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Phenotypic and genetic differences in soil bacterial communities among successional stages

Rowena A. G. Hamlet

PHENOTYPIC and GENETIC DIFFERENCES IN SOIL BACTERIAL COMMUNITIES AMONG SUCCESSIONAL STAGES

A Thesis

By

Rowena A. G. Hamlet

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Approved as to style and content by:

Anita L. Davelos Baines
Dr. Anita L. Davelos Baines
Chair of Committee

Kristine Lowe
Dr. Kristine L. Lowe
Committee Member

Jonat Lieman
Dr. Jonathan H. Lieman
Committee Member

May 2009

DEDICATION

I would like to thank God for showing me that I can believe in him, and accept science at the same time. I would also like to thank my mother, Sylma Hamlet, older brother Raunel Hamlet, and younger sister, Racquel Hamlet, for all of their help, patience, support and prayers during my academic endeavours. A special thanks to my extended family and church family (BME Christ Church, St. James, (Toronto, Canada) and Baptist Temple (McAllen, USA)), and friends both here in Texas and at home in Canada. Thank you all for encouraging me. The study in this thesis was the work of two and a half years, while pursuing a career that would literally have me running in circles. Thank you Mr. Christie! We can get started now. I dedicate this book to the lady that was left with HER 3 children, no money, no job, no car, and the house-hold contents... Thank you so much for everything, Mom. Your hard work was well worth it and much appreciated.

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ABSTRACT

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The association between soil microbes and plants can influence plant growth and survival as well as alter soil microbial community dynamics. The purpose of this study was to determine how the length of this interaction between plants and bacteria affects the bacterial soil community structure. Soil microbial communities associated with plant communities at different successional stages at the Santa Ana National Wildlife Refuge were investigated. The study sites included three revegetated sites (4 months, 21 months, and 221 months since revegetation) and a control site (native brush, never revegetated). Five soil samples were randomly collected at each site. Soil microbial communities at each site were characterized for density, nutrient utilization, and genetic profiles. There was no significant difference in density among the revegetated sites. Microbial communities associated with plant communities at earlier successional stages used significantly more nutrients and had higher activities than communities at later stages. Amplified Ribosomal DNA-Restriction Analysis (ARDRA) profiled the bacterial community to determine genetic differences in community structure within and among the sites. Restriction analysis using five restriction enzymes revealed more variation within and among bacterial communities associated with plant communities at earlier successional stages than at later stages. Soil microbial communities associated with younger revegetated sites were still in flux as they were undergoing succession and had not yet achieved a climax community.

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CHAPTER I

INTRODUCTION

Both long-lived plant species and annual plant species can alter soil microbial communities. Soil microbes and pathogens can affect individual plants as well plant community dynamics. Understanding how these interactions may affect the structure and function of both plant and bacterial communities could help improve land management for both agricultural and restoration applications.

Plant-Microbe Interactions

Soil microbes are vital to nutrient cycling and the decomposition of plant material, and play a significant role in plant health through nitrogen fixation, inhibiting the development of plants disease and nutrient uptake. Many interactions that take place between plants and heterotrophs, such as microbes, are essential to understanding how the structure of the ecosystem is regulated, population dynamics, the flow of energy and the cycling of nutrients through time (Crawley, 1983). Microbial interactions play a significant role in plant fitness. Nitrogen-fixing bacteria convert nitrogen to ammonia that is then absorbed by plants (Chanway et al., 1991). This improves nutrient uptake and cycling. This is evidence that bacteria have the ability to aid and improve the survival of plants and increases root-shoot ratios. Plant interactions may range from antagonistic, as in Citrus Canker, where the bacterium *Xanthomonas axonopodis* causes lesions on

leaves, stems, and fruit. Also, interactions may be positive or mutualistic as it is when bacteria fix nitrogen for leguminous plants.

Factors involved in spatial temporal dynamics of natural vegetation and effects of soil pathogens on spatial temporal dynamics of plant communities are likely to be overlooked because of lack of studies concerning soil microbial activity and succession. Failures of seedling survival are not readily attributed to soil pathogens and soil microbes unless studied in detail both in the field and in controlled experimental conditions (Packer and Clay, 2000). The same is true for studies pertaining to the contribution of soil pathogens and microbes to directional succession (Van der Putten et al., 1993) and cyclic succession on the maintenance of plant species in old field grasslands (Bever, 1994). Cyclic succession, mostly observed during secondary succession, is change in a small number of species over time (Jackson, 1993). These changes may or may not be initiated by a large scale disturbance, like a natural disaster, but are more likely due to small scale disturbances such as an insect infestation.

