sambrook et al 1989). Enzymatic degradation is perhaps the greatest single source of RNA loss encountered during any extraction procedure (Ogram et al 1995). Everything that will come in contact with RNA in a sample must be cleaned or treated to remove RNases. Protein denaturants such as diethylpyrocarbonate (DEPC), guanidinium isothiocyanate, phenol and detergents such as sodium dodecyl sulfate (SDS) are typically used to inactivate RNases (Ogram et al 1995). Incubation at temperatures above 65°C can also denature RNases.

An important consequence of nuclease activity is that different regions of the rRNA molecule are subject to varying degrees of degradation. This can affect hybridization by partially destroying probe target sites while leaving others fully intact. For example, Gobel et al observed a hundred fold reduction of rRNA hybridization signal intensity when using a *Mycoplasma pneumoniae* extract, which was repeatedly frozen and thawed when compared to a cellular extract from freshly grown cells. The difference was attributed to nuclease activity (Gobel et al 1987).
Raskin et al noticed that the more highly conserved regions of the 16S rRNA molecule (general probe sites) were less structured and therefore more susceptible to cleavage by nucleases (Raskin et al 1997). Risatti et al observed that since hybridization results obtained using a universal probe are generally used to normalize the responses obtained with specific probes, samples with partial RNA degradation may result in unrealistically elevated fractional response (Risatti et al 1994).

2.7f Presence of DNA in RNA Extracts

It is possible that DNA may by co-extracted with RNA during extractions even though RNA extractions typically occur at a pH of 5.2. DNA should not be as soluble as RNA in the aqueous phase at this low pH. Observations by Raskin et al found that in environmental extracts, substantial amounts of DNA sometimes co-extracted with the RNA. This contaminating DNA could cause inflated estimates of populations even under stringent hybridization and washing conditions (Raskin et al 1997). To study this effect, samples containing the DNA were RNased to remove any RNA and then blotted and hybridized with a probe. The hybridization response was three orders of magnitude lower than the response obtained for samples that were not RNased prior to hybridization (Alm and Stahl 1996). The results suggested that by using stringent hybridization and washing conditions the nonspecific binding of the probe to the DNA could be limited. The samples can also be DNase treated prior to hybridization to remove contaminating DNA (section 3.4a).
2.7g Humic Contamination of RNA Extracts

The most predominant contaminate in nucleic acid extracts from environmental samples is humic substances; a group of organic molecules ranging in molecular weight from a few hundred to over 300,000 that are very difficult to degrade (Aiken et al 1985). Humics are thought to be derived from the breakdown of plant components such as lignin by microorganisms (Ogram 1988). They are very darkly colored as a result of their chemically unsaturated nature and are largely responsible for the dark colors of soils and peats (Bacon 1968).

There are three classes of humics. The first group is extractable with water. A second group, the fulvic and humic acids, are extractable with alkaline solutions. Fulvic acids remain in solution upon acidification, while the humic acids will precipitate out of solution upon acidification. A third group of humics is humin, which is non-extractable with alkali (Bacon 1968).

Because humic acids are precipitated when alkaline extracts of soil are acidified, they co-precipitate with RNA in extracts at pH 5.2. This leaves the RNA extract very dark and standard spectrophotometric means of measuring RNA quantities useless. Humics also interfere with subsequent molecular procedures such as PCR amplification (Moran et al 1993). Additional methods may be employed to remove the humics from the RNA among them precipitation with guanidium isothiocyanate or polyethylene glycol (PEG) and passing the extract through a Sephadex column (see section 3.3i for details).
2.8 Monitoring Microbial Soil Microbial Communities Using 16S rRNA Analysis

The literature base shows that molecular methods can more precisely determine what microorganisms are present in an environmental sample than can classical methods of monitoring microorganisms in the environment (sections 2.3, 2.4 and 2.5). Molecular methods do not rely on culturing methodologies, which have been shown to grossly underestimate the true microbial diversity within a sample (sections 2.1 and 2.2). Also, molecular methods allow one to determine potential evolutionary relationships between microorganisms in a sample using the molecular clock analysis of substitution rates between bases (section 2.4).

Several methodologies have arisen which encompass the use of molecular methods. Some methods rely on protein sequences others on nucleic acid sequences. Many scientists feel that the best indicator of phylogeny is the 16S rRNA molecule, which embodies several characteristics of a molecular clock (section 2.5). The 16S rRNA molecule has become the basis for determining new taxonomic relationships. When monitoring microorganisms in the environment, detection of 16S rRNA is preferable over DNA because the rRNA molecule affords natural signal amplification and may therefore be indicative of the activity of the microbial community. The 16S rRNA also exhibits very rapid turnover and will quickly diminish if a microorganism is no longer viable. The DNA molecule on the other hand is only present in a single copy and may persist in some environments after a microorganism is no longer viable (section 2.5).
Many of the methods used to detect 16S rRNA or the 16S rDNA gene in an environmental sample employ the use of PCR amplification prior to cloning, sequencing or restriction digestion and gel separation (section 2.6a-c). While these methods are advantageous and necessary for samples with low biomass, there are several potential biases that may occur when using PCR amplification. Biases include differential amplification, the formation of PCR artifacts, and potential contamination by exogenous DNA.

In order to circumvent the potential biases incurred using PCR, direct probing can be utilized. Fluorescent in situ hybridization is a direct probing method and can be useful in determining the spatial relationships between microbial communities in a sample (section 2.6d). However, due to the limited sample size that can be viewed at any time and the tedious nature of counting the cells in the viewing field, this method is not very useful for large-scale sampling. Furthermore, in many environmental samples, such as soil, there is a strong autofluorescence background, which limits ones ability to quantify and identify microorganisms.

Direct probing of extracted RNA bound to a membrane (dot-blot analysis, section 2.6e) has been used to quickly quantify and qualify shifts in a microbial communities in activated sludge systems, rumen samples and aquifers. Dot-blot analysis does not require prior amplification, although PCR products and cDNA can be used, and therefore circumvents the potential biases PCR amplification may incur. Previous community analysis in soil systems using direct probing of extracted RNA without amplification has been reported for: marsh sediments (Devereux 1996), deep-
Obtaining suitable yields of RNA from agricultural soils is very problematic (section 2.7). Cells adhere to soil particles making the cells difficult to lyse. The RNA may be quickly degraded upon release from the cell in a soil environment. Salts and humics co-precipitate with the RNA and can make direct probing particularly difficult. However, if these problems could be overcome, direct probing of RNA from soil microbial communities could be an excellent method for monitoring microbial community shifts in soil environments.

The goal of this research was to optimize a protocol to rapidly obtain from soil high yields of RNA suitable for membrane hybridizations with hierarchical probes without prior amplification or cloning of the RNA. An objective of the research was to optimize hybridization and washing condition for the hierarchical probes. Another objective was to apply the above methodologies to the Ames Plantation research to test the hypothesis; that changes occur in the microbial community structure that compensate for changing from annual row crops to short rotation woody biomass crops in order to maintain and complete biogeochemical cycles.

2.9 Background on the Ames Plantation Study

The Ames Plantation Study is a joint study by Tennessee Valley Authority (TVA), The University of Tennessee’s Plant and Soil Science Department and The University of Tennessee’s Center for Environmental Biotechnology (CEB). The study was designed to assess the environmental impact of changing from growing annual
row crops to growing perennial tree crops. During the study the soil physical, chemical and biological changes will be monitored to determine if perennial tree crops will be more economically and environmentally beneficial than are the row crops currently in place.

According to Tyler et al, current socioeconomic and environmental demands indicate a need for examination of alternative agricultural systems; one of which includes integrating trees through the adoption of agroforestry principles. Congruent with this is the need for an alternative fuel source to coal; a fuel source that will have no net CO₂ emissions, low SO₂ emission and low NO emission (Tyler et al 1996). Another factor is the Public Utilities Regulatory Policies Act (PURPA), which requires utilities to purchase the surplus electricity generated from small electric power producers (Thorton et al 1996). Since the passage of PURPA the biomass power industry has grown from about 200MW in 1979 to more than 6000MW in 1990 (Bain 1993). The Department of Energy (DOE) is predicting far greater implementation of biomass power by the year 2010, projecting that up to 20GW of capacity will be on line (Thorton et al 1996). An analysis of the potential land base for producing biomass crops in the U.S. has shown that the Northeast, South Central and Southeastern states are the most suitable for production of biomass crops (Wright and Hughes 1993). TVA has an interest in creating an alternative to their coal-fired operations to reduce CO₂, SO₂, and NO emissions. Also, biomass crops increase the carbon content of the soil in which they are planted, thus serving as a sink for carbon. TVA also proposes that implementing growing biomass crops would create jobs and
introduce a new agricultural option for area farmers. DOE and TVA are investigating using 145.3 million hectares of biomass crops to supply 10% of their power requirements in the future. Most of this land (~92%) is currently being used for growing row crops and as pastures (Tyler et al 1996).

Tyler et al proposed evaluating the effects on an agricultural system where short rotation woody biomass crops (SRWCs) are rotated with conventional row crops. The study will document the soil physical, chemical and biological changes that occur over time in a soil after converting from row crops to SRWCs. The study will monitor the soil nitrogen requirements under each crop and the economics and environmental benefits from changing from row crops to SRWCs (Tyler et al 1996). Conversion of row crops to SRWCs is expected to affect the water quality by reducing soil erosion as well as levels of nitrate, phosphorus, pesticides and herbicides in surface runoff and groundwater (Ranney and Mann 1994). But research on the impact of this type of conversion on water, air and land resources have been very limited and such assessments are needed before large scale conversions of cropland to SRWCs is implemented.
3. MATERIALS AND METHODS

3.1 Ames Plantation Study

3.1a Experimental Design

Three sites were selected for studying the effects of converting row crops to SRWCs. One of the sites is the Ames Plantation, located in the loess belt of west Tennessee near Grand Junction. The site is on the University of Tennessee’s Agricultural Experiment Station at Ames. Aerial photographs, previous soil surveys and detailed topographic maps of each site were used to establish replicate watersheds so the groundwater chemistry and the physical, chemical and biological properties of the soil would be similar in all test plots. At the Ames Plantation site, the soils and slopes were particularly uniform (Joslin and Schoenholtz 1997).

Sycamore trees (Plantinus occidentalis L.) were planted as seedlings in 1983 and harvested in 1993. Regrowth from the remaining stumps will be harvested again in 1998. At another test area at Ames Plantation, a second crop of sycamore trees was planted in 1995 in an area totaling 5 hectares. The sycamore trees plots were compared with a silage corn/winter wheat field that was covered with fescue grass to prevent erosion in between growth of the two major crops (Joslin and Schoenholtz 1997).

The soil biological parameters that were compared between the conventional crops and SRWCs include populations of bacteria, fungi and other microfauna and mesofauna, including arthropods, nematodes and earthworms. The bacteria and fungi
were monitored by the Plant and Soil science group using: chloroform fumigation, Phospholipid Fatty Acid Analysis (PLFA) and DNA analysis for relevant functional genes.

As part of this thesis research, the bacteria and fungi were also monitored using 16S rRNA analysis. The soil from test plots was extracted and the 16S rRNA was bound to a membrane using the dot-blot technique. A nested probe analysis was performed to see if the microbial community present in the sycamore test plots differed significantly from the microbial community present in the corn field test plots.

The Ames Plantation Project consisted of four sample types; (1) a no-till corn silage/ winter wheat field covered by fescue grass at the time of sampling, (2) a young sycamore tree (*Plantinus occidentalis* L.) plantation planted in February 1995 (i.e., three years old at the time of sampling), (3) an old sycamore tree (*Plantinus occidentalis* L.) plantation planted in 1983 and harvested in 1993 (i.e., five years of regrowth at the time of sampling from a 15 year old tree base), and (4) an undisturbed mixed hardwood forest. The test plots at Ames Plantation were arranged as follows in Figure 1. The cornfield and young sycamore tree plots were adjacent to one another. The hardwood forest bordered the young sycamore trees. The older sycamore tree plantation was located approximately 1.5 miles away, but in a similar soil type. Each treatment was subdivided into three plots. The cornfield plots measured 50 by 100 meters and were separated by a raised berm to prevent the flow of water between the plots. The young sycamore trees were similarly arranged.
The old sycamore trees were planted with spacing similar to the young sycamore trees, but the plots were smaller (15 by 17 meters).

As previously mentioned, at the time of sampling the cornfield was covered with fescue grass to prevent erosion until the corn was planted. The young sycamore trees possessed a small amount of ground cover consisting of dried leaves from the previous two years growth. In contrast, the old sycamores possessed a very heavy ground cover of detrital leaves and scrubby bushes. The hardwood forest had a deep organic horizon of degraded material comprised of degraded leaves and herbaceous cover.
3.1b Statistical Analysis

The Ames Plantation data was analyzed to determine if statistical differences existed between the four test plots; the cornfield, the young sycamore trees, the old sycamore trees and an established hardwood for the bacterial populations as determined by hybridization results using the eleven probes; Univ1390, Eub338, Euk, Arch915, Alflb, Bet42a, Gam42a, SRB385, HGC69a, LGC and CF319a. Each test plot was randomly sampled nine times (n=9) except for the hardwood forest (n=3) where only three samples were taken.

The 16S rRNA was extracted from the samples and blotted to eleven replicate membranes. The eleven probes listed above were used to detect the RNA bound to the membrane (a different probe for each membrane). The radioactive signal from the probe was detected using a phosphor-imaging system. The amount of probe bound to the RNA was quantified by the phosphor-imaging system and reported as counts. The counts are unitless and do not directly correspond to anything other than the intensity of the probe signal. The counts obtained for each sample was compared to the counts obtained from a standard curve of RNA of known concentrations blotted concurrently on each membrane. This resulted in a report of RNA concentrations in nanograms for each sample using each probe.

The data was imported into Statgraphics Plus for Windows (version 2.0, Manugistics) for statistical analysis. First the data was checked to see if any outliers were included in each sample set. For example, all cornfield sample results obtained with the universal probe were analyzed to see that the data was normally distributed...
and all values were within three standard deviations of the mean. A cutoff value of three standard deviations from the mean was used because soil samples can be very heterogeneous so the least stringent condition was utilized. Next a box and whiskers plot was created which also highlights potential outliers. If either method suggested an outlier, the original datafile containing the membrane pixels was checked to ensure that the high or low number was not created due to an anomaly on the membrane. Some spotting occurred on a few of the membranes, perhaps due to non-specific binding of the probe. At times the spots did not interfere with the actual dot-blots, but in three cases, a spot was found on top of a dot-blot so that data was excluded in the calculation of the mean.

Once all the data was double-checked, several statistical analyses were performed using Statgraphics for each probe hybridization dataset. The first analysis was a basic Summary of Statistics for each test plots data. The average, variance and standard deviation were computed for each test plot. The Summary of Statistics also listed the minimum and maximum for each test plot and the standard skewness and kurtosis. The standard skewness and kurtosis can be used to indicate if the sample was from a normally distributed sample set. Standard skewness and kurtosis values that are highly positive or highly negative can indicate that the sample set is not evenly distributed.

Next, an Analysis of Variance (ANOVA) was run to compare the variance between the test groups. The ANOVA then calculated a probability (p-value) that the variances between the test groups were equal. If the p-value was less than 0.05 then
there was a statistically significant difference between the means at a 95% confidence level.

Lastly, a multiple range test was run using the Fisher’s least significant difference procedure to see which test groups’ mean was significantly different from one another. The multiple range test compared the means of 1) the cornfield to the hardwood 2) the cornfield to the old sycamore trees 3) the cornfield to the young sycamore trees 4) the hardwoods to the old sycamore trees 5) the hardwoods to the young sycamore trees and 6) the old sycamore trees to the young sycamore trees. In this pairwise fashion, each test plot was compared to all other test plots and significant differences between their means were highlighted. With the Fisher’s least significant difference procedure, there is a 5% risk of calling each pair of means significantly different when the actual difference equals zero. After all the data presented in nanograms was analyzed, the relative percentage of each sample was similarly compared and the significant differences were determined for each test plot for each probe type.