Microbes have been strongly linked to the success, or progress, of some plant species as a results both plant and microbes thrive and have an increased presence within the community (Lichter, 1998). The success of revegetation efforts may be significantly influenced by microbial activity in soils (Belnap et al., 2001). Changes in microbial activity can alter soil fertility, and structure, and thus impact the ability of soil to store and deliver resources to plants. The absence of microbes, such as nitrogen-fixing bacteria, can severely limit seedling establishment, plant growth, and plant survival. Mycorrhizae and symbiotic nitrogen fixing bacteria may enhance host and nonhost plant establishment, increase productivity, enhance exploitation of soils for water and nutrients,

increase nutrient quality of foliage, and increase rates of succession on reseeded sites (Collins et al., 2008).

In turn, plants may influence the density and composition of the soil microbial community. Existing data show that both long-lived plant species (e.g., perennial prairie plants) (Eom et al., 2000) and annual species (agricultural crops, green manures) (Eom et al., 2000) can alter soil microbial communities. Plant successional studies that consider bacterial communities have shown a general pattern of high numbers or biomass of bacteria early in succession with declines in later stages (Klein et al. 1995; Belnap et al., 2001). However, none of these studies examined the dynamics of specific components of the bacterial community. Soil microbial communities associated with plant communities at different successional stages at the Santa Ana National Wildlife Refuge were investigated. How the length of the interaction between plants and microbial communities can alter bacterial community structure was investigated by determining the density of at each of the sample sites, determining nutrient utilization by the microbial community, and establishing profile by determining the genetic structure of the microbial communities among the sites.

Succession

It is well documented that soil microbes are beneficial, if not vital, to the survival of plants (Chanway et al. 1991; Wilson and Hartnett, 1997; Van der Heijden et al., 1998; Hartnett and Wilson, 1999). Succession is the process of one community replacing another until a climax community is achieved. Early successional species may facilitate colonization and growth of later species. The gradual changes that occur during succession could result not only in differences in soil microbial community composition,

but also the plant community it interacts with. Primary succession begins on rocks or an area without existing or previous vegetation. Initially, pioneer species establish in an area or environment that may be extreme and not necessarily optimal in regard to resources. Secondary succession occurs on sites with established vegetation and re-establishes a community after a temporary disturbance. It can be a relatively discrete event in time that causes an abrupt change in the ecosystem, community, or population structure, and may also change resource availability, substrate availability, or the physical environment. Natural events that range in intensity may be considered disturbances: fire, tornadoes, animals, disease/pathogens/viruses and humans (Pickett and White, 1985). These events can range from an insect infestation and animal grazing to natural disasters up to and including floods and hurricanes. The sizes of the affected area, as well as the frequency (how often disturbance occurs), are factors that could impede or restrict ecological development of both microbial and plant communities (Pickett and White, 1985). Species composition over time reveals change; however, more often than not there is little indication as to why these changes occurred (Pickett and White, 1985). Understanding the indicators that influence and participate in these changes would help to make predictions regarding the ecosystem.

In general, soil communities undergoing succession progress and go through several intermediate phases and therefore,are in a constant state of flux (Pickett and White, 1985). Achieving stability or what would be considered a climax community, could take years to accomplish because new plants and microbes enter into the community changing the dynamics.

As a remnant subtropical forest surrounded by cleared agricultural land Santa Ana National Wildlife Refuge (SANWR) is a 2,080-acre natural preserve located in the Lower Rio Grande Valley of Texas, seven miles south of Alamo in Hidalgo County. The refuge is noted for its unusual birds, mammals, butterflies, and plants. Native brush includes 1,200 types of plants. Trees include mesquite, sabal palm, Texas ebony, prickly pear, and Montezuma bald cypress. The abundance of the varying vegetation is necessary as the refuge is home to 700 vertebrate species; including 500 bird species, and 11 different biological communities (Jahrsdoerfer et al., 1988).

In recent years an initiative to revegetate some agricultural areas in South Texas to the native brush has been made with mixed success (Castillo, M. pers. comm., 2007). Revegetation has occurred at different time intervals beginning in 1989, to as recent as 2003 at the Ranchito Tract (Figure 1, Figure 2). This land, located in Cameron County, TX is owned by the SANWR.

Understanding the microbial communities and how they could influence, positively or negatively, the establishment and growth of vegetation (and ultimately their ability to survive) may be critical for the success of revegetation programs such as the one at Santa Ana.

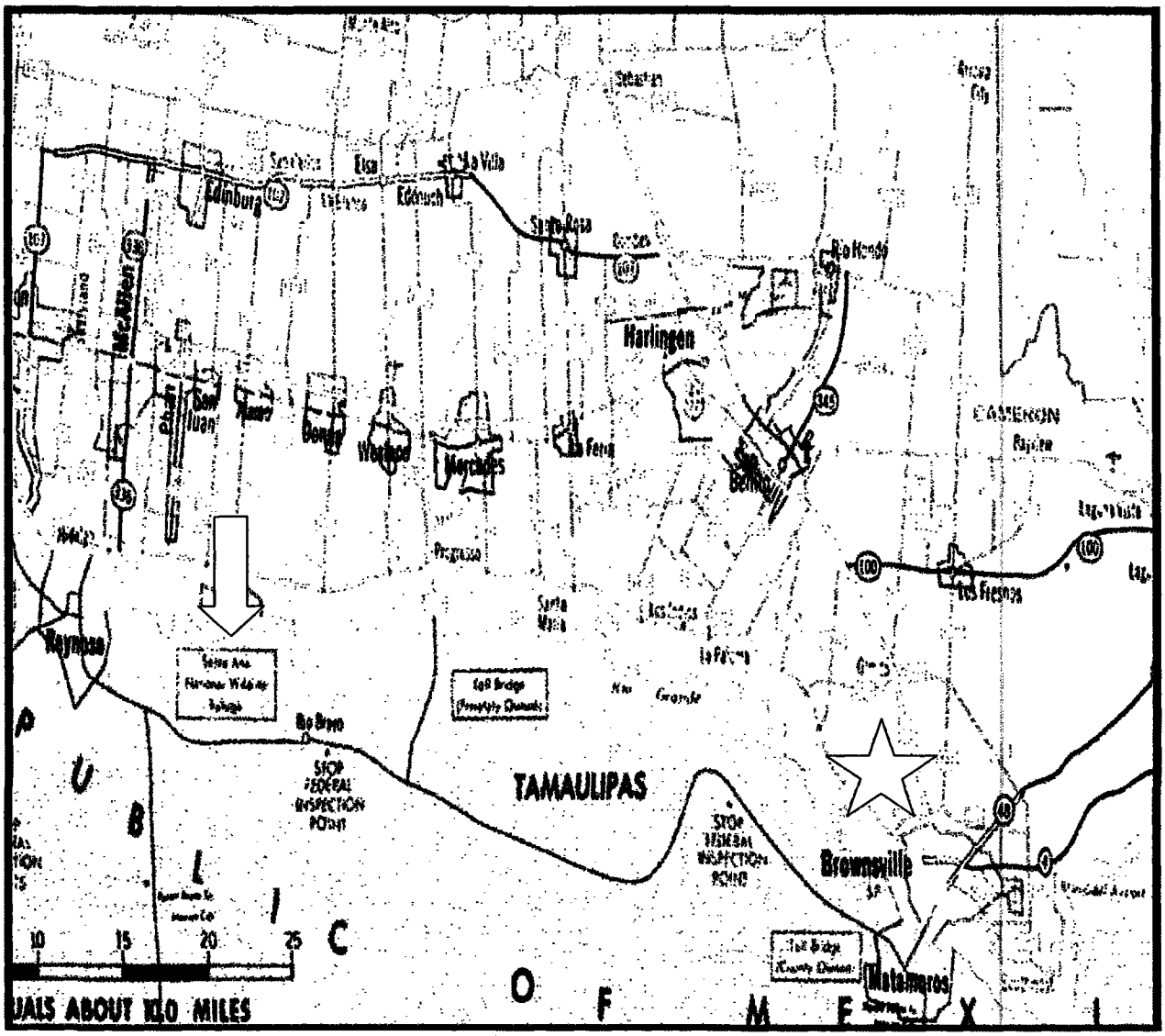


Figure 1: Map of the Lower Rio Grande Valley. Arrow shows the location of the Santa Ana National Wildlife Refuge in Hidalgo County. Star shows the location of the Ranchito tract in Cameron County where samples were collected.