3.1c Collection and Storage of Samples

The samples were collected in March 1998. A numerical grid was devised for each plot to ensure that each plot was randomly sampled. For the cornfield and young sycamore plots, each 10 square meters of a plot was numbered 1 through 150. The old sycamore plots were divided into 3 square meter regions and numbered 1 through 25. The plot numbers were randomly pulled from a bag and marked on a grid diagram of the sample test plots. The corresponding numerical position in each plot was then
sampled. Each plot was sampled three times for a total of nine samples per treatment. Except for the hardwood forest, where only three samples were taken.

Samples were obtained using a hand-held corer approximately 5cm in length and 5.5cm in diameter. The device was cleaned with chloroform between each plot to ensure that no cross-contamination occurred between treatments or plots. The samples were then put in labeled bags and placed on dry ice. After transportation to the University of Tennessee, the samples were immediately transferred to a –80°C freezer for storage until time for extraction.

3.2 Preparation of 16S rRNA Standards from Pure Cultures

Eight organisms were selected to represent the domains or groups of interest; an Archaea, a Eukaryote, and seven representatives of the Eubacteria domain; the Alpha, Beta, Gamma and Delta subgroups of the Proteobacter, High and Low G+C DNA content and Cytophaga/Flavobacterium/Bacteriodes groups. The specific organisms selected to represent each domain, group or subdivision are listed in Table 1 along with their growth media and growth temperatures. The recipes for the organisms’ growth media can be found in Appendix 3. The probe used to detect each organism is also listed in Table 1. The sequence, target and reference for each probe can be found in Table 3 (section 3.6a).

The cultures were harvested at mid-log phase to obtain the maximum quantity of rRNA. Twenty milliliters of each culture was placed in a 30ml Oak Ridge Tubes that had been scrubbed clean and finally rinsed with de-ionized water and then autoclaved.
for at least 1 hour to degrade RNases. The cultures were centrifuged at 8,000 rpm in a

Table 1 - Reference Organisms and Their Growth Conditions

<table>
<thead>
<tr>
<th>Group or Domain (Probe in Table 3)</th>
<th>Representative Organism</th>
<th>Culture Source</th>
<th>Growth Media</th>
<th>Growth Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha (Alflb)</td>
<td><em>Brevimonas species</em></td>
<td>CEB Culture #256</td>
<td>ATCC Media #3</td>
<td>room temperature</td>
</tr>
<tr>
<td>Beta (Bet42a)</td>
<td><em>Alcaligenes eutrophus</em></td>
<td>B30P4</td>
<td>PAS Media with 5% POL</td>
<td>room temperature</td>
</tr>
<tr>
<td>Delta (SRB385)</td>
<td><em>Desulfovibrio vulgaris</em></td>
<td>ATCC 29579</td>
<td>ATCC Media 1249</td>
<td>37°C</td>
</tr>
<tr>
<td>Gamma (Gam42a)</td>
<td><em>Pseudomonas putida</em></td>
<td>IPL5</td>
<td>PAS Media with 5% POL</td>
<td>room temperature</td>
</tr>
<tr>
<td>High G+C DNA Content (HGC69a)</td>
<td><em>Arthrobacter globiformis</em></td>
<td>ATCC 8010</td>
<td>ATCC Medium #3</td>
<td>26°C</td>
</tr>
<tr>
<td>Low G+C DNA Content (LGCa,b,c)</td>
<td><em>Bacillus species</em></td>
<td>ATCC 14807</td>
<td>ATCC Medium #3</td>
<td>30°C</td>
</tr>
<tr>
<td>Cytophaga/Flavobacterium</td>
<td><em>Flavobacterium species</em></td>
<td>ATCC 29790</td>
<td>ATCC Medium #3</td>
<td>30°C</td>
</tr>
<tr>
<td>Archaea (Arch915)</td>
<td><em>Halobacterium halobium species</em></td>
<td>ATCC 43214</td>
<td>ATCC Medium #217</td>
<td>30°C</td>
</tr>
<tr>
<td>Eukarya (Euk)</td>
<td><em>Glycine soya</em></td>
<td>RNA isolated by Brad Day</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dr. Gary Stacey's Laboratory/UTK</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Beckman J2-21 centrifuge using a pre-cooled JA-20 rotor. The media was then
decanted and the resulting pellet was extracted using the Pure Culture Extraction
Method described in section 3.3c. After treatment with DNase, as described in
Section 3.4a, the resulting RNA pellet was resuspended in 50-100 μl of DEPC treated
dH₂O and transferred into an RNase-free Eppendorf tube and stored at -80°C until
use.

The RNA was quantified using a Beckman D-70 Spectrophotometer. The optical
density at 260 and 280 nanometer wavelengths was measured using DEPC-treated
water to zero the spectrophotometer. The A₂₆₀/A₂₈₀ value gave an indication of the
purity of the RNA, as the value should be 2.0 for highly pure RNA. Contamination
with protein or phenol will decrease the A₂₆₀/A₂₈₀ ratio. An estimate of the amount
of RNA was obtained by multiplying the A₂₆₀ value by 40μg/μl and the dilution
factor. For example, RNA with an A₂₆₀ equal to 0.1106 would contain
approximately 1.47μg/μl when 3μl of sample was diluted into 1000μl of DEPC
dH₂O.

An aliquot of each standard was diluted in DEPC dH₂O to a final concentration of
1μg/100μl. This working stock was used to make standards at 200, 100, 50, 10, 5 and
1ng/100μl concentrations. All standards were stored at -80°C.

To check the integrity of the RNA, each RNA standard was run on a 1% Low
EEO agarose gel (Fisher) made with 0.5X TBE. Approximately 10μg of standard
was mixed with dH₂O to a final volume of 10μl. The standard was then mixed with
5μl of RNA Loading Dye and heated for 15 minutes at 65°C to denature the RNA
prior to loading onto the gel. The gel was run at 90 volts for 1.5 hours. The RNA was visualized using Sybr Gold dye (Molecular Probes) and the Molecular Dynamics Storm 840 ImageQuaNT imaging software.

### 3.3 Comparison of Different Extraction Protocols

Several protocols and mixtures thereof were tried on various soil types and soil volumes. Most trials consisted of simultaneous extraction of a particular soil type and size using several of the different methods and then comparing the resultant pellet size and ability to hybridize with the Universal probe when the RNA was applied to a membrane using the dot-blot protocol detailed in Section 3.6. The methods used are described below in Sections 3.3a through 3.3g. Soils used in the trials included soil from Columbus Air Force Base (CAFB) contaminated with BTX, lysimeter soil mixed with transformer oil, activated sludge from a CEB reactor, and lake bottom sediment from Lake Michigan.

Four methods were chosen which yielded the greatest quantity of hybridizable 16S rRNA from pure culture extractions or in the trials using CAFB soil, lysimeter soil, activated reactor sludge or the Lake Michigan sediment to determine which extraction method would yield the greatest quantity of hybridizable 16S rRNA from the Ames Plantation Soils. The methods are identified as the Modified Guanidine Thiocyanate method (Alm/Stahl 1998, Section 3.3a), Ogram Method (Ogram 1995, Section 3.3b), Fleming Extraction Method (Fleming 1998, Section 3.3c) and Fleming Pure Culture Method (Modified Fleming 1993, Section 3.3d).
A radiolabeled transcript was produced using an in vitro transcription kit (MEGAscript Ambion). The method outlined by the manufacturer was followed (see Section 3.3h). After counting an aliquot of the transcript an equal volume of the transcript was added to twelve tubes containing 5g of Ames Plantation soil each. The transcript was gently mixed throughout the soil and left setting on ice for 5 minutes. Three replicate tubes were subjected to each method as detailed below in Sections 3.3a through 3.3d. At each extraction step an aliquot equal to 1/100th of the total aqueous volume was removed and placed in Beckman ReadySafe Scintillation Cocktail and counted using the Beckman LS5000TD scintillation counter. The amount removed and the total aqueous volume recovered at each step was recorded. A calculation was made to determine the total amount of transcript recovered at each step.

\[
\text{Total CPM of Transcript Added Initially} = \left( \frac{\text{CPM of Aq. Aliquot} \times \text{Vol. of Aq. Aliquot} \times \text{Total Vol. of Aq. Portion Recovered}}{\text{Total Vol. of Aq. Portion Recovered}} \right)
\]

A plot for the amount of transcript recovered versus the procedural step was constructed to determine at what point the recovery of the transcript either failed or succeeded.

3.3a Extraction of rRNA from soil by Modified Guanidine Thiocyanate Extraction (Alm and Stahl 1996)

In a 50 ml Falcon tube, 1 g of polyvinylpolypyrrolidone (Holben 1992) was added to 25 ml of lysis buffer (6.29M guanidine thiocyanate, 25mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1M b-mercaptoethanol) (Chomczynski 1987) was gradually added
and mixed with 15g (wet weight) of Ames Plantation Soil. Five grams of zirconium glass beads were added and vortexed for 5 minutes. The mixture was centrifuged for 10 minutes at 3,000 RPM in a Beckman TJ-21 Centrifuge with a TH-4 Swing Bucket Rotor.

The lysis solution was decanted into a fresh 50ml Falcon tube. An equal volume of phenol (pH 5.2): chloroform (4:1v/v) was added, mixed and centrifuged as above. The supernatant was transferred to a fresh tube using a disposable pipette and an equal volume of chloroform: isoamyl alcohol (24:1v/v) was added, mixed and centrifuged as above.

The supernatant was transferred to a 150ml Corex bottle (baked 4 hrs. at 180°C) using a disposable pipette and taking care not to disturb the interface. Precipitation of the nucleic acids occurred by addition of 20μl of 2M MgCl₂, 7.5ml of 10M ammonium acetate (final concentration 0.5V of 7.5M ammonium acetate), and 2 volumes of ethanol. The bottle was then inverted 10 times to mix the solution and placed at −20°C overnight.

The extract was centrifuged for 30 minutes at 4°C and 10,000 RPM. The supernatant was carefully decanted and any residual liquid was pipetted away. The resultant pellet was transferred to an eppendorf with 400μl DEPC treated dH₂O. It should be noted that unlike DNA pellets, the RNA should not be allowed to dry entirely, as this makes resuspension extremely difficult. Furthermore, one should always work as quickly as possible with RNA. The samples were stored at −80°C until enzymatic treatment (Section 3.4).
3.3b Isolation of RNA from Low-Biomass Sediments (Ogram 1995)

Fifteen grams of soil from the Ames Plantation Plant was homogenized with a sterilized mortar and pestle and added to a 30ml Oak Ridge tube that had been autoclaved to degrade RNases (1hr, 121°C, 15psi, dry cycle). Eight milliliters of Extraction Buffer (0.2M Sodium Phosphate Buffer, 0.1M EDTA, pH 8.0) was added and mixed thoroughly. To this tube, 1.0ml of 10% SDS was added and mixed by shaking briefly.

The tube was then placed in a boiling water bath for 5 minutes. Periodically, the tube was removed and carefully shaken to ensure even warming. Next, the tube was place in the -80°C freezer for 30 minutes. Afterwards, the sample was thawed for 15 minutes at 65°C, mixing occasionally to ensure total thawing. The sample was then centrifuged at 10,000 RPM for 15 minutes at 10°C. The lysate was decanted into a fresh tube to which 10ml of Denaturing Solution (4M-guanidium isothiocyanate, 0.5% sarcosyl, 25mM sodium citrate, pH 7.0) had been added to inactivate any remaining nucleases.

The remaining soil pellet was resuspended in 5ml of Extraction Buffer and again boiled for 5 minutes. The freeze/thaw cycle was not repeated. After centrifuging as before, the resulting supernatant was combined with the aqueous portion from the first lysis step.

To remove proteins and other cellular components, 10ml of phenol (pH 5.2) and 5ml of chloroform: isoamyl alcohol (24:1 v/v) was added and the sample carefully mixed by shaking. This solution was centrifuge at 6,000 RPM for 10 minutes at 4°C.
The aqueous portion was transferred to a fresh tube using a sterile transfer pipette, taking care not to disturb the interface.

To ensure any remaining phenol was removed, the aqueous solution was re-extracted with 10ml of chloroform: isooamyl alcohol (24:1 v/v). After mixing and centrifuging, as above, the aqueous portion was transferred using a sterile transfer pipette to a 30ml Corex tube (baked 4 hrs. at 180°C).

The nucleic acids were precipitated with 0.1Vol 3M NaAc (pH 5.2) and an equal volume of isopropanol at room temperature. Precipitation occurred overnight at 4°C. It is important that the isopropanol be at room temperature to limit the amount of SDS and salts that might co-precipitate along with the nucleic acids in this method. Experiments showed that this precipitation cannot occur at -20°C without massive amounts of coprecipitates falling out of solution.

The nucleic acid containing solution was centrifuged at 10,000 RPM for 30 minutes at 4°C to pellet the precipitate. The isopropanol was then very carefully decanted and pipetted away from the pellet. For some sample types, the precipitate may look like oily droplets adhered to the side of the bottle, for others, a huge dime-sized pellet may be obtained.

The nucleic acids were resuspended and transferred using 400μl of DEPC treated dH₂O to an RNase-free eppendorf. The extract was then stored at -80°C until enzymatic treatment (Section 3.4).
3.3c Extraction of RNA from Soil Microcosms (Fleming et al. 1998)

Ten ml of extraction buffer (100mM Tris HCl, 1.4M NaCl, 20mM EDTA and 1% SDS, pH 8.0) was added to 15g of soil from Ames Plantation in a 30ml Oak Ridge tube that had been autoclaved (1hr, 121°C, 15psi, dry cycle). Five ml of phenol (pH 8.0) and 5ml chloroform was added, mixed and heated at 60°C for 5 minutes with intermittent shaking to ensure equal heating. The solution was then shaken on a wrist-action shaker for 5 minutes followed by placing the samples on ice for 5 minutes. The tubes were then centrifuged at 10,000 RPM for 15 minutes at 4°C. To remove any traces of phenol, the aqueous portion was transferred to a fresh 30ml Oak Ridge tube and 10ml chloroform: isoamyl alcohol (24:1 v/v) was added. The tubes were again shaken, placed on ice and centrifuged as above.

The aqueous portion was transferred to a 30ml Corex tube (baked 4 hrs. at 180°C) and 5µl of linear acrylamide (Ambion), 10ml of isopropanol and 0.1 volume 3M NaAc were added to precipitate the nucleic acids. The tubes were mixed by inversion ten times and stored overnight at −20°C. The nucleic acids were pelleted by centrifugation at 10,000 RPM for 30 minutes at 4°C. The supernatant was decanted and the resulting pellet was resuspended in 400µl of DEPC treated dH₂O and transferred to an RNase-free eppendorf tube. The RNA was stored at −80°C until DNAse treatment (section 3.4a).

3.3d Isolation of RNA from a Pure Culture (Modified from Fleming et al. 1993)

Fifteen grams of soil was placed in a 30ml Oak Ridge tube that had been autoclaved (1hr, 121°C, 15psi, dry cycle). Ten ml of lysis buffer (0.05M NaAc,
0.05M NaCl, 0.003M EDTA and 1.5% SDS (pH 5.2)] was added and mixed with vigorous shaking. To this, 5ml of phenol pre-warmed to 60°C and 5 ml of chloroform: isoamyl alcohol (24:1 v/v) was added, mixed and incubated at 60°C for 5 minutes. The tubes were then shaken on a wrist-action shaker for 5 minutes. Then cooled on ice for 5 minutes and centrifuged at 10,000RPM for 20 minutes at 4°C. The upper aqueous phase was removed with a sterile disposable pipette to a clean 30ml autoclaved Oak Ridge tube. An additional 10ml of chloroform: isoamyl alcohol (24:1 v/v) was added to re-extract the aqueous phase.

The solution was mixed on the wrist-action-shaker for 5 minutes and put on ice for 5 minutes. The solution was then centrifuged as above and the aqueous portion was transferred using a sterile disposable pipette to a 30ml Corex tube (baked 4 hrs. at 180°C).