Restoration of Plant Communities

With regards to revegetation efforts, determining the best options for success is crucial. Using seedlings, direct seeding, or plants for revegetation are possible. Other factor are important in the impact of the revegetative mechanism will have on the soil microbial community and dynamic. The soil microbes, as well as their (rate) activity. are important and may provide vital information that will enable successful revegetative efforts. Restoration and revegetation of an ecosystem are based on the knowledge of abiotic and biotic interactions that affect plant establishment (Pyke and Archer, 1991). However knowledge of these interactions and how revegetation process affect the success of revegetated communities, is limited (Pyke and Archer, 1991). A series of potential interactions need to be taken into account to enable a successful revegetation program. Determining whether or not there will be subsequent competition between plant species is also important to revegetation, as species would need to coexist in the community. Revegetation can vary from partial restoration (rehabilitation) to total restoration of the original ecosystem. Partial restoration occurs when ecologists or land manager successfully restore a portion of the former species, whereas with total restoration many if not all species are re-established. An alternate option would be to replace the former community with an entirely new one that would yield a similar and/or much improved vegetation (Cooke and Suski, 2008). Understanding the mechanisms underlying the process of succession, and how they may impact competition and community composition, is also important.

Competition for resources may influence the presence, absence, or abundance of species in a community and determine their spatial arrangement. The importance of

competition within the ecosystems of deserts and prairies has been scrutinized because of an ongoing debate over the acceptance that both autogenic and allogenic succession influences the activity of soil microbial communities (Brussard et al., 2007). Autogenic succession is driven by factors internal to the community, such as the lifespan of a given species. Allogenic succession is driven by external factors including varying disturbance regimes. Alternatively, abiotic stress, such as the presence or absence of an element in the soil, rather than competitive interactions, may determine community structure and function in both plant and soil communities (Fowler, 2005). Competition among plant species has may increase the amount of time necessary to achieve reproductive maturity and decrease growth rates, frequency, and viable seed production (Packer, 2005). Different species within communities have different adaptations and therefore different patterns of growth and reproduction, both spatially and temporally (Packer, 2005). They are limited by resources and environmental factors, which inadvertently influence phenotype (Belnap et al., 2001). Instability in weather or resources could essentially result in decreased annual variation in the productivity of individual species (Packer, 2005). All of these factors may affect plant community structure and the success of restoration projects.

Soil and Climate

There is a small range of soil types in within the LRGV, including clay soils, saline soils, and silty soils (Murray et al., 2006). The permeability of the soils is in direct relation to its proximity to water; soils close to the Rio Grande River are more permeable (Box, 1961). Sternberg (1999) described the varying loams (soil composed of sand, silt and clay) of the "Valley" as a sandy plain that is bisected by a 'clayey' floodplain." Due

to the variation in soil, other abiotic factors (N, Fe, Ca etc.) could vary as well. The presence of particular plant species or functional groups (abiotic factor) in communities can stimulate the activity and functional diversity of soil bacteria.

Lower Rio Grande Valley Brush

Agriculture and urban development has destroyed 95% of native brushland habitat in the Lower Rio Grande Valley (LRGV) of South Texas (Jahrsdoerfer, 1988; Pyke, 1991). Efforts to restore the natural habitat have been in progress since 1982 by The United States Fish and Wildlife Service (USFWS), Texas Parks and Wildlife (TPWD), The Nature Conservancy of Texas (TNC), The LRGV National Wildlife Refuge, and the Santa Ana National Wildlife Refuge. Brushlands in the LRGV are typically dominated by native species such as *Fraxinus berlandieriana (*White ash*), Ulmus crassifolia (*Elm*), Celtis laevigata (*Sugarberry*), Chloroleucon ebano (*legume), *Ehretia anacua (*Koda*)* also known as *Prosopis glandulosa, Guaiacum angustifolium (American shrub), Sabal texana (Sabal palm), Choremolaena odorata(perennial and shrub,* belonging to daisy family*), Rivina humilis (*Pigeonberry , *Malpighia glabra (*Barbados cherry*)*, and *Sertaria leucopilia* also known as *Panicum maximum (*Guinea grass*)*. Restoration attempts have been done using direct seeding and seedling planting (Pyke, 1991). Revegetation methods varied due to cost, according to rates of success which had been influenced by the associated plant and soil microbe communities (Sternberg, 1999; Vora, 2008). Twenty to fourty-seven native brushland species were planted in weed-free agricultural fields in designated areas to initiate the revegetation effort (Vora, 2008). Herbicides were used to prevent invasive non-native grass species such as *Cynodon dactylon (*Bermuda grass*), Pennisetum ciliare* (Buffel grass), and *Pennisetum purpereumm* (Elephant grass)