To precipitate the nucleic acids, 0.1 volume of 3M NaAc and 1 volumes of isopropanol was added and mixed by inversion ten times. Precipitation was allowed to occur overnight at -20°C. The tubes were then centrifuged at 10,000 RPM for 30 minutes at 4°C. The supernatant was carefully decanted and the resulting pellet resuspended and transferred to a RNase-free eppendorf with 400μl of DEPC treated dH2O. The RNA solution was stored at -80°C until DNAse treatment (section 3.4).

3.3e Extraction of Ribosomal RNA from Soil with High Humic Content (Hahn et al 1990)

Ten grams of soil was suspended in 30 ml of 0.1M Na4P2O7 buffer in an autoclaved Oak Ridge tube and shaken for 15 minutes on a wrist-action shaker. The
slurry was then centrifuged for 10 minutes at 10,000 RPM at 4°C. The supernatant was decanted into a fresh tube and centrifuged 10 minutes at 15,000 RPM at 4°C. The supernatant was decanted and the resultant pellet saved. The original soil was reextracted with 20 ml of TE-buffer (10mM Tris, 1mM EDTA, pH 8.0). It was then shaken for 15 minutes on the wrist-action shaker and centrifuged for 10 minutes at 10,000 RPM at 4°C. The supernatant was added to the pellet from the first tube. This was centrifuged 10 minutes at 15,000 RPM at 4°C. The supernatant was decanted and the pellet was saved.

The pellet was washed two times with TE-Buffer and centrifuged each time for 10 minutes at 15,000 RPM at 4°C. Next, 5 ml of 7.5M Guanidine HCl/ 1M Tris, pH 7.0 was added to the pellet. After thoroughly mixing, the solution was sonicated for three 30 sec bursts interspersed with 30 second rest intervals. To remove insoluble residues, the solution was centrifuged for 10 minutes at 3000g, 4°C. The supernatant was saved in a fresh tube.

The nucleic acids were precipitated with 0.1 vol 3M NaAc (pH 5.2) and 2.5 volumes of ethanol overnight at -20°C. To pellet the nucleic acids the solution was centrifuged for 20 minutes at 10,000 RPM and 4°C. The pellet was resuspended in 400μl DEPC treated dH2O and extracted two times with 200μl of phenol (pH 8.0) and 200μl of chloroform: isoamyl alcohol (24:1 v/v). After a final extraction with 400μl of chloroform: isoamyl alcohol (24:1 v/v), the nucleic acids were reprecipitated as above with ethanol.
If the pellet was still brown, and it usually was, it was resuspended in 400μl of the 7.5M guanidine HCl/1M Tris solution and reprecipitated as above. After centrifugation, the resulting pellet was reextracted with phenol/chloroform as above. This was repeated until a colorless pellet was obtained. The pellet was then resuspended in 400μl DEPC treated dH2O and stored at -80°C until enzymatic treatment (Section 3.4).

3.3f Rapid and Direct Method for Extraction of RNA from Soil (Borneman et al 1997)

The following method uses the Fast Prep RNA kit (BIO101 Vista, CA Cat. # 6030-600) and the Savant (Farmingdale, NY) bead-beater system to extract RNA from soil. Five hundred mg of soil was added to 990μl of 200mM sodium phosphate buffer (pH 8.0) and 410μl of chaotropic RNA stabilizing reagent (CRSR) in a green FastPrep tube containing beads. The mixture was shaken in the FastPrep machine for 45 seconds at 6m s⁻¹. The mixture was placed on ice for 1 minutes, then centrifuged for 5 minutes at 13,000g. The supernatant (~500μl) was transferred to a 1.5ml Eppendorf tube and 250μl of CRSR, 50μl 80% glacial acetic acid, 500μl of phenol acid reagent (PAR) and 100μl chloroform: isoamyl alcohol (CIA) (24:1 v/v) was added and mixed. The acidification of the solution causes the DNA and proteins to be more soluble in the organic phase, while the RNA remains in the aqueous phase. After centrifuging for 5 minutes at 13,000g, the aqueous portion was transferred to a new eppendorf tube. The nucleic acids were precipitated on ice for 30 minutes with 0.1V 3M NaAc and an equal volume of isopropanol. The resultant pellet was
resuspended in 20μl of SAFE reagent and stored at –20μl until the RNA was applied to a membrane and tested for hybridization with the Universal probe.

3.3g Devereux Method For Nucleic Acid Extraction (Devereux Laboratory Protocol 1993, Personal Communication)

Ten grams of soil was combined in a small bead-beater cup with 10g of baked zirconium beads, 1.75ml of phenol (pH 5.2), 500μl 10% SDS, 7.5ml rRNA extraction buffer (50mM NaAc, 50mM EDTA, pH 5.2). The sample was beaten on ice for 3 cycles of 15 seconds on and 1 minute off. The lysate was transferred to a 30ml Oak Ridge tube and the bead-beater cup was rinsed with 2ml of buffer and this was added to the tube. The lysate was centrifuged in a JA-20 rotor for 10 minutes at 8,000 RPM and 4°C. The supernatant was transferred to a fresh tube and stored on ice. The soil pellet again washed with 5ml of rRNA buffer. After centrifugation, the supernatant was combined with the first supernatant.

This method was tried only with the CAFB soils and the following steps were omitted due to the CAFB soils low humic concentration, but should be included if the soil has a high humic content. Three grams of acid-washed polyvinylpolypyrrolidone (PVPP) (appendix 1) is added to the supernatant and the tube vortexed for 5 minutes then incubated on ice for 20 to 30 minutes. The solution is then centrifuged for 10 minutes at 8,000 RPM and 4°C. The supernatant is transferred to a new tube and reserved on ice while the PVPP is back extracted with 5ml of rRNA buffer. After centrifugation the supernatants should be combined.
The resultant supernatant (with or without PVPP treatment) was incubated for 10 minutes at 60°C with an equal volume of phenol (pH 5.2). The solution was then vortexed and centrifuged for 15 minutes at 10,000 RPM and 4°C. The supernatant was transferred to a new tube and 1/6\textsuperscript{th} volume of 5M NaCl, and 1/9 volume of CTAB (hexadecyltrimethylammoniumbromide) was added, mixed and incubated at 65°C for 5 minutes. The solution was then extracted with an equal volume of phenol: chloroform: isooamyl alcohol (25:24:1 v/v/v). After centrifugation at 10,000 RPM for 15 minutes, the supernatant was transferred to a new tube. The supernatant was reextracted with chloroform: isooamyl alcohol (24:1 v/v) to remove any remaining phenol. The supernatant was transferred to a 30ml Corex tube (baked 4 hrs. at 180°C) and 0.1 volume of 3M NaAc and two volumes of ethanol were added to precipitate the nucleic acids. The tube was then centrifuged at 10,000 RPM for 30 minutes at 4°C. The ethanol was carefully decanted. The pellet was resuspended in 100µl of DEPC treated dH₂O and stored at −80°C until enzymatic treatment (section 3.4).

3.3h Preparation of an In-Vitro Transcript

An in-vitro transcript was prepared in the following manner. The components in Table 2, from a MEGAscript Kit (Ambion), were mixed in 6 replicate tubes and incubated at 37°C for 6 hours. The transcript was precipitated overnight at −20°C with 15µl ammonium acetate stop solution, 115µl nuclease free water and an equal volume of ethanol.
Table 2- Preparation of an In-Vitro Transcript

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Reaction Buffer</td>
<td>2μl</td>
</tr>
<tr>
<td>ATP, CTP, GTP, UTP (cold) - 75mM each</td>
<td>2μl of each</td>
</tr>
<tr>
<td>UTP 75mM *800 μCi/mmol</td>
<td>1μl</td>
</tr>
<tr>
<td>pTRI-Xef control DNA (1.85Kb Xenopus Elongation Factor Transcript)</td>
<td>2μl</td>
</tr>
<tr>
<td>Nuclease free water (for a final volume of 20μl)</td>
<td>4μl</td>
</tr>
<tr>
<td>Enzyme Mix</td>
<td>2μl</td>
</tr>
</tbody>
</table>

After centrifugation, the transcripts were pooled and resuspended in 100μl of DEPC treated dH₂O. One microliter was counted using the Beckman LS5000 TD Scintillation Counter. To ensure the transcript was of the proper size, ten microliters of the transcript solution were run on a denaturing gel containing 1% agarose and formaldehyde (see appendix 1).

3.3i Purification of Extracts Containing Humics

The following methods were tried to remove humic contaminants from RNA extracts; spun-column gel filtration, precipitation with Guanidium HCl and precipitation with Polyethylene glycol (PEG). The purification with Guanidium HCl method is detailed in section 3.3f.

The spun-column gel filtration method was taken from a paper by Moran et al (1993). Sephadex G-75 was equilibrated overnight with sterile DEPC treated dH₂O. A 3-ml sterile syringe was packed with a small plug of sterile glass wool. The wool was pre-wetted with DEPC treated dH₂O and a 2.5ml column of Sephadex G-75 was
prepared. A 0.5ml volume of Sephadex G-75/DEPC treated dH₂O slurry was transferred to the syringe and then centrifuged 5 minutes at 1,400 x g in a Beckman TJ6 swing basket rotor centrifuge. An additional volume of Sephadex G-75 was added to the syringe and centrifuged. This was repeated until a column of 2.5ml of Sephadex G-75 had been obtained. The column was equilibrated with two 300μl applications of sterile DEPC treated dH₂O.

The column was placed in a 50ml Falcon tube to catch the eluent and a 200μl volume of soil extract was added. The column was centrifuged for 5 minutes at 1500 x g. The eluent was transferred to an eppendorf tube and 0.1volume 3M NaAc and 2X volumes of cold ethanol were added to precipitate the RNA at -20°C.

The method for purification of plasmid DNA by precipitation with polyethylene glycol (PEG) in Maniatis (1982) was used to clean humic contaminates from soil extracts. An equal volume of 1.5M NaCl containing 13% (w/v) PEG 8000 was mixed with a soil sample extract in 400μl DEPC treated dH₂O. The solution was left for 2 hours at 4°C. The solution was centrifuged for 5 minutes at 12,000 RPM at 4°C. The resultant pellet was resuspended in 400μl of DEPC treated dH₂O and subsequently extracted with equal volumes of phenol (pH 5.2), phenol/chloroform (1:1 v/v) and reextracted with an equal volume of chloroform. To precipitate the RNA, 0.1 volume of 10M ammonium acetate and two volumes of cold ethanol was gently mixed with the aqueous solution and left setting 10 minutes at room temperature. The solution was centrifuged at 12,000 RPM for 5 minutes to pellet the RNA. The pellet was
resuspended in 400μl DEPC treated dH₂O and the sample was stored at -80°C until enzymatic treatment (Section 3.4).

3.4 Enzymatic Digestion of Samples

3.4a DNase Treatment

To ensure that RNA extracts were free of DNA, all extracts were enzymatically treated with DNase. Pure culture extracts had 0.1 volumes of 100mM MgCl₂/10mM Dithiothreital (DTT) and 1μl DNase I, RNase-free (Boehringer Mannheim, 10U/μl) added to them. They were then vortexed for 30 seconds prior to incubating in a 37°C water bath for 15 minutes at 37°C. The extracts were cleaned with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v). After vortexing for 30 seconds and centrifuging for 5 minutes at 14,000 RPM and 4°C, the aqueous portion was transferred to a fresh RNase-free eppendorf taking great care not to disturb the interphase. It was then re-extracted with equal volumes of chloroform: isoamyl alcohol (24:1 v/v). The aqueous portion was precipitated with 0.1 Volumes 3M NaAc and 2.5 volumes ice-cold ethanol, inverted and stored at -20°C for at least 2 hours to overnight before centrifuging for 20 minutes at 14,000 RPM and 4°C. The resulting pellet was resuspended in 50-100μl of DEPC treated dH₂O.

Soil extracts were treated similarly with the following exceptions. The amount of MgCl₂/DTT co-factor used was increased to 0.2 volumes. Also, 3μl of DNAsel was used. The incubation time was also increased from 15 minutes to 1 hour. Lastly, an additional phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) extraction was
necessary to obtain a cleaner interphase. The extracts were resuspended in 1500μl of 
DEPC treated dH₂O and stored at -80°C.

3.4b RNase Treatment

For a negative control, each soil sample had an aliquot of RNA extract removed 
for RNase treatment to remove all RNA. One-tenth of the original soil extract was 
removed and 4μl of DNase-free, RNase (Boehringer Mannheim, 500μg/ml) was 
added. The sample was vortexed for 30 seconds prior to incubating for 1 hour at 
37°C. The extract was cleaned with an equal volume of phenol: chloroform: isoamyl 
alcohol (25:24:1 v/v/v). After vortexing for 30 seconds and centrifuging for 5 minutes 
at 14,000RPM and 4°C, the aqueous portion was transferred to a fresh RNase-free 
1.5ml tube, taking great care not to disturb the interphase. It was then re-extracted 
with equal volumes of chloroform: isoamyl alcohol (24:1 v/v). The aqueous solution 
was precipitated with 0.1 Volumes 3M NaAc and 2.5 volumes ice-cold ethanol, 
inverted and stored at -20°C for at least 2 hours to overnight before centrifuging for 
20 minutes at 14,000RPM and 4°C. The resulting pellet was resuspended in 1500μl 
of DEPC treated dH₂O for a final concentration equal to a 1/10th dilution of the 
original sample.

3.5 Application of RNA to Membrane (Dot-Blot Protocol)

The dot blot manifold (BioRad) was carefully scrubbed and rinsed. It was then 
soaked for a minimum of 1 hour in a solution of 1N NaOH. Afterwards, it was re-
rinsed and assembled as per the manufacturer’s directions. The membranes used
were 0.45μM Biotrans (+) Nylon Transfer Membrane (ICN Biomedicals Inc.). The membrane was rinsed for 5 minutes in 2X SSC prior to assemblage of the manifold.

The samples and standards were prepared as follows: 100μl of sample or a standard, as the case may be, was added to a 1.5ml tube containing a mixture of 200μl formamide (Fisher), 71.8μl 37% formaldehyde (Fisher) and 20μl 20X SSC. The formamide had been deionized (See Appendix 3) and stored at −20°C until use. The tubes were mixed briefly and incubated at 68°C for 15 minutes to denature the RNA molecules. Afterwards, the 1.5ml tubes were immediately quenched on ice and kept on ice until blotting occurred to prevent the RNA from renaturing.

The solutions were applied to the membrane by loading all 391.8 μl from each eppendorf into a separate well on the dot-blot apparatus. (Please see page 101 in the Results Section for the organization of the Ames Plantation sample blots.) Any unfilled wells were filled with 20X SSC. A slight vacuum, not exceeding 4psi, was applied to the membrane and the blotting solution passed through leaving behind the RNA. For specificity, 500μl of 20X SSC was applied to each well and the vacuum applied to pull the SSC through the membrane along with anything soluble in the 20X SSC. Once all liquid had passed through the membrane, it was dried an additional 5 minutes under a slight vacuum.

The apparatus was disassembled and the membrane transferred to a piece of Whatman filter paper using forceps. The membrane was baked for 1 hour at 80°C to bind the RNA to the membrane. Afterwards, the membrane was transferred to a Kapak/Scotchpak Heat Sealable bag (6.5 x 8 inches) and 10ml of hybridization
solution (0.5M NaH₂PO₄, 1mM EDTA, 7% SDS [pH 7.2]) was added and the bag was sealed. Pre-hybridization occurred for a minimum of 4 hours before addition of a probe. For the Ames Plantation samples, the membranes were pre-hybridized for 2 days. Pre-hybridization and washing occurred at the temperature specified for the probe to be used in each study. For determination of the optimal probe hybridization temperatures see Section 3.6d and page 88.

3.6 Hybridization with Hierarchical Probes

3.6a Preparation of Probes

The probes in Table 3 were used in analyzing microbial community shifts. The hierarchical probes were obtained in one of two ways. Genosys commercially synthesized the Univ1390, Eub338, Arch915, Alf1b, Bet42a, Gam42a, and Delta (SRB385) probes. A working stock of 1µg/µl stock was prepared and stored at -80°C. Working stock probes were prepared by diluting the 1µg/µl stock in TE to a final concentration of 5ng/µl.

The Euk, HGC69a, LGCa,b,c and CF319a probes were prepared using the Beckman Oligo 1000 DNA Synthesizer as per the manufacturer’s directions. Each probe was prepared to a 30nmolar concentration with final detritylation. After the probe was synthesized, it was deprotected to remove the cyanoethyl groups from the phosphate backbone, thus converting the phosphate triester linkage into the diester linkage found in native DNA. Deprotection also removes the isobutyl and benzoyl protecting groups from the exocyclic amines of the bases.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Target</th>
<th>Sequence (5' to 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univ1390</td>
<td>All Domains</td>
<td>GACGGGCGGTGTGTACAA</td>
<td>Zheng et al (1996)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA, position 1390-1408</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eub338</td>
<td>Bacteria</td>
<td>GCTGCCTCCCCGTAGGAGT</td>
<td>Amann et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>16SrRNA, position 338-355</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16S rRNA, position 1195-1209</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arch915</td>
<td>Archaea</td>
<td>GTGCTCCCCCGCAAATTCTT</td>
<td>Stahl and Amann (1991)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA, position 915-934</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alflb</td>
<td>α-Subdivision of Proteobacteria</td>
<td>CGTTCGYTCTGAGCCAG</td>
<td>Manz et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA, position 19-35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bet42a</td>
<td>β-Subdivision of Proteobacteria</td>
<td>GCCTTCCCACCTCGTTT</td>
<td>Manz et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>23S rRNA, position 1027-1043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gam42a</td>
<td>γ-Subdivision of Proteobacteria</td>
<td>GCCTTCCCACATCGTTT</td>
<td>Manz et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>23S rRNA, position 1027-1043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRB385</td>
<td>δ-Subdivision of Proteobacteria</td>
<td>GGGCGTCGCTGCTGTCAGG</td>
<td>Amann et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA, position 385-402</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGC69a</td>
<td>Gram-positive bacteria with high DNA G+C Content</td>
<td>TATAGTACCACCGCGT</td>
<td>Roller et al. (1994)</td>
</tr>
<tr>
<td>LGCa, b, c</td>
<td>Gram-positive bacteria with low DNA G+C Content. 16S rRNA, position 979,982,982 for a,b,c respectively.</td>
<td>Sequences were obtained from Dittmar Hahn via personal communication. Not yet published.</td>
<td>R. Amann Unpublished</td>
</tr>
<tr>
<td>CF319a</td>
<td>Cytophaga-Flavobacterium cluster of the CFB phylum</td>
<td>TGGTCGTGTTCAGTAC</td>
<td>Manz et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>16S rRNA, position 319-336</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
500 µl each of ammonium hydroxide (J.T. Baker) and methylamine (Aldrich) was added to the synthesis vial and mixed via an attached syringe. The solution containing the probe was heated at 65°C for 5 minutes and then cooled on ice.

The solvents were allowed to evaporate at room temperature for 20 minutes prior to full removal by vacuum centrifugation (SpeedVac, Savant). Once all solvents had been removed, the probe was suspended in 50 µl of TE and placed −80°C for storage.

The hierarchical probes were prepared as in the Table 4 below. After mixing, the probes were incubated at 37°C for 30 minutes in a Perkin Elmer Cetus DNA Thermal Cycler set on a soak cycle.

The probe was purified using a Stratagene nuc-probe column pre-wetted with 90 µl of STE (0.1M NaCl, 10mM Tris-HCl, 1mM EDTA, pH 8.0) buffer. After pre-wetting the column, all of the probe mixture was added and carefully pushed through the column using air pressure from a 10ml lure-loc syringe. The column was then washed with 90 µl of STE (pH 8.0) buffer to remove any remaining probe.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>sterile dH2O</td>
<td>35 µl</td>
</tr>
<tr>
<td>5X Forward Reaction Buffer (Gibco BRL)</td>
<td>10 µl</td>
</tr>
<tr>
<td>probe (20 ng/µl final concentration)</td>
<td>X µl</td>
</tr>
<tr>
<td>Most probes were at a 5ng/µl working stock concentration.</td>
<td></td>
</tr>
<tr>
<td>T4 Polynucleotide Kinase (Gibco BRL)</td>
<td>1 µl</td>
</tr>
<tr>
<td>32P gamma ATP (166 µCi/µl) (ICN Biomedicals, Inc.)</td>
<td>1 µl</td>
</tr>
</tbody>
</table>
A 5µl aliquot of the purified probe mixture was placed in 10ml of Beckman Ready Safe liquid scintillation cocktail for counting by a Beckman LS5000TD scintillation counter. The probe counts were at least $10^7$ counts per minutes/µl. An amount equal to $10^8$ CPM/µl of the purified probe mixture was added to each 10ml of hybridization solution in the bags containing blots. Hybridization occurred overnight at the temperature specified for each probe (see Section 3.6d and page 88).

3.6b Washing of Blots to Remove Unbound Probe

Each blot was rinsed in washing buffer (2X SSC and 0.1% SDS) for 20 minutes at a specified temperature (see section 3.6d). The washing buffer was decanted and replaced with fresh washing buffer and the rinsing was repeated for two more washes. The blot was placed on a piece of Whatman #2 filter paper for 15 minutes to air dry before placing the blot in a sealable bag for visualization as in section 3.6c.

3.6c Detection and Quantification of RNA

One of two methods, x-ray film or phosphor-imaging, was used to visualize the blots. The blot was placed in a film cassette holder to view a blot by x-ray film. In a dark room, an intensifying screen was placed over the blot and a piece of Kodak Biomax MS film was placed over the intensifying screen. Another intensifying screen was laid over the film and the cassette was closed and placed at $-80^\circ$C for exposure times varying from 1 hour to 48 hours depending on the probe intensity and the amount of RNA present. The film was then developed and quantified using a Hewlett Packard ScanJet IIC flat bed scanner and Sigma gel imaging software.
All Ames Plantation blots were visualized using the Molecular Dynamics Storm 840 phosphor-imaging system. The blots were placed in one cassette and one Kodak Storage Phosphor Screen SO230 imaging screen was used. The cassette sat at room temperature for a period of 2 hours. The phosphor screen was then scanned using the Storm 840 machine. Each blot was quantified individually using Molecular Dynamic's ImageQuaNT imaging software (Version 4.2a).

The intensity of each dot on the membrane, measured in counts for the Storm System, was quantified using a portion of the membrane void of any sample for the background correction factor. The data obtained were imported into an Excel spreadsheet. A standard curve of the counts versus the concentration of reference standard blotted was prepared for each blot. The amount of RNA present for each sample was calculated using this standard curve for comparison. For the Ames Plantation samples, the RNase negative control sample was used to subtract the background obtained from non-specific binding of the probe to humics or other contaminants for each sample. A comparison of each group probe relative to the total RNA, obtained using the Universal probe, was made. This comparison gave a value of the relative percentage of each group to the total population.

3.6d Determination of Optimal Wash Temperature

The optimal temperature for hybridization was determined by one of two methods. The first method involved preparing four to six replicate blots with a series of each reference standard organism’s RNA ranging from 200 to 1 ng/100μl. Each
blot was then hybridized and washed at a temperature bracketing the theoretical $T_d$ (calculated using the formula for calculating $T_d$ in Section 2.6 g) shown in Table 5.

For example, the theoretical temperature for the Gam42a was calculated to be 48°C. Six Gam42a probes were prepared by end-labeling with $^{32}$P-$\gamma$ATP and purified as in Section 3.6a. The replicate blots were placed in separate bags and pre-hybridized with hybridization solution for a minimum of 4 hours. After counting the Gam42a probe preparations, an equivalent amount of probe was added to each bag and the bags were resealed and hybridization occurred overnight. One blot was washed at 40°C, one blot was washed at 45°C, and so forth for the following temperatures: 50, 55, 60, 65°C.

The probe was visualized using Kodak Biomax MS film incubated with the blots for a period of 24 hours at -80°C. After the film was developed, a comparison of the specificity of the probe at the various temperatures could be assessed. From these observations, the optimal temperature for probe specificity could determined, i.e. the temperature at which the probe bound the most to its target and the least to non-target organism’s RNA. In the case of Gam42a, it was found that at 55°C, the probe lost some of its binding capacity for the beta group reference organism’s RNA.

Competitive hybridization was accomplished by adding an equal volume of non-$^{32}$P labeled Bet42a probe to the bags containing $^{32}$P labeled Gam42a. This removed the signal almost completely from the beta reference organism RNA when using the Gam42a probe. Competitive hybridization also reduced non-specific binding of the Bet42a probe to gamma reference organism RNA.
### Table 5 - Calculated $T_d$ and Observed Optimal Hybridization Temperature for Probes

<table>
<thead>
<tr>
<th>Probe</th>
<th># Bases</th>
<th>G+C</th>
<th>% G+C</th>
<th>Calculate $d T_d$</th>
<th>Hybridization Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univl390</td>
<td>18</td>
<td>11</td>
<td>61.11</td>
<td>67.78</td>
<td>55</td>
</tr>
<tr>
<td>Eub338</td>
<td>18</td>
<td>12</td>
<td>66.66</td>
<td>68.35</td>
<td>55</td>
</tr>
<tr>
<td>Euk</td>
<td>16</td>
<td>10</td>
<td>62.50</td>
<td>62.65</td>
<td>50</td>
</tr>
<tr>
<td>Arch915</td>
<td>20</td>
<td>13</td>
<td>65.0</td>
<td>73.93</td>
<td>55</td>
</tr>
<tr>
<td>Alflb</td>
<td>17</td>
<td>10</td>
<td>58.82</td>
<td>64.16</td>
<td>55</td>
</tr>
<tr>
<td>Bet42a</td>
<td>17</td>
<td>9</td>
<td>52.94</td>
<td>61.75</td>
<td>55</td>
</tr>
<tr>
<td>Gam42a</td>
<td>17</td>
<td>9</td>
<td>52.94</td>
<td>61.75</td>
<td>55</td>
</tr>
<tr>
<td>Delta</td>
<td>19</td>
<td>15</td>
<td>78.95</td>
<td>77.49</td>
<td>55</td>
</tr>
<tr>
<td>(SRB385)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HGC(69a)</td>
<td>18</td>
<td>9</td>
<td>50</td>
<td>63.23</td>
<td>55</td>
</tr>
<tr>
<td>Low G+Ca</td>
<td>18</td>
<td>9</td>
<td>50</td>
<td>63.23</td>
<td>50</td>
</tr>
<tr>
<td>LGCb</td>
<td>18</td>
<td>10</td>
<td>55.55</td>
<td>65.51</td>
<td>for a 1:1:1 mixture of the three probes</td>
</tr>
<tr>
<td>LGCc</td>
<td>18</td>
<td>10</td>
<td>55.55</td>
<td>65.51</td>
<td></td>
</tr>
<tr>
<td>CF319a</td>
<td>18</td>
<td>10</td>
<td>55.55</td>
<td>65.51</td>
<td>57</td>
</tr>
</tbody>
</table>
A second method for determining the optimal washing temperature was also utilized. To characterize a probe’s binding ability at various temperatures, 6 replicate mini-blots were made using the target organism’s RNA. The replicate mini-blots were placed in one bag for hybridization with the end-labeled probe that was to be characterized. After hybridization had occurred, the six mini-blots were placed in six individual scintillation vials containing 3ml of 2X SSC, 0.1%SDS wash buffer. The vials were washed for 10 minutes at 35°C. Afterwards the mini-blots were transferred to six new scintillation vials containing 3mls of wash buffer and washed at 40°C for 10 minutes. This was repeated for every 5°C increment up through 70°C. Five ml of scintillation cocktail was added to the wash buffer in each vial and the vials were counted using a Beta scintillation counter. The last vial contained the mini-blot so any probe that had not yet been washed off could be counted as well. A plot of the amount of probe released at each temperature was prepared and the point at which one-half of the probe remained bound was used as the optimal washing temperature. In order to check for probe specificity, a blot containing all reference organisms was prepared and the recently characterized probe was allowed to hybridize and was washed at the determined optimal washing temperature. After visualization with Kodak Biomax MS film, one could assess the specificity of the probe.
4. RESULTS

4.1 Standard Recovery of RNA Transcripts from Ames Plantation Soils using Four Different Methods.

A $^{32}$P-labeled RNA transcript was added to Ames Plantation Soils and the amount of transcript recovered at each extraction step was measured using a beta-scintillation counter (Figure 2). The extraction methods compared were the Ogram method (section 3.3b), the Fleming method (section 3.3c), the Pure Culture method (3.3d) and a Modified Guanidine Isothiocyanate method (3.3a). The percent recovery of the RNA transcript is plotted against the extraction step in Figure 2. It should be noted that the methods contained different extraction steps. For example, the Ogram method and the Modified Guanidine Isothiocyanate method have soil washing steps before the introduction of phenol/chloroform, whereas the Pure Culture method and Fleming method do not. Therefore, the recovery has been plotted with the initial point corresponding to the initial point in the recovery process. Also, four replicate samples were analyzed for each method and the error bars are included for each data point even though the bars are not visible for the Modified Guanidine Isothiocyanate and Pure Culture methods.

The Pure Culture method was not effective in removing the transcript from the soil. The initial recovery of transcript in the organic phase was not observed, perhaps due to adherence of the transcript to soil particles. Although the soil was not measured by Scintillation counting, a Geiger counter reading revealed that the
Figure 2. Comparison of the recovery of a $^{32}$P-labeled RNA transcript from Ames Plantation soil using four different methods; the Ogram method, the Fleming method, the Pure Culture method and the Modified Guanidine Isothiocyanate method.
transcript remained in the soil. The Pure Culture method uses a buffer composition of 0.5M NaAc, 0.05M NaCl, 0.003M EDTA and 1.5% SDS at pH 5.2. SDS and phenol/chloroform are used to lyse cells along with heating at 60°C and shaking on a wrist-action shaker.

The Modified Guanidine Isothiocyanate method was only slightly better at initially recovering the transcript from soil, but the transcript was not effectively pelleted out of the final aqueous solution. The Modified Guanidine Isothiocyanate method uses an initial soil washing using PVPP and lysis buffer (6.29M guanidine thiocyanate, 25mM sodium citrate, 0.5% sarcosyl, 0.1M β-mercaptoethanol). This solution is combined with zirconium beads and vortexed to lyse cells. After centrifugation the aqueous solution is extracted using phenol/chloroform. The precipitation differs from the other methods in that 2M MgCl₂, 7.5M ammonium acetate and ethanol is used to precipitate the nucleic acids.

The Fleming method initially recovered approximately eighteen percent of the transcript, but the transcript was not efficiently pelleted as evidenced by the retention of the transcript in the aqueous solution after precipitation. The Fleming method uses a lysis buffer consisting of 100mM Tris HCl, 1.4M NaCl, 20mM EDTA and 1% SDS at pH 8.0. The lysis buffer and phenol/chloroform is heated at 60°C then placed on the wrist-action shaker to lyse the cells. Precipitation of the nucleic acids occurs by addition of linear acrylamide, isopropanol and 3M NaAc.

The Ogram method demonstrated the greatest recovery of the transcript (approximately 75%) after a second soil wash. However, the transcript was lost
during subsequent extraction steps, in particular during the precipitation of the nucleic acids where approximately 10% of the transcript was lost to the aqueous phase leaving a total recovery of approximately 37%. The Ogram method uses a lysis buffer (0.2M sodium phosphate buffer, 0.1M EDTA, 10% SDS at pH 8.0) and boil/freeze/thaw cycles to lyse the cells. The RNA is protected from degradation during extraction by the addition of 4M guanidium isothiocyanate, 0.5% sarcosyl, 25mM sodium citrate at pH 7.0. The nucleic acids were precipitated by the addition of 3M NaAc and isopropanol.