from growing (Jahrsdoerfer, 1988). It was also determined during early replanting efforts that *Clematis drummondii (*leather flower*)* appeared to impede the growth of some plants as it has a tendency to grow over newly planted species (Jahrsdoerfer, 1988; Box, 1937). This is a climbing vine that covers fences and shrubs and is highly drought-tolerant. Some of the aforementioned invasive species are also documented as preventing the growth of some species by developing a dense mantle over vegetation, therefore blocking necessary sunlight (Jahrsdoerfer, 1988). The Rio Grande Valley is one of the fastest growing regions in the United States, thus restoration efforts of native plant species could prove to be difficult. Maintaining the integrity of the existing brush could also prove to be a task for the same reasons (Sternberg, 1999).

CHAPTER II

MATERIALS & METHODS

Study Sites

The Santa Ana National Wildlife Refuge (SANWR) is located in Hidalgo County, Texas (Figure 1). This site is managed and maintained by the Texas Parks & Wildlife Department (TPWD) and United Stated Fish and Wildlife Service (USFWS) (Castillo, M., pers. comm., 2007). Being one of the most biologically diverse National Wildlife Refuges (NWR) in the United States (US), the grounds at Santa Ana are home to approximately 1100 types of plants. The Ranchito Tract, owned by the SANWR, is a part of a revegetation project to reclaim agricultural lands. Samples were collected from the Ranchito Tract located in Cameron County, TX. Four sites were selected for this study: 3 revegetated sites and one control site. Each site was revegetated at a different time, whereas the control site consisted of only native undisturbed brush that had never been used for agricultural purposes.

At the time the samples were collected (May 2007), the most recent revegetated site was site 15-S (4 Month Site). This site was 69.86 acres in size and was revegetated using seedlings of *Poaceae* (grass), *Prosopis glandulosa (*Sugarberry*), Rivina humilis (*Pigeonberry*),Malpighia glabra (*Barbados cherry*),Cynodon dactylon (*Bermuda grass*)*, and *Pennisetum ciliare* (Buffel grass) on January 30, 2007. Site 124 (41Month Site) was

81.69 acres. It had been revegetated on December 26, 2003, also using seedlings of *Poaceae*, *Prosopis glandulosa* (Sugarberry), *Rivina humilis* (Pigeonberry), *Malpighia glabra* (Barbados cherry), *Cynodon dactylon* (Bermuda grass), and *Pennisetum ciliare* (Buffel grass). The smallest site, 102 (221 Month Site) for the purposes of this sudy) at 16.80 acres, was the oldest revegetated site and was seeded using *Celtis laevigata* (Sugarberry), *Chloroleucon ebano* (legume), *Ehretia anacua* (Koda), *Rivina humilis* (Pigeonberry) , *Malpighia glabra* (Barbados cherry), and *Panicum maximum* (Guinea grass). This site had been revegetated on December 12, 1989. Each revegetated site was at different successional stage. The control site was 206.88 acres, and contained native brush such as *Fraxinus berlandieriana* (Mexican ash), *Ulmus crassifolia (Cedar elm), Celtis laevigata, Chlorileucon ebano* (legume), *Ehretia anacua* (shrub) also known as *Prosopis glandulosa* and various species of *Poaceae,* and had never been disturbed. The 4 Month Site, located at the northern end at the Ranchito Tract (Figure 2), was bare with the exception of the transplants. The 41 Month Site, also located at the northern end of the Ranchito Tract (Figure 2), had sparse to little vegetation. The oldest plots both had a fair amount of vegetation and were located at the southern region of the Ranchito Tract (Figure 3).

Soil sampling

Soil samples were collected at the Ranchito Tract in May 2007. Five samples were obtained from each study site. Random soil samples from the three revegetated sites of different ages and 1 non revegetated site were collected with a soil corer that penetrated the soil surface approximately 30 centimeters (cm). The soil corer was approximately 3-4 cm in diameter. Collected samples were placed in a Ziploc freezer

bag and stored in a cooler on ice until our return to the lab. The samples were stored at 4°C until they were processed.