4.2 Probe Hybridization Studies

Two types of studies were performed to determine the optimal temperature for hybridization of the probe to RNA bound to a membrane and washing of the membrane to remove any probe that was not bound at the correct specificity. Preliminary studies had been performed previously (data not shown) for the Alflb, Bet42a, Gam42a, SRB385, Arch915, Eub338 probes and narrowed the test range. These probes were tested by washing at temperatures within the pre-determined test range and the results were visualized using film (see sections 3.6d and 4.2a). A second method was used to characterize the optimal hybridization and wash temperature for the Univ1390, Euk, HGC69a, LGCa,b,c and CF319a probes. These probes had not been previously studied by members of our laboratory and were characterized by measuring the amount of probe released from the membrane with increasing temperature (see sections 3.6d and 4.2b).
4.2a Optimal hybridization and wash temperature as visualized by film.

The following probes were characterized by washing at several temperature in a pre-determined range and visualizing the results by film; Alflb, Bet42a, Gam42a, SRB385, Arch915, Eub338. As an example the results for the Alflb probe are presented in Figure 3 and Figure 4. The results for the other probes can be found in Appendix 1 (section A1-1). Four organisms were blotted in replicate onto a single membrane. The membrane was then sectioned into four pieces, each containing the four organisms, and the quadrants were hybridized and washed at four separate temperatures, 47°C, 50°C, 52°C and 55°C. The organisms blotted (from left to right) were Alcaligenes eutropha B30P4 (a member of the beta subgroup), Pseudomonas putida IPL5 (a member of the gamma subgroup), a Pseudomonas putida PpG7 culture contaminant (apparently an alpha) and Bradyrhizobium japonicum USDA110 (a member of the alpha subgroup). The PpG7 culture should have been a member of the gamma subgroup, but these results and subsequent tests revealed the PpG7 culture stock had become contaminated with a member of the alpha subgroup. The Alflb probe hybridized strongly with both alpha members and slightly with the beta and gamma members at temperatures below 55°C. At 55°C, the probe did not bind with the beta and gamma members and the desired specificity had been obtained for the probe. Loss of signal from the alpha members occurred at temperatures above 55°C.

In Figure 4, the specificity of Alflb at a hybridization and wash temperature of 55°C was tested with a broader range of microorganisms. The organisms blotted (from left to right) were B. japonicum USDA110 (an alpha), Brevimonas diminuta
(a) 47°C  (b) 50°C  (c) 52°C  (d) 55°C

Figure 3 (a-d). Example determination of hybridization temperature using the Alf1b probe specific for the Alpha subdivision of the Proteobacteria. Organisms blotted (left to right) B30P4 (beta), IPL5 (gamma), PpG7 culture contaminant (alpha), USDA110 (alpha). The amount of RNA blotted was (top to bottom) 50, 100, 250, 500 ng.

Figure 4. Assessment of Alf1b Specificity at 55°C. Organisms blotted (left to right) (1) USDA110, (2) *Brevimonas* (3) B30P4, (4) *D. vulgaris*, (5) F1, (6) IPL5, (7) PpG7 contaminant, (8) ATCC 23975, (9) ATCC 33015, (10) ATCC 19549, (11) *Drosophila*, (12) Halophile sp. The amount of RNA blotted was (top to bottom) 500, 250, 100, 50 ng.
(an alpha), *A. eutropha* B30P4 (a beta), *D. vulgaris* (a delta), *P. putida* F1 (a gamma), *P. putida* IPL5 (a gamma), PpG7 contaminant (an alpha), *P. putida*-ATCC 23975 (a gamma), *P. putida*-ATCC 33015 (a gamma), *Zoogloea*-ATCC 19544 (a gamma), *Drosophila melanogaster* (a eukaryote), *Halobacterium halobium* (an archaeae). The Alf1b probe was specific for the alpha members at 55°C with the exception of the *Drosophila* RNA where slight hybridization occurred. The *Drosophila* extract was not axenic and the eukaryotic RNA appeared to be contaminated with eubacterial RNA. Thereafter, RNA extracted from *Glycine soya*, which was grown under aseptic conditions, was used as the eukaryotic RNA standard. The optimal hybridization and wash temperatures for Bet42a, Gam42a, SRB385, Eub338 and Arch915 were also found to be at 55°C. Each probe was group specific at this temperature.

**4.2b Hybridization Studies Using Scintillation Counter**

Probes, which had not been previously studied, were characterized as to their optimal hybridization and wash temperature by measuring the amount of probe released from the blot as the temperature increased. The point at which one-half of the probe had been released was determined to be the optimal temperature. Probes characterized in this manner were the Univ1390, Euk, HGC69a, LGCa,b,c and CF319a probes. The Univ1390 probe results will be used as a representative sample and are shown in Figure 5. The results for the other probes are presented in Appendix 1 (section A1-2).

The amount of probe released was measured by scintillation counting and plotted against the concurrent washing temperature. For the Univ 1390 probe, one-half of the
Figure 5. Determination of Univ1390 probe optimal hybridization temperature by measuring the amount of probe released from the membrane with increasing temperature.
probe was released at the 55°C temperature. The optimal temperature obtained for the Euk and LGC probes was 50°C. The HGC probe optimal temperature was calculated to be 55°C and the CF319a probe’s optimal hybridization and washing temperature was calculated to be 57°C.

4.2c Other considerations in hybridizations

It should be noted that Bet42a and Gam42a required competitive hybridization to achieve specificity since the probes differ by only one base. An equal amount of non-radioactively labeled or “cold” probe of Bet42a was added to the radioactively labeled or “hot” probe of Gam42a during hybridization or vice versa. Absolute specificity could not be achieved using competitive hybridization, but the signal was greatly reduced for the nonspecific member for the Beta and Gamma groups (see Figure 6).

Another study showed the importance of probe purification after the end-labeling reaction. Prior to purification with a Stratagene nuc-probe column, significant amounts of background non-specific hybridization was detected when using end-labeled probes. After purification with the Stratagene nuc-probe column, the background level of non-specific hybridization was greatly reduced (see Figure 7).

4.3 Assessment of Reference Organism’s Extracted 16S rRNA Quality

Prior to performing dot-blot analysis on the Ames Plantation Soil Sample’s RNA, the 16S rRNA extracted from the reference organisms was run on a gel to confirm the 16S rRNA quantity and quality (section 3.2). The gel was visualized using Sybor
Figure 6. (left) Addition of “cold” Bet42a probe and “hot” Gam42a probe. (right) Addition of “hot” Bet42a probe and “cold” Gam4a probe. Reference Organisms blotted left to right are *A. eutropha* B30P4 (a beta), *P. putida* IPL5 (a gamma).

Figure 7. Comparison of membranes hybridized with the Bet42a probe without purification (left) and with post end-labeling purification (right).
Gold and the Molecular Dynamics Storm machine. A digitized image is shown in Appendix 1 section A1-3.

All standards contained a distinct 16S rRNA band and exhibited little degradation, which would be evidenced by smearing of the bands. The *Bacillus* sp. was barely visible and was reextracted and reconfirmed prior to creation of a standard (data not shown). Likewise, the *D. vulgaris* band intensity was quite low and another *D. vulgaris* RNA extract was used for preparation of reference standards.

4.4 Ames Plantation Data

The Ames Plantation samples were extracted and replicate blotted onto eleven membranes. The hierarchical probes were allowed to hybridize overnight and were washed at their optimal temperature. The results are presented in the following sections.

4.4a Dot-blot Images and Results

All membranes were blotted in an identical manner and the format is presented in Figure 8. The membrane hybridized with the Univ1390 probe will be used as the representative membrane image. All other membrane images are shown in Appendix 1 (section A1-4). Note that many of the images did not transfer well and are not indicative of the image used in quantifying the results.

The cornfield, young sycamore and old sycamore test sites contained three plots from which three replicate samples were taken. The hardwood forest was sampled only three times total. The extracts from these replicates are blotted horizontally.
<table>
<thead>
<tr>
<th></th>
<th>Plot 1</th>
<th>Plot 2</th>
<th>Plot 3</th>
<th>Standard Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CF

CF RNA

YS

YS RNA

OS

OS RNA

HW

HW RNA

Alpha Beta Gam Delta HGC LGC CFB Arch Euk
Reference Organisms Blotted to Check Probe Specificity

Figure 8. Example of an Ames Plantation Samples dot-blot membrane with extracted RNA detected by hybridization with the Univ 1390 probe. CF= cornfield, YS= young sycamore trees, OS= old sycamore trees, HW= hardwood forest, RNA= = RNAsed negative control. Amount of RNA blotted for standard curve is (top to bottom) 200, 100, 50, 10, 5, 1 ng. The reference organisms were at an approximate concentration of 100ng.
Below each extract is an aliquot, which has been RNase treated to remove any RNA. This serves as a negative control and was used to detect any non-specific hybridization of the probe to non-nucleic acid material within the sample.

In Figure 8, to the right of the RNased Hardwood control samples, are all of the reference organisms blotted to confirm the probe specificity. For the membrane presented in Figure 8, the Univ1390 probe should hybridize with all reference organisms and the probe did bind to all reference organisms.

In Figure 8, to the right of the sample dot-blots a standard curve is blotted vertically. The concentrations of the RNA applied to the membrane are 200, 100, 50, 10, 5, and 1 ng. The probe signal intensities, obtained from the phosphor-imager, were plotted against the respective standard concentration. A standard curve was used to generate a regression equation for each membrane blot. Figure 9 is a graphical representation of a standard curve and contains the regression equation given by the data for the Universal Probe. Using this equation, the concentration of the RNA in each sample was obtained on the Universal Blot. A similar graph was created for each of the other blots hybridized with the group probes. The regression equation calculated for each probe’s standard curve was used to determine the RNA quantities on each probe’s blot. The background signal from non-specific hybridization was subtracted from each sample’s signal.

The final results are presented in Table 6. All RNA detected by the various domain, group or subgroup probes is expressed as a relative percentage of the total 16S rRNA. The Univ1390 probe was used to measure the total microbial community
Figure 9. Graphical representation of a standard curve used to calculate the amount of RNA in Ames Plantation Samples. The linear regression equation was obtained using Excel. The quantity of RNA in nanograms (X) for each sample was then determined by solving the equation for (X) given the counts for each sample (Y).
Table 6. Part (i) Percentage of domain, group or subdivision relative to the total community RNA as measured by the Universal probe. Part (ii) total nanograms of rRNA as measured by the Universal probe per gram of soil extracted.

<table>
<thead>
<tr>
<th>(i) Domain/Group or Subdivision</th>
<th>Corn Field Plots</th>
<th>Young Sycamore Trees Plots</th>
<th>Old Sycamore Trees Plots</th>
<th>Hardwood Forest Plots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eubacteria</td>
<td>69.62 ± 25.68</td>
<td>69.53 ± 18.97</td>
<td>53.04 ± 16.69</td>
<td>49.88 ± 4.97</td>
</tr>
<tr>
<td>Eukarya</td>
<td>18.68 ± 9.58</td>
<td>6.40 ± 4.74</td>
<td>14.59 ± 7.08</td>
<td>1.15 ± 1.03</td>
</tr>
<tr>
<td>Archaea</td>
<td>0.27 ± 0.16</td>
<td>0.38 ± 0.21</td>
<td>0.25 ± 0.21</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td><strong>Sum Total of the 3 Domains</strong></td>
<td><strong>88.57 ± 32.47</strong></td>
<td><strong>76.31 ± 20.67</strong></td>
<td><strong>65.97 ± 19.42</strong></td>
<td><strong>51.19 ± 4.10</strong></td>
</tr>
<tr>
<td>Alpha</td>
<td>21.64 ± 7.56</td>
<td>27.09 ± 11.09</td>
<td>35.01 ± 10.33</td>
<td>16.81 ± 8.14</td>
</tr>
<tr>
<td>Beta</td>
<td>7.63 ± 3.52</td>
<td>6.04 ± 1.58</td>
<td>10.35 ± 6.95</td>
<td>4.35 ± 1.34</td>
</tr>
<tr>
<td>Gamma</td>
<td>2.86 ± 1.76</td>
<td>4.61 ± 2.48</td>
<td>3.82 ± 2.76</td>
<td>1.26 ± 0.03</td>
</tr>
<tr>
<td>Delta</td>
<td>8.73 ± 5.62</td>
<td>12.70 ± 8.43</td>
<td>9.78 ± 5.72</td>
<td>10.40 ± 5.97</td>
</tr>
<tr>
<td>High G + C</td>
<td>1.36 ± 3.07</td>
<td>5.21 ± 6.63</td>
<td>9.31 ± 8.73</td>
<td>12.82 ± 7.04</td>
</tr>
<tr>
<td>Low G + C</td>
<td>2.46 ± 2.64</td>
<td>0.75 ± 1.69</td>
<td>0.94 ± 1.22</td>
<td>3.93 ± 3.34</td>
</tr>
<tr>
<td>Cytophaga/Flavobacterium/ Bacteroides</td>
<td>0.87 ± 0.96</td>
<td>0.77 ± 0.59</td>
<td>0.76 ± 0.75</td>
<td>0.71 ± 0.16</td>
</tr>
<tr>
<td><strong>Sum Total of Eubacterial Groups</strong></td>
<td><strong>47.48 ± 12.77</strong></td>
<td><strong>53.26 ± 12.26</strong></td>
<td><strong>69.96 ± 15.96</strong></td>
<td><strong>50.28 ± 13.29</strong></td>
</tr>
<tr>
<td>(ii) Total (ng/g soil)</td>
<td>158.53 ± 63.25</td>
<td>86.11 ± 23.80</td>
<td>206.00 ± 88.97</td>
<td>196.81 ± 6.99</td>
</tr>
</tbody>
</table>
16S rRNA in each sample and is indicative of the total microbial biomass in the soil. The total nanograms of 16S rRNA per gram of soil extracted is presented at the bottom of Table 6 and is diagramed in Figure 10. Part (I) of Table 6 lists the relative percentage of the three domains measured by the domain probes. The sum of all the relative percentages of the three domain probes is also presented. In theory, this sum should be 100% if the domain probes are detecting all organisms. Likewise, the sum of all of the individual eubacterial group probes (presented in Table 6) should equal 100% of the Eubacterial Domain probe. This will be discussed further in the discussion section.

Statistical analysis using a Fisher’s least significant differences test of the biomass results was performed and the mean biomass level for each test plot was compared to the other test plots (results in Appendix 2). The Fisher’s test of the Univ1390 probe data determined that the cornfield, old sycamore tree plot and the hardwood plots all possess significantly higher biomass than that found in the young sycamore tree plot. The cornfield contained nearly twice as much microbial biomass and the old sycamore tree plots and hardwood samples contained more than twice the microbial biomass found in the young sycamore tree plots. There was no significant difference between the biomass levels of the cornfield, old sycamore tree and hardwood forest samples although the old sycamore trees and hardwood forest appeared to possess a slightly higher biomass level. The variability within each test plot’s biomass level was the least for the hardwood forest samples and greatest for the old sycamore trees.
Figure 10. Total microbial biomass in nanograms of RNA per gram of soil extracted for each Ames Plantation Test Plot as measured by the 16S rRNA Univ1390 Probe.
A comparison between each test plot of the relative percentages determined by the three domain probes; Euk, Eub338, Arch915 is exhibited in Figure 11. Also shown on this graph is a group called “other”. The relative percentages of the three domain probes should equal 100% if the domain probes define the total microbial community. The “other” group includes the percentage of the total microbial community that is not defined by the three domain probes. The archaea composed a very minor proportion of the total microbial community. The archaeal composition never exceeded 0.5% in the test plots and no differences were found between the test plots. The Eukaryotes comprised a minor proportion of the hardwood forest microbial community at approximately one percent. This value was much lower than the values for the cornfield and old sycamore tree plots, which contained average Eukaryotic percentages of 18.7% and 14.6%, respectively. The young sycamore trees possessed a eukaryotic proportion of 6.4%, which was significantly lower than the cornfield and old sycamore tree values.