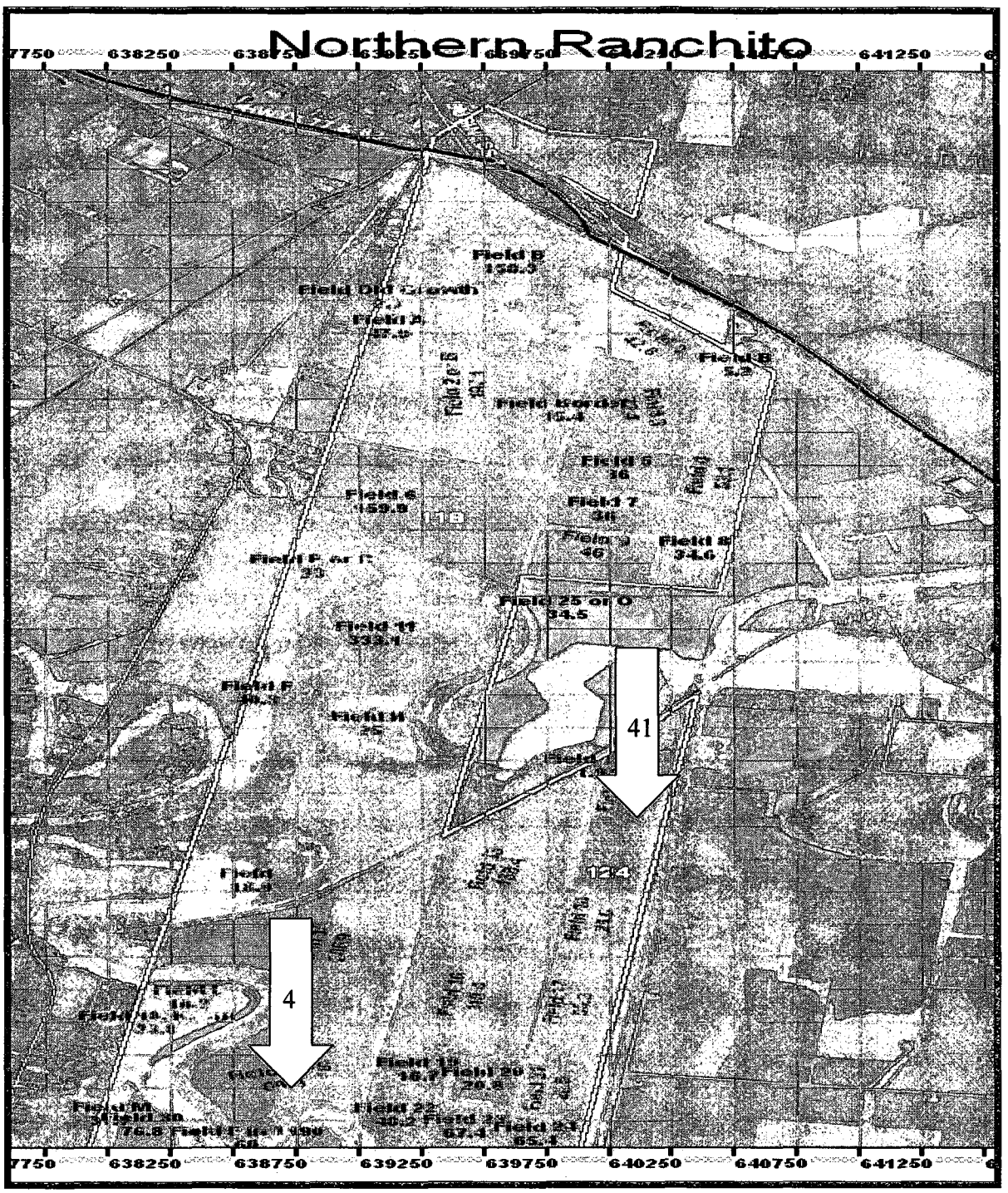


Figure 2: 4 Month Site and 41 Month Site are located at the Northern end of the Ranchito Tract. Figure 2 provided by M. Castillo, SANWR.

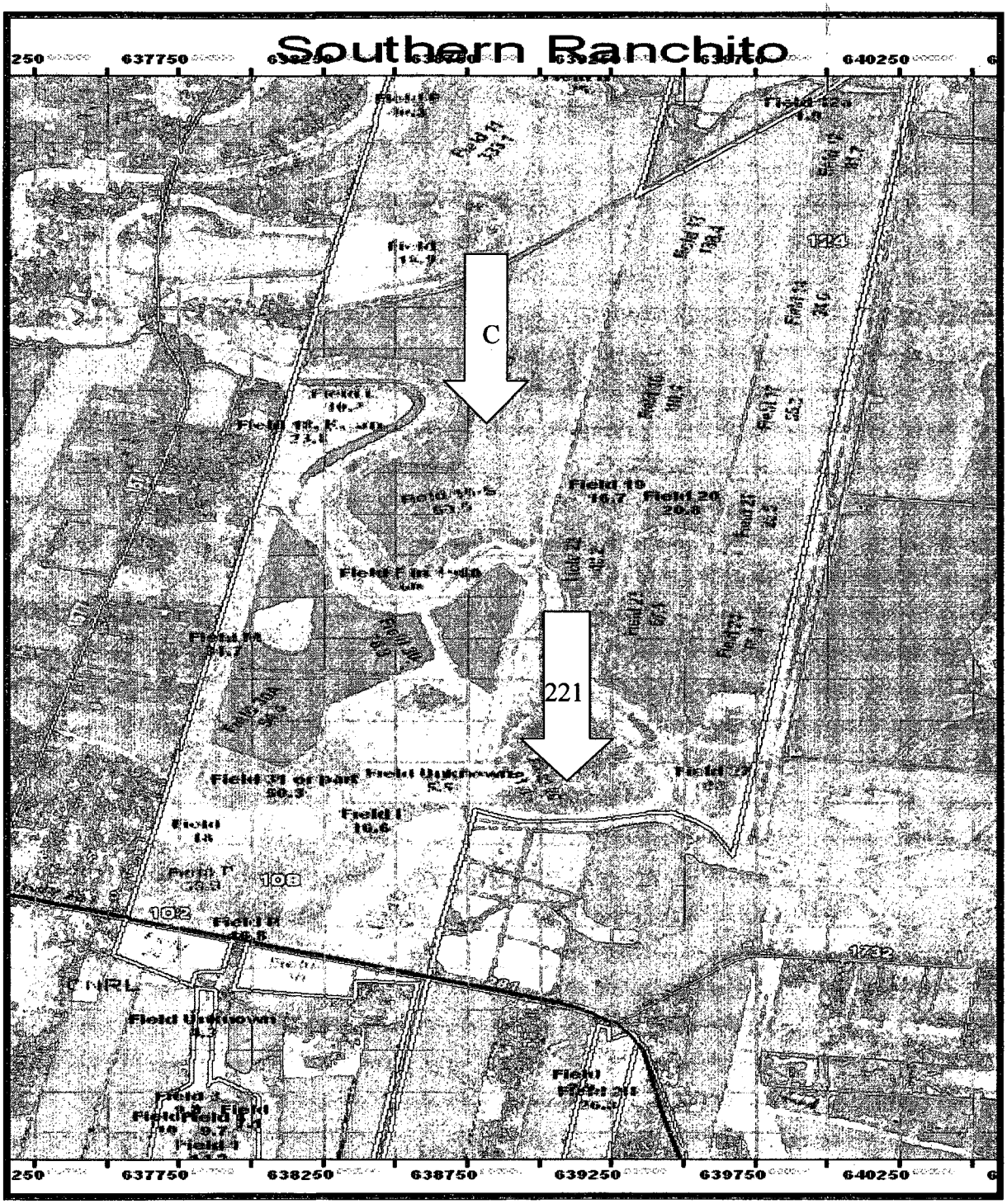


Figure 3: Control Site and 221 Month Site are located at the Northern end of the Ranchito Tract. Figure 2 provided by M. Castillo, SANWR.

Soil Analyses

Using the Model STH Series Combination Soil Outfit, provided by LaMotte, a soil analysis was conducted on each sample to determine variation within and among sites with regards to abiotic factors. Nitrate, Calcium, Iron, Magnesium, Sulfate, Ammonia, Manganese, Aluminum, Potassium, and Nitrogen were measured in pounds per acre. Following the manufacturer's instructions, a general soil extraction was carried out. Obtaining the measure of these elements present in the soil, was able to provide an understanding to what the soil community chemical content might entail. Soil microbial communities vary in composition, thus having a range of requirements for survival (Campbell, 1997). Plants would also influence the concentration of the macronutrients (Nitrogen, Phosphorus, Potassium, Calcium, Magnesium, and Sulfur), which are elements required in high quantities (Dighton et al., 1996). Micronutrients (Manganese and Iron) and trace nutrients (Aluminum) are elements required in smaller concentrations.