The Eubacteria were the most prevalent domain in each of the test plots ranging from a high of 70% for the cornfield plots to a low of 50% for the hardwood samples. The young sycamore tree plots and the old sycamore tree plots eubacterial values were 70% and 53%, respectively. There were no significant differences in the total Eubacterial biomass as measured by the Eub338 probe between any of the test plots.

Figure 12 is a graphical representation of the relative percentages of the total microbial community for the Proteobacter group of the Eubacteria domain. Four probes were used to detect different subdivisions of the Proteobacter group; Alflb,
Figure 11. Percentage of each domain, Eukarya, Eubacteria and Archaea relative to the total microbial community RNA as measured by the Univ1390 probe. "Other" represents the remaining microbial population not detected by the three domain probes, but detected by the Univ1390 probe.
Figure 12. Comparison of the relative percentage of the Proteobacter subdivisions; Alpha, Beta, Delta and Gamma for each Ames Plantation Plot.
Bet42a, Gam42a and SRB385. These probes detect the alpha, beta, gamma and delta subdivisions of the Proteobacter group, respectively.

The relative percentages of each Proteobacter group revealed that the old sycamore tree plots contained significantly more members of the alpha subdivision (35%) than did either the cornfield plots (22%) or the hardwood forest samples (17%), but did not contain significantly more than did the young sycamore trees (27%). There were no significant differences in the number of beta subdivision members between any of the test plots and the values ranged from a high of 10% in the old sycamore trees to a low of 4% in the hardwood forest samples. Similarly, there were no significant differences in the delta subdivision composition between the test plots. The young sycamore trees possessed the greatest number of delta subdivision members (13%) and the cornfield possessed the least number of delta subdivision members (9%). The hardwood forest samples contained significantly less gamma subdivision members (1.26%) than did the young sycamore trees plots (4.6%). The cornfield (2.86%) and old sycamore trees (3.82) did not differ in their gamma subdivision composition from each other or from the young sycamore trees and the hardwood forest.

The High G+C and Low G+C DNA content groups and the Cytophaga/Flavobacterium/Bacteriodes group of the Eubacteria domain were detected using the HGC69a, LGCa,b,c and CF319a probes, respectively. The relative contribution of these groups to the total microbial community are presented in Figure 13.
Figure 13. Comparison of relative percentages of the Eubacteria Groups; High G+C DNA Content (HGC), Low G+C DNA Content (LGC) and Cytophaga/Flavobacterium/Bacteriodes (CFB) for each Ames Plantation Plot.
The Fisher's least significant difference test showed no significant differences between any of the test plots for the CFB group. This group comprised less than 1% of the total microbial community in all test plots. The High G+C group was significantly higher in the old sycamore tree plots (9%) and hardwood samples (13%) relative to the cornfield plots (1.3%), but not higher than in the young sycamore tree plots (5.25%). The Low G+C group was significantly higher in the hardwood forest samples (4%) relative to the old sycamore trees (0.9%) and the young sycamore trees (0.74%), but not higher than in the cornfield plots (2.45%).

An overview of the total microbial community for each test plot is shown in Figures 14 a through d. The relative percentage of the Eukaryotic and Archaeal domains are shown as individual pie pieces. The Eubacterial domain is divided into its representative parts based on all of the eubacterial group and subdivision probes used in the analysis. The "unknown" pie piece depicts the amount of Eubacterial domain not represented by the other eubacterial probes and was calculated by subtracting the sum total of all eubacterial group and subdivision probe data from the eubacterial domain probe data.

Also included in the results is Table 7, which contains a portion of the physical and chemical data obtained for the Ames Plantation soil samples. The samples were analyzed by the Plant and Soil Sciences Department at the University of Tennessee and the data was obtained from Michael Kirchner from soil samples obtained concurrently with the soil samples analyzed for 16S rRNA. The table is included so that inferences can be made as to possible reasons for microbial community shifts.
a. Ames Plantation Corn Field Soil Microbial Community Structure

Figure 14. Comparison of the relative percentages of the total microbial community for various Ames Plantation samples. The relative percentages are indicated.
b. Ames Plantation Young Tree Plots Soil Microbial Community Structure
c. Ames Plantation Old Tree Plots Soil Microbial Community Structure
d. Ames Plantation Hardwood Forest Soil Microbial Community Composition
Table 7. Soil Chemical Analyses Data. Obtained from Michael Kirchner, UTK Plant and Soil Sciences Department.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Corn Field Plots</th>
<th>Young Sycamore Trees</th>
<th>Old Sycamore Trees</th>
<th>Hardwood Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Std. Dev.</td>
<td>Average</td>
<td>Std. Dev.</td>
</tr>
<tr>
<td>Carbon%</td>
<td>1.06</td>
<td>0.25</td>
<td>0.96</td>
<td>0.17</td>
</tr>
<tr>
<td>Sulfur%</td>
<td>0.03</td>
<td>0.04</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Nitrogen%</td>
<td>0.32</td>
<td>0.11</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>pH</td>
<td>1.94</td>
<td>2.99</td>
<td>1.42</td>
<td>3.02</td>
</tr>
<tr>
<td>NO3-(ppm)</td>
<td>256.61</td>
<td>104.76</td>
<td>1525.62</td>
<td>1362.07</td>
</tr>
<tr>
<td>Ca(ppm)</td>
<td>1041.76</td>
<td>142.99</td>
<td>254.16</td>
<td>354.94</td>
</tr>
<tr>
<td>Cu(ppm)</td>
<td>134.92</td>
<td>22.34</td>
<td>122.74</td>
<td>42.25</td>
</tr>
<tr>
<td>Fe(ppm)</td>
<td>15.74</td>
<td>21.21</td>
<td>121.93</td>
<td>54.25</td>
</tr>
<tr>
<td>K(ppm)</td>
<td>1.57</td>
<td>0.20</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Mg(ppm)</td>
<td>182.17</td>
<td>156.72</td>
<td>277.75</td>
<td>43.43</td>
</tr>
<tr>
<td>Na(ppm)</td>
<td>66.22</td>
<td>27.50</td>
<td>43.43</td>
<td>14.63</td>
</tr>
<tr>
<td>Zn(ppm)</td>
<td>20.48</td>
<td>7.77</td>
<td>27.50</td>
<td>34.17</td>
</tr>
<tr>
<td>P(ppm)</td>
<td>134.92</td>
<td>22.34</td>
<td>122.74</td>
<td>54.25</td>
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</tbody>
</table>

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5. DISCUSSION

A goal of this research was to optimize a protocol to rapidly obtain from soil high yields of RNA suitable for membrane hybridizations with hierarchical probes without prior amplification or cloning of the RNA. This goal was achieved by combining various aspects of several different RNA extraction protocols. Results demonstrated that the Ogram method recovered the highest amount of RNA from soil samples (section 4.1). The recovery efficiency from the Ames Plantation soils was slightly higher than that obtained by Ogram et al during their experiment (Ogram et al, 1995). Thirty-seven percent RNA recovered from the Ames Plantation soil compared to twenty-six percent RNA recovered from deep subsurface sediments. The amount of RNA recovered per gram of soil extracted was comparable to amounts achieved by Ogram, Felske and Holben, approximately 0.2 μg/g soil (Ogram et al 1995, Felske et al 1996, Holben et al 1988).

Hybridization studies (data not shown) also demonstrated that the Ogram method was capable of recovering RNA from a variety of soil types including oligotrophic sandy soils (CAFB soils), lake sediment samples (Lake Michigan sediments) and lysimeter soil. The detection limit for RNA in soil using the Ogram method was found to be 10⁻⁴ cells/g soil. This was similar to the detection limit Holben et al found using DNA extracted from cells. RNA is more abundant in a cell and should afford a natural amplification of the signal, but in soil systems, nutrients may be limited thus reducing the amount of RNA in a cell. The soil microorganisms may adapt by forming dwarf cells, cysts or spores and produce less RNA (Zarda et al 1997). RNA
extracted from cells is also more susceptible to degradation than is DNA, thus, reducing any natural amplification after processing of samples is completed.

The Ogram method co-extracted humics from some soil types. Several methods were attempted to remove the humics including PVPP treatment, guanidine HCl precipitation and passage through a Sephadex column. Although these methods removed some of the humic contaminants, they also reduced the total yield of RNA. By using an RNased control, the membrane hybridizations could be quantitative without the full removal of the humic contaminants. Considerations in the extraction of RNA from soil will be discussed in section 5.1.

An objective of the research was to optimize hybridization and washing conditions for the hierarchical probes. During this project, the optimal hybridization and washing conditions for eleven hierarchical probes were determined using two different methods (Section 4.2). This included five probes that had not been previously used in our laboratory for community analysis. Factors relevant to hybridization and the importance of optimization for correct quantification will be discussed in section 5.2.

Another objective was to apply the above methodologies to the Ames Plantation research to test the hypothesis; that changes occur in the microbial community structure that compensate for changing from annual row crops to short rotation woody biomass crops in order to maintain and complete biogeochemical cycles. Data obtained from the hybridization of hierarchical probes to membrane bound RNA extracted from Ames Plantation soil demonstrated that different microbial
communities exist in each of the four test plots at the Ames Plantation site (Section 4.4). These differences could be quantitatively assessed in terms of relative percentages of the total community using the Universal probe for comparison. The observed differences and their potential relationships to biogeochemical shifts will be discussed in Section 5.3.

5.1 Considerations in the Extraction of rRNA from Soil

As previously discussed in Section 2.7, the isolation of rRNA from soil depends on several factors including cell lysis, and the extraction and purification of rRNA away from soil particles. Cell lysis is achieved either by mechanical means or by chemical measures. Several different procedures were compared which used different methods to lyse cells. Results showed that lysis methods using boil/freeze/thaw cycles combined with SDS were as efficient as bead-beating methods. A potential problem encountered in using large volume chambers for bead-beating is the potential for degradation of the RNA by RNases. The large volume chambers are difficult to clean and thoroughly remove RNases. Small volume bead-beaters such as the Savant Fast-Prep system use sterile tubes and the potential for degradation by RNases is limited, but the sample size is also limited. Therefore, the sample may not be representative of the heterogeneous population typical of soil samples.

Buffer composition was also found to be important in removing RNA from soil. Results demonstrated that the Ogram method which used a lysis buffer containing 0.2M sodium phosphate and 0.1M EDTA was better at recovering RNA from soil.
than other methods which used sodium citrate, sodium acetate or Tris-HCl. The phosphate in the Ogram lysis buffer may block active sites on the soil, which would bind the RNA once the cells have been lysed. The Ogram buffer also contains more EDTA which is a metal chelator and may also assist in ensuring that the RNA is free to leave the soil by binding metals in the soil which might otherwise bind to the RNA and trap it within the soil.

One problem encountered in using a buffer with a high salt content is the co-precipitation of salts with the RNA during the precipitation procedure. By increasing the temperature at which precipitation occurs from −20°C to 4°C, co-precipitation of salts was greatly reduced. An attempt was made to remove salt co-precipitates from RNA extracts by dialysis, but this did not sufficiently remove salt co-precipitates and required the RNA to be in solution for a long period where RNases could degrade the RNA. Furthermore, yields of RNA after dialysis were very low (data not shown).

Increasing the centrifugation time may enhance the recovery of the RNA after precipitation. A study by Ambion detailed in their Technical Bulletin demonstrated that increasing the centrifugation time increases RNA recoveries (Ambion #160). The Ogram method suggested centrifuging for 30 minutes. Increasing the centrifugation time may increase the recovery of RNA from soil. A problem encountered during centrifugation was the inability to obtain speeds greater than 10,000RPM because the Corex bottles used during the precipitation step were susceptible to breaking. The bottles were repeatedly heated to 180°C and then cooled to reduce RNase contamination. The repeated expansion and contraction of the glass may have made
the bottles brittle and several broke during the centrifugation step even though extreme caution was taken to balance the bottles. Another type of transparent tube that could be centrifuged at higher RPMs, would enhance the recovery of the RNA. Several types of tubes have been tried, but none have succeeded in replacing the Corex bottles.

Another factor affecting the recovery of RNA from soil is the buffer to soil ratio. Several studies showed that the recovery of RNA was not linear when comparing the recovery of RNA from 10, 15, 20 and 30 grams of soil using the exact same method (data not shown). There seems to be a limit to the amount of RNA that can be effectively removed from a mass of soil when the buffer amount stays constant. It is possible that the soil contains active sites, which are blocked by components in the buffer such as phosphate or EDTA. If the number of active sites in the soil exceeds the ability of the buffer to block the active sites in the soil, then those active sites are free to bind the RNA. Therefore, by increasing the buffer to soil ratio, more of active sites will be blocked and more RNA can be recovered. Elizabeth Alm and David Stahl also observed this phenomenon (Alm and Stahl 1996).

Recovery of RNA from soil depends on the soil type. For example, a method that works well in a low organic content soil may not work as well in a high organic content soil. Therefore, it is important that the efficiency of a method be tested prior to extracting RNA from real samples.

5.2 Optimization of Hierarchical Probes' Hybridization Conditions

An objective of the research was to optimize hybridization and washing
conditions for the hierarchical probes. Two methods were used to determine the optimal hybridization and washing conditions. One method used film to visualize the temperature at which specificity had been obtained. This was the temperature at which the probe bound only to members within its domain, group or subdivision depending on the probe’s target sequence. At temperatures above this point, the probe did not bind as well to its target sequence and the signal began to diminish. This method worked well for probes that had been partially characterized since the temperature range had been reduced by previous studies and did not require the production of ten or more replicate blots. However, for new probes, the second method of scintillation counting and simultaneous washing of replicate blots is less time consuming.

Characterization of probes using scintillation counting worked very well for the Univ1390 probe. The study used six replicate mini-blots and the release curve obtained could be used to determine the temperature at which approximately one-half of the probe had been released from the blot. This temperature was used as the hybridization and washing temperature. The other probes characterized by this method (HGC, LGC, CF319 and Euk) did not have smooth curves like the Univ1390 probe. These probes were produced in the laboratory using a Beckman Oligo-1000 DNA Synthesizer. The probes produced using this system may actually be a consortium of probes containing some mismatches. The LGCa,b,c probe was definitely a 1:1:1 ratio of three different probe sequences. Each sequence would possess a slightly different ability to bind to target sequences and would therefore
create a composite probe release curve. The probe's optimal hybridization temperature was estimated using the composite curve by determining the point at which one-half of the probe had been released from the blot. Each probe was checked for specificity at the optimal hybridization temperature and all probes were found to be specific for their target sequence at the hybridization temperature determined by either of the above methods.

5.3 Monitoring Microbial Community Shifts in Ames Plantation Soils

A primary objective was to apply the above methodologies to the Ames Plantation research to test the hypothesis; that changes occur in the microbial community structure that compensate for changing from annual row crops to short rotation woody biomass crops in order to maintain and complete biogeochemical cycles. From the pie charts in Figure 14, one can see that the microbial communities within the four test plots are not identical. Changes did occur in the microbial community structure when changing from annual row crops to short rotation woody biomass crops. Suggesting a community effort to maintain and complete biogeochemical cycles. This later interpretation remains to be tested with data on biogeochemical activity.

The overall microbial biomass associated with a gram of soil was on average significantly lower in the young sycamore tree plots (Figure 10). The biomass was twice as high or higher in the cornfield, old sycamore tree plots and in the hardwood. Some potential explanations for the reduced microbial biomass in the young sycamore trees are an observed lower pH (5.22), carbon and nitrogen in the young sycamore tree plots relative to the other plots (Table 7). The young sycamore tree
plots were not nutrient amended with fertilizer, as was the cornfield plots. According to Smith et al, the concentration and activity of soil microbial biomass is controlled by several factors including the amounts of organic matter, clay content, temperature and water (Smith et al 1993). The level of soil organic matter controls the absolute concentration of microbial biomass. The decomposition of organic matter yields CO$_2$, NH$_4^+$, NO$_3^-$, PO$_4^{3-}$ and SO$_4^{2-}$ (Smith et al 1993). Microorganisms use surface organic matter or residue as the primary source for new nitrogen and carbon inputs into the soil ecosystem. The young sycamore tree plots were visibly devoid of groundcover from grass or detrital leaves that could be used as a nutrient source and they also exhibited the least microbial biomass.

The cornfield plots were adjacent to the young sycamore tree plots and the cornfield plots' soil composition was similar to the young sycamore tree plots in several respects. The carbon, nitrogen, potassium, and magnesium concentrations were very comparable (Table 7). The pH of the cornfield was the highest of any of the plots at 5.98. The microbial biomass level in the cornfield plots was nearly double that found in the young sycamore tree plots and slightly less than that found in the old sycamore tree plots and the hardwood samples (Figure 10). A potential explanation for this is the organic matter input from crop residue. The cornfield plots were covered with fescue at the time of sampling and the previous crop residue had not been removed, therefore the organic matter input was higher in the cornfield plots than in the young sycamore tree plots. The cornfield plots had also been given a fertilizer treatment.
The old sycamore tree plots and the hardwood forest samples contained approximately the same microbial biomass levels (Figure 10). The pH, carbon, nitrogen and other chemical contents of the old sycamore tree plots and the hardwood forest samples did not differ significantly except in the level of potassium which was slightly higher in the hardwood forest samples (Table 7). The groundcover and thus organic matter input was very similar at the two sites as well.

For the three domains the primary difference between the test plots occurred in the relative percentage of the amount of Eukarya, which was significantly higher in the cornfield and old sycamore tree plots relative to the hardwood forest and young sycamore tree samples (Figure 11). A potential reason for this is the observed increase in the number of fine roots within the rhizospheres of the cornfield and old sycamore tree soil relative to the young sycamore tree and hardwood forest soil noticed during the sampling period.

The relative percentages of the Archaea in all of the test plots did not differ and were very low, comprising less than 1% of the total microbial community. The soils sampled were 0-5cm in depth and were well aerated, therefore, the limited Archaeal input is not surprising since Methanogens are anaerobic and the extremophile halophiles and thermophiles would not like the low salt concentrations and moderate soil temperatures. The slight response is probably due to anaerobic microniches, which could sustain methanogens.

Perhaps most interesting is the number of undefined members of the microbial community represented by the "other" category in Figure 11. As discussed
previously, the “other” category was obtained by adding the relative percentages of
the three domain probes; Euk, Arch915 and Eub. This “three domain sum” was then
subtracted from 100; an assumption was made that the Universal probe detected
100% of the microbial community. The resultant value is the relative percent of the
community not detected by the three domain probes or the undefined members. In
general, the amount of undefined members increased as the ecosystem complexity
increased.

In the cornfield samples approximately 10% of the total microbial community
detected by the Universal probe, was not detected by the three domain probes. By
comparison, in the young sycamore tree plots and old sycamore tree plots 24% and
34%, respectively, of their microbial communities were undefined by the three
domain probes. In the hardwood forest samples, 50% of the total microbial
community was undefined by the three domain probes. A possible explanation, for
this phenomena, is the Universal probe may be more specific, i.e., better able to
detect microorganisms, than the three domain probes. Since the Universal probe
targets the most highly conserved region of rRNA, the Univ1390 probe is highly
specific. The Eub, Arch915, and Euk domain probes target less highly conserved
regions of the rRNA and are, therefore, less specific and may miss detecting some
microorganisms. Therefore, the three domain probes would not be 100% additive as
they should be in theory (Zheng et al 1996). Another potential reason is there may be
greater microbial diversity in the hardwood forest samples, microbial diversity that
has yet to be detected by standard microbiological techniques of culturing and
sequencing (Stahl 1997). Therefore the domain probes, that were generated based on sequences available in the current databases, may not be specific for all unknown species residing within soil communities. As new sequences become available to the databases, more specific domain probes should be generated to incorporate novel sequences.

The relative amount of the Eubacteria in each of the test sites was similar and differed primarily in composition. The Alpha subdivision of the Proteobacteria contains many types of bacteria, which can form intracellular associations with eukaryotic cells, and includes the *Bradyrhizobium* group, *Rhizobiaceae*, nitrogen fixers, and the plant pathogen, *Agrobacterium*. The Alpha subdivision comprised the largest proportion of the Eubacteria in any test plot ranging from 17% to 35% of the total microbial community (Table 6 and Figure 12). The microbial communities associated with the old sycamore tree plots contained significantly more members of the Alpha group (35%) than did the cornfield plots or the hardwood forest samples (22% and 17%, respectively), but not significantly more than did the young sycamore tree plots (27%). The relative percentage of the total community represented by the Alpha subdivision was quite similar for the old sycamore tree plots and the young sycamore tree plots and may indicate the Alpha subdivision members may be using an exudate produced from these trees as a nutrient source.

The Beta and Delta subdivisions contained the next largest proportion of the microbial community depending on the test plot (Table 6 and Figure 12). The Beta subdivision of the Proteobacteria contains ammonia-oxidizing bacteria,
Alcaligenaceae, Burkholderia, Gallionella, Thiobacillus and some Zoogloea. There were no significant differences in the relative proportion of Beta subdivision members in any of the test plots. The relative percentages ranged from 4.35% in the hardwood forest to 10.35% in the old sycamore tree plots with the cornfield and young sycamore tree values falling in between at 7.6% and 6.04%, respectively. The amount of ammonium ions in the soil samples were lower in the cornfield plots and in the young sycamore tree plots relative to the old sycamore tree plots and the hardwood forest soils (Table 7). But no difference was seen in the amount of nitrate in the test plots. The ionic species of iron were listed only as iron, but the levels did not differ between the test plots. The amount of ammonia, nitrite, nitrate can influence the species of ammonia-oxidizers present in a soil sample. Similarly, the availability of iron can influence the presence of Thiobacillus and Gallionella.

The Delta subdivision of the Proteobacteria contains members of several different phenotypes. Among them, the sulfur and sulfate-reducing bacteria (SRB), Myxobacteria and Bdellovibrios and Geobacter. There were no significant differences in the relative percentages of Delta members in the test plots and the values ranged from 3.7% in the cornfield plots to a high of 12.7% in the young sycamore tree plots (Table 6 and Figure 12). The old sycamore trees plots contained 9.8% and the hardwood samples contained 10.4%. Since the agricultural soils were primarily aerobic, it is unlikely that the delta members present in the soil are representative of the SRBs which are anaerobic. However, as in the case for the methanogens, anaerobic microniches could be present in the soil. Another limiting
factor is that the amount of sulfur in the soils was 0.2% (Table 7). Geobacter could be present in the soil and play a role in iron cycling. As discussed previously, the iron level did not differ between the plots (Table 7). The Bdellovibrios are aerobic predators of other Gram-negative organisms and may play a role in the reduction of other Proteobacter subdivisions. Although there were no significant differences, there seemed to be a potential trend towards more Delta subdivision members as the pH decreased.

The High G + C DNA content group of the Eubacteria is representative of Actinomycetes which includes Arthrobacter and Brevibacterium, as well as, Streptomyces and Mycobacterium. Actinomycetes may be anaerobic or aerobic. Arthrobacter and Brevibacterium are aerobic members of the Actinomycetes and are typical soil microorganisms that can be involved in lignin degradation. The Hardwood samples contained significantly more High G+C organisms than did the cornfields plots, 13% versus 1.3%, respectively (Table 6 and Figure 13). The young sycamore trees and old sycamore trees were more similar in the relative percentages of High G+C organisms with 5.2% and 9.3%, respectively.

The Low G+C DNA content group of Eubacteria contains several groups including Bacillus, which is aerobic, Lactobacillus, Streptococcus, and Clostridium, which are predominantly anaerobic. The soil samples contained a surprising low amount of Low G+C type organisms. The hardwood forest samples contained significantly more Low G+C type organisms relative to the old sycamore tree and young sycamore tree plots (4% versus 0.75% and 0.94%, respectively, Table 6 and
The cornfield samples contained 2.5% Low G+C type organisms. The cornfield plots and the hardwood samples may be more highly aerated than the old sycamore tree and young sycamore tree plots and contain more *Bacillus*-type organisms or may be less aerated and contain more clostridial type organisms. Given the lack of representatives from other anaerobic groups, the former case is more likely. However, anaerobic microniches may exist in the soil.

The *Cytophaga/Flavobacterium/Bacteriodes* group can be split into the aerobic members *Cytophaga* and *Flavobacterium* and the anaerobic members of the *Bacteriodes* group. The relative percentages of the CFB group found in the test plots were very low and comprised less than 0.87% (Table 6 and Figure 13). Again, given the aerobic nature of the samples, this number probably represents members of the *Cytophaga* or *Flavobacterium* groups.

The total amount of Proteobacteria in the community represented by the summation of the relative percentages of the Alpha, Beta, Gamma and Delta subdivisions can be used as a measurement of Gram-negative organisms in the test plots. The total amount of the High G+C, Low G+C and CFB groups can be used as a measurement of the Gram-positive organisms in the test plots. In the old sycamore tree plots, nearly 60% of the total microbial community was represented by Gram negative organisms. This value was significantly higher than that found in the cornfield samples (42%) and in the hardwood tree plots (33%), but not higher than for the young sycamore tree plots (48%). The relative percentage of Gram-positive organisms was much higher in the hardwood samples (17.5%) relative to the
cornfield and young sycamore tree plots (4.7% and 6.7% respectively). In old sycamore tree plots, the total microbial population was comprised of approximately 11% of Gram-positive organisms. Therefore, a shift towards more Gram-positive organisms and less Gram-negative organisms was observed with increasing forestation.

The soil microbial community results obtained using the dot-blot analysis compared favorably to soil microbial community analysis assessed by PCR amplification of cDNA, the production of clone libraries and sequencing of rDNA. Studies have been conducted on Wisconsin soils, grassland soils in the Netherlands, Australian soils, Southwestern United States soils and on soils from Amazonia (Borneman et al 1996, Felske et al 1998, Liesack and Stackebrandt 1992, Kuske et al 1997, Borneman and Triplett 1997). Approximately 100 clones or less were analyzed in each study. The results indicate that the microbial diversity greatly exceeds the known bacterial domains. Vigdis Torsvik suggested the level of diversity in a deciduous forest soil was so high that the true diversity could only be determined with great difficulty. Furthermore, the diversity resided in a portion of the community that could not be isolated by standard techniques (Torsvik et al 1990). Nearly 40% of the clones, from the Wisconsin soils, did not belong to any major taxa (Borneman et al 1996). Several distinct groups of unidentified bacteria were also found in the Netherland grassland soils (Felske et al 1998). Out of 83 clones analyzed from the Australian soils, 22 clones grouped in a novel main line of descent (Liesack and Stackebrandt 1992). Similarly, 36 out of 56 clones formed five distinct clades of
novel descent in the Southwestern U.S. soils (Kuske et al 1997). In a study on the effects of deforestation in Amazonia, Borneman et al found 18 out of 100 clones could not be matched with any previously identified bacteria in the Eubacterial Domain. Furthermore, forest samples included in the study contained more unclassified microorganisms than did pasture samples (Borneman et al 1997).

These results are similar to those found in the Ames Plantation soils. The Hardwood forest samples possessed the greatest amount of unidentified organisms. This undefined portion of the microbial community may consist of *Planctomyces*, which have been found by Zarda et al in terrestrial environments (Zarda et al 1997). Sequence analysis performed by Liesack and Stackebrandt on Australian soils found 7 out of 83 clones to be Planctomycetes in origin (Liesack and Stackebrandt 1992). Kuske et al also identified *Planctomyces* in their Southwestern soil samples (Kuske 1997). These results show that *Planctomyces* may be ubiquitous in soil microbial communities.

The unidentified component of the microbial community may also be fungal in origin. The fungal component may be underrepresented because fungal hyphae have little DNA and little cytoplasm along the length of their hyphae (Harris 1994). This phenomenon may cause problems in enumerating the fungal input into a microbial community.

Consistent with the Ames Plantation data, few soils analyzed by clone libraries contained members of the Archaeal domain. Indeed the above studies all lacked
Archaeal clones with the exception of the Amazonia Forest, which contained 2 clones (out of 100 clones) belonging to the *Crenarcheota* subdomain (Borneman 1997).

All of the clone studies found the Eubacterial domain to be the most abundant in the soil microbial communities. The proportions of the bacterial groups within this domain varied greatly with the soil origins. The study of Wisconsin soil found 98% of the clones were from the Eubacterial domain. Nearly 40% were not from the currently named major taxa. Of the remaining clones, 16% were Proteobacter, 22% were CFB and 22% were Low G+C (Borneman et al 1996). The Netherland grassland soil study found 37 out of 72 clones matched *Bacillus* in the ribosomal database. The remaining clones matched members of the Alpha subgroup (Felske et al 1998). The CFB and Low G+C groups were not as highly represented in the Ames Plantation soils.

The study of the arid Southwestern soil sequenced 56 clones. Thirty-six were from novel clades. Of the remaining 20 clones, 2 were Beta members, 1 was a Gamma member, four were from the Alpha group and three were from the High G+C group. Only one clone was from the Low G+C group in this soil (Kuske et al 1997). The Australian soil yielded 57 out of 83 clones from the Alpha group and 22 from a novel line of descent (Liesack and Stackebrandt 1992). The study by Borneman et al on Amazonian soils compared forest soils with pasture soils. This study found High G+C organisms only in pasture soil (3 out of 100 clones)(Borneman et al 1992). This is contrary to the results shown in the Ames Plantation study where more High G+C
organisms were detected in the Hardwood forest samples than in the cornfield samples.

From these clone studies it is evident that it is difficult to predict the soil microbial community composition a priori. The community composition of a soil is influenced by many factors including pH, organic matter content, water content, chemical constituents and disturbances whether induced by nature or man. The diversity found in soil, as Felske suggests, cannot be expected to be understood by analysis of a few hundred clones (Felske et al 1998). Hybridization of RNA directly extracted from the soil limits biases based on PCR amplification, cloning and sequencing. The technique also gives one the ability to check a broad range of domains, groups and subgroups rapidly and in a less costly manner than does sequencing individual clones. Furthermore, the technique can be combined with statistical analysis of the data. This allows one the ability to more definitively say which portion of the microbial community is shifting in response to changes in soil composition.

5.4 Conclusions

The extraction of 16S rRNA from agricultural soils can be achieved with a buffer of high sodium phosphate and EDTA concentrations and using boil/freeze/thaw cycles. Reducing the temperature at which nucleic acid precipitation occurs can reduce the amount of salt co-precipitation. The humics that co-precipitate with the RNA can be removed by several different methods, but this reduces the RNA yield. An RNased negative control can be used to determine background signals to
circumvent hybridization problems encountered by non-specific binding of probes to humic contaminants.

After optimal hybridization conditions have been determined, hierarchical probes can be used to assess microbial community composition by direct probing of membrane bound 16S rRNA. The use of direct probing as a method to assess microbial community composition was successfully applied to the research question at Ames plantation that community shifts would occur as a response to changing agricultural practices in order to maintain biogeochemical cycles. The results from the Ames Plantation project showed an increase in Gram-positive microorganisms and a decrease in Gram-negative organisms as the agricultural practices shifted from annual row crop management to short rotation tree crop management. As the short rotation woody crop began to mature and to look more like a hardwood forest, the shift became more pronounced. Subdivisions of the Proteobacteria, in particular the Alpha subdivision, and other groups in the Eubacterial domain also demonstrated community dynamics as a result of changing agricultural practices. These changes could be statistically assessed by the dot-blot method. The changes may be related to changing chemical concentrations and organic matter input in the environmental systems. Correlations between the biogeochemical data and the microbial community data will aid in modeling the ecological impact of changing agricultural practices from field crops to woody crops.
REFERENCES
REFERENCES


143


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Education, and Extension Service National Research Initiative Competitive Grants Program.