Bacterial Densities

Five grams (g) of each sample (20 samples total) were placed in individual weigh boats and covered with sterile cheese cloth. The soil was allowed to dry overnight. Samples were ground using a mortar and pestle and each sample was added to 50 mL of sterile distilled water. Samples were suspended by a reciprocal shaker at 250 revolutions per minute (rpm) for an hour (in a refrigerator) at 4°C. Serial dilutions were plated out, in triplicate, to grow the culturable bacterial community. The culturable bacterial community was grown on both Starch Casein Agar (SCA) and Oatmeal Agar (OA). Starch Casein Agar is a selective media known to specifically promote the growth of *Streptomyces* (Davelos Baines, A. L., pers. comm., 2007). Oatmeal Agar is a high

nutrient agar which is good for a wide range of bacterial groups. Though it is not possible to grow the entire bacterial community on plates, the bacteria that do grow are a representative of a portion of the community. This is normal for this type of experiment as other studies have shown that, less than 1% of soil microbes will grow on laboratory media (Torsvi et al., 1990). Plates were incubated at 28°C for 5 days and then counted. Colony forming units (cfu) were counted and the density of the community was estimated by multiplying by the dilution factor.

Statistical analysis was performed using SPSS© (Statistical Package for the Social Sciences). An analysis of variance (ANOVA) was performed to compare density of culturable bacteria among sites (P=0.0501). SPSS assumes that all the variables are representative of a normal distribution (Edwards, R., pers.comm., 2008). Significant differences among means were determined using Scheffe post hoc test (P=0.001).

Nutrient Utilization

BIOLOG EcoPlates™ were used to estimate nutrient utilization among microbial communities at different successional stages. The 10^5 dilution (from the density experiment) from the serial dilution described above was used to inoculate BIOLOG EcoPlates®, which can determine carbon utilization/metabolic activity (Garland et al., 1997; Glimm, et al., 1997). These plates gave both quantitative and qualitative results with regards to physiological (metabolism) activities of microorganisms in the different soil communities. Each plate had 96 wells containing 31 different carbon sources. Each carbon source was present in triplicate. The remaining 3 wells were water controls. The 10^5 dilution was mixed then poured into a sterile petri dish. An eight channel Finnpipette (Thermo Electron Corporation) was used to transfer 100 μL of the sample to each

individual well in the microplate. Each microplate was incubated for 3 days at 28°C. A BioRad® Model 680 microplate reader was used to measure the results every 24 hours at 590 nm. Mean number of carbon sources utilized by each sample (mean, since the experiment was done in triplicate), mean nutrient sources utilized by site, nutrient utilization trends, mean activity per sample, mean activity per site, and the level of activity below and above the mean, was also noted and recorded.

The readings obtained from day 3 were analyzed and used to construct a dendrogram using NT-SYS (Rohlf, F. J., 1998). A dendrogram demonstrates the similarities, if any, between samples. Clustering of samples would indicate that there were similarities in their use of substrates; however, that does not mean that they used the same substrates. In addition to assessing the similarities of substrate/nutrient utilization, the sum of the activity was determined by totaling the raw data from the microplate readings. This would more so pertain to the phenotypic similarity between the samples that are clustered. Calculations must be done to correct for water control. Since each plate has each carbon source, and water control in triplicate, the mean of each of these is taken. This results in 32 numbers. The mean value for the water controls is subtracted from the means for each carbon source. This can result in negative values at which time any negative values are set to zero.This allowed us to assess the activity for not only the individual samples, but the entire site as well (Rohlf, F. J., 1998).

Amplified Ribosomal-DNA Restriction Analysis

Amplified Ribosomal-DNA Restriction Analysis (ARDRA) is a molecular profiling method based on restriction endonuclease digestion of amplified bacterial 16S rDNA (Gich et al., 2000). A restriction enzyme recognizes specific sequences and

divides the DNA into fragments at these recognition sites (Gich et al., 2000). Separation of digestion products were run on 0.8% gels. The resulting banding patterns showed differences and similarities between the samples, and subsequently the sites.

One gram of each sample was grown in 10 mL of nutrient broth. Cultures were placed in a test tube rack and grown for seven to ten days at room temperature (25°C). Cultures were spun down at 14,000 rpm in a centrifuge for 5 minutes. The supernatant was removed and DNA extracted from the pelleted cells using a maximum yield protocol Wizard® Genomic DNA Isolation