APPENDICES
Appendix 1-Experimental Results

A1-1 Probe Hybridization Studies

A1-1a Using Film

(I) Arch915- Archaeal Probe Hybridization Studies
(Ia) Hybridization Temperature

40°C

50°C

52°C

55°C

(Ib) Assessment of Arch915 Probe Specificity at 55°C
(II) Eub338 - Eubacterial Probe Hybridization Study

(IIa) Hybridization Temperature (l to r-USDA110, PpG7, IPL5, B30P4)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Image</th>
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</thead>
<tbody>
<tr>
<td>47°C</td>
<td>![47°C Image]</td>
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<tr>
<td>50°C</td>
<td>![50°C Image]</td>
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<tr>
<td>52°C</td>
<td>![52°C Image]</td>
</tr>
<tr>
<td>55°C</td>
<td>![55°C Image]</td>
</tr>
</tbody>
</table>

B30P4
Brevimonas
D.vulgaris
Drosophila
IPL5
PpG7
USDA
Zoogloea

(IIb) Assessment of Eub338 Probe Specificity at 55°C (l to r-USDA110, Brevimonas, B30P4, D.vulgaris, F1, IPL5, PpG7, 2375, 33015, 19544, Drosophila, Halophile)
(III) Alflb Alpha Probe Hybridization Study

(III a) Hybridization Temperature (I to r- B30P4, IPL5, PpG7, USDA110)

47°C

50°C

52°C

55°C

(III b) Assessment of Alflb Specificity at 55°C

alphas

alpha
(IV) SRB385 Delta Probe Hybridization Study

(IVa) Hybridization Temperature (hybridization with D. vulgaris)

52°C

55°C

57°C

60°C

(IVb) Assessment of SRB385 Delta Probe Specificity at 55°C

D. vulgaris
(V) Bet42a Beta Probe Hybridization Study (no competitive hybridization)

(Va) Hybridization Temperature (l to r- USDA110, PpG7, IPL5, B30P4)

- 40°C
- 42°C

- 45°C
- 47°C

(Vb) Assessment of Bet42a Probe Specificity at 45°C

Only Beta and Gamma Group’s Hybridized (did not use competitive hybridization)
(VI) Gam42a Gamma Probe Hybridization Study (no competitive hybridization)

(VI a) Hybridization Temperature (l to r- B30P4, IPL5, PpG7, USDA110)

40°C 42°C 45°C 47°C (a) 50°C
$52^\circ C$

$55^\circ C a$

$55^\circ C b$ (top row - B30P4, bottom row left Zoogloea, right IPL5 for next three blots)

$57^\circ C$

$60^\circ C$
(VIb) Assessment of Gam42a Probe Specificity at 55°C

Hybridized with Beta and Gammas (did not use competitive hybridization)
A1-2 Hybridization Studies Using Scintillation Counter

A1-2a

(I) Univ1390 Universal Probe Hybridization Study (shown in results)
(II) High G+C Content Probe Hybridization Release Curve

High G + C Probe Hybridization Temperature Study

![Graph showing high G+C probe hybridization temperature study with temperature in °C on the x-axis and probe released in counts/minute on the y-axis. The graph shows a trend where the probe released increases as the temperature increases from 40 to 75 °C.]
(III) LGCa,b,c- Low G+C DNA Content Probe Hybridization Release Curve

Low G + C Probe Hybridization
Temperature Study

![Graph showing probe released counts per minute vs. temperature (°C)](image-url)
(IV) CF319a - Cytophaga/Flavobacterium/Bacteriodes Probe Hybridization Release

Curve

CF319a Probe Hybridization Temperature Study

![Graph showing the relationship between temperature and probe released counts/minute.](image-url)
(V) Euk- Eukaryotic Probe Hybridization Release Curve

Euk Probe Hybridization
Temperature Study

![Graph showing the relationship between probe released (counts/minute) and temperature (°C). The graph indicates a linear increase in probe release as temperature increases.](image-url)
A1-3 Assessment of 16S RNA Quality From Reference Organisms

0.5X TBE/1% Agarose gel run at 90V for 1.5 hours

<table>
<thead>
<tr>
<th>Lane</th>
<th>Reference Organism</th>
<th>Original Concentration(µg/µl)</th>
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<tbody>
<tr>
<td>1</td>
<td><em>Brevimonas diminuta</em></td>
<td>5.46</td>
</tr>
<tr>
<td>2</td>
<td><em>A. eutrophus</em> B30P4</td>
<td>1.47</td>
</tr>
<tr>
<td>3</td>
<td><em>P.putida</em> IPL5</td>
<td>4.18</td>
</tr>
<tr>
<td>4</td>
<td><em>D.vulgaris</em></td>
<td>1.45</td>
</tr>
<tr>
<td>5</td>
<td><em>A. globiformis</em></td>
<td>1.52</td>
</tr>
<tr>
<td>6</td>
<td><em>B. subtilius</em></td>
<td>0.693</td>
</tr>
<tr>
<td>7</td>
<td><em>Flavobacterium</em> sp.</td>
<td>6.21</td>
</tr>
<tr>
<td>8</td>
<td><em>Halobacterium halobium</em></td>
<td>4.78</td>
</tr>
</tbody>
</table>
A1-4 Ames Plantation Data
See Results Section for Membrane Format

Eub338 Probe- Eubacteria Domain Dot-Blot Image (a *P. putida* is used as the Reference Organism)

<table>
<thead>
<tr>
<th>Corn</th>
<th>Corn RNA-</th>
<th>Y.Syc.</th>
<th>Y.Syc.RNA-</th>
<th>Old Syc.</th>
<th>Old Syc. RNA-</th>
<th>Hardwood</th>
<th>HW RNA-</th>
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</table>

Probe Specificity Check

Euk Probe- Eukarya Domain Dot-Blot Image (*G.soya* –Ref. Organism)
Bet42a Probe - Beta Subdivision Dot-Blot Image (*A. eutrophus*-Ref. Organism)

Gam42a - Gamma Subdivision Dot-Blot Image (*P. putida*-Ref. Organism)
SRB385 Probe- Delta Subdivision Dot-Blot Image (D. vulgaris-Ref. Organism)

HGC69a Probe- High G + C DNA Content Group Dot-blot Image (A. globiformis-Ref. Organism)
LG Ca,b,c Probe- Low G + C DNA Content Group Dot-Blot Image (Bacillus sp.-Ref. Organism)

CF319 Probe- Cytophaga/Flavobacterium/Bacteroides Group Dot-Blot Image (Flavobacterium sp. -Reference Organism)
### Appendix 2-Statistical Results

#### Univ1390 Probe (ng/dot-blot)

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<td>39.3633</td>
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<td>1.39948</td>
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### ANOVA

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<th>F-ratio</th>
<th>P-Value</th>
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<td>153.833</td>
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#### Multiple Range Test

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<tr>
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<tr>
<td>CF-YS</td>
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(*) denotes a statistically significant difference at a 95% confidence level.

#### Euk Probe (ng/dot-blot)

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<td>0.01</td>
<td>0.84</td>
<td>-0.516289</td>
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<tr>
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### ANOVA

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<td>0.0017</td>
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<td>Within Groups</td>
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#### Multiple Range Test

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<td>OS-YS</td>
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(*) denotes a statistically significant difference at a 95% confidence level.
### Eub Probe (ng/dot-blot)

#### Summary of Statistics

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<tr>
<td>Hardwood</td>
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<td>1.35191</td>
<td>18.15</td>
<td>20.83</td>
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<td>-0.6595</td>
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<td>Old Trees</td>
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<td>50.92</td>
<td>7.14</td>
<td>8.42</td>
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<td>18.33</td>
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#### ANOVA

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#### Multiple Range Test

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(*) denotes a statistically significant difference at a 95% confidence level

### Arch915 Probe (ng/dot-blot)

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### Bet42a Probe (ng/dot-blot)

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#### Multiple Range Test

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(*) denotes a statistically significant difference at a 95% confidence level
## SRB385 Probe (ng/dot-blot)

### Summary of Statistics

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### Multiple Range Test

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## Gam42a Probe (ng/dot-blot)

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(*) denotes a statistically significant difference at a 95% confidence level.
**LCGa,b,c Probe (ng/dot-blot)**

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**ANOVA**

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**Multiple Range Test**

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**HGC69a Probe (ng/dot-blot)**

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**ANOVA**

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**Multiple Range Test**

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(*) denotes a statistically significant difference at a 95% confidence level
**CF319a Probe (ng/dot-blot)**

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**ANOVA**

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**Multiple Range Test**

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(*) denotes a statistically significant difference at a 95% confidence level
### Eub Probe (Relative Percentages)

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(*) denotes a statistically significant difference at a 95% confidence level

### Arch915 Probe (Relative Percentages)

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(*) denotes a statistically significant difference at a 95% confidence level
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(*) denotes a statistically significant difference at a 95% confidence level
### SRB385 Probe (Relative Percentages)

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(*) denotes a statistically significant difference at a 95% confidence level.
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## HGC,6a Probe (Relative Percentages)

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(*) denotes a statistically significant difference at a 95% confidence level
Appendix 3- Recipes

A3-1 Media Recipes

ATCC 3 Nutrient Broth

8g of Nutrient Broth Media (BBL) per 1 liter Deionized water.

pH to 7.0-7.2 and autoclave at 121°C, 15 psi for 15 minutes.

ATCC 217 Halophile Media

10g Yeast Extract 1g/L K₂HPO₄

0.5g MgSO₄

25% NaCl (wt/vol)

1L Deionized H₂O

pH 7.0-7.2 and autoclave at 121°C, 15 psi for 15 minutes.
ATCC 1249 Modified Baar's Medium for Sulfate Reducers

Component I

2.0g MgSO₄
5.0g Sodium citrate
1.0g CaSO₄
1.0g NH₄Cl
400ml Distilled Water

Component II

0.5g K₂HPO₄
200ml Distilled Water

Component III

3.5g Sodium Lactate (2.69ml DL-Lactic Acid Sodium Salt)
1.0g Yeast Extract
400ml Distilled Water (Prepare in a 1L bottle)

Each component is adjusted to pH 7.5 and autoclaved at 121°C, 15 psi for 15 minutes. Wrap and sterilize a funnel. Using the funnel, aseptically mix the three components. Aseptically dispense 20ml of media into a serum bottle. Sparge the media with 97% N₂ and 3%H₂ for a minimum of 30 minutes. Carefully plug and crimpotop seal the serum bottle, using care to sparge the headspace of any oxygen. Filter sterilize a solution of 5% ferrous ammonium sulfate, Fe(NH₄)₂(SO₄) and add 0.1ml per 5ml of medium prior to inoculation.
CEB PAS Media

**Component I-PA Concentrate**

per 1 Liter of Deionized Water

56.8g K$_2$HPO$_4$

22.0g KH$_2$PO$_4$

27.7g NH$_4$Cl

**Component II- PAS Salts**

per 1 liter of Deionized Water

19.5g MgSO$_4$

5.0g MnSO$_4$.H$_2$O1.0g FeSO$_4$.2H$_2$O

0.3gCaCl$_2$.2H$_2$O

Acidify with H$_2$SO$_4$ to pH 2.5 and filter sterilize

**Component III-5% Yeast Extract (wt/vol)** **Component IV-5% Stock Solution (wt/vol)**

of Polyoxyethylene 10 lauryl Ether (POL) SIGMA Co.

For PAS Broth

Mix 78ml of Component I-PA Concentrate with 911ml of Distilled H$_2$O and

autoclave 121°C, 15 psi for 15 minutes. After the broth has cooled, add 10ml of

Component II-PAS salts and 1ml of Component III-5% Yeast Extract. If an additional

carbon source is needed, add Component IV-5% POL stock for a final concentration

of 0.2% POL.
A3-2 Solutions Recipes

CTAB

Place 4.1g NaCl and 10g CTAB in a beaker. Add 70ml dH2O and stir over low heat for 1 to 2 hours or until dissolved. Bring the volume to 100ml and autoclave.

DEPC (Diethylpyrocarbonate) Treated dH2O

Use 500µl of DEPC (Sigma) per 1L dH2O. Stir solution overnight, then autoclave for at least 45 minutes to remove all DEPC. Use caution when using DEPC as it is a suspected carcinogen. Always wear gloves.

Deionization of Formamide

Mix 50ml of Formamide and 5g of mixed-bed, ion-exchange resin (Bio-Rad AG 501-X8, 20-50). Stir for 30 minutes at room temperature and filter twice through a Whatman No. 1 filter paper. For RNA blotting mixture, dispense 34ml into a 50ml Falcon tube and store at -20°C.

Hybridization Solution

138g 0.5M NaH2PO4, 0.74g EDTA, 140g SDS, 2L dH2O
pH to 7.2 with NaOH Pellets

MgCl/DTT

1.017g MgCl and 0.077g Dithiothreital in 50ml of DEPC dH2O, filter sterilize through a 0.2micron Anodisk syringe filter into a sterile Falcon Tube. Store at -20°C.
Phenol Equilibration

Note: Always use extreme caution when working with phenol. Always work in the hood when using phenol and wear appropriate safety clothes: solvent resistant gloves, goggles, lab coat and apron. Phenol can cause severe burns. If phenol is spilled on the skin immediately douse the region with polyethylene glycol which should always be kept in the hood nearby. Then rinse in water for at least 15 minutes. Remove any clothing that may be saturated with phenol.

Tape the bottom of a jar of solid phenol to prevent possible breakage. In a 65°C water bath, which has been placed in the hood, loosen the cap and melt the phenol. Add 0.1% (w/w) 8-hydroxyquinoline to the melted phenol and swirl to dissolve. Add an equal volume of 10X TE (pH 8.0 for DNA extractions and 5.2 for RNA extractions). It is best to work over an autoclave tub at this point. Carefully, invert the jar several times to mix. Let the phenol separate into two phases. Aspirate off the top aqueous layer. Add 5X TE at the correct pH, repeat mixing and reequilibration to two separate phases. Add 1X TE at the correct pH and repeat mixing and reequilibration. Repeat this last step until the desired pH is obtained. The pH can be ascertained by removing a portion of the bottom phenol layer and placing a drop on a pH strip. Equilibrated phenol can be stored at 4°C for one month without oxidizing. For long term storage, divide into smaller tubes and store at –20°C.
PVPP

Place 200g of PVPP in a 4L beaker. Add 2L 3M HCl and stir in a hood for 3 to 4 hours.
Filter the solution through a 100 micron nylon screen in a buchner funnel under low
vacuum. Add 2 L 200mM KPO₄, pH 7.4 and stir 1 to 2 hours. Filter as before. Repeat
until the pH is 7.0 (approximately 5 times). Filter and dry overnight in a drying oven.
Store dry at room temperature.

RNA Denaturing Gel

Mix 37.5ml DEPC dH₂O, 12ml 5X MOPS, 0.7g agarose. Heat into solution and then
cool to 60°C. Add 10.8 ml Formaldehyde. Load the samples with RNA Loading Buffer: (2X Gel Loading Buffer: 80% Formamide, 0.1% Bromophenol Blue, 0.1%
Xylene cyanol, 2mM EDTA). Run at 60V for 1.5 to 2 hours. Visualize bands using
either Sybr Gold or ethidium bromide stains.

RNA Wash Buffer:

While stirring, add 100ml 20X SSC and 10ml of 10% SDS (wt/vol) to 890 ml of
dH₂O. Note: Do not mix 20X SSC and SDS together before adding to the water
because the SDS will fall out of solution. If this occurs the solution can be heated to
resolubilize the SDS. Be sure to let the wash buffer cool to room temperature before
use.

10% SDS (Sodium Dodecyl Sulfate)

100g SDS per 1L dH₂O
**20X SSC**

175.3 g of NaCl, 88.2 g of Sodium Citrate, 1 L dH₂O, pH to 8.0, Autoclave 15 minutes at 121°C, 15 psi.

**STE**

0.1M NaCl, 10 mM Tris HCl, 1mM EDTA (pH 8.0)

**TE**

100mM Tris, 1mM EDTA, pH to 8.0, Autoclave 15 minutes at 121°C, 15 psi
VITA

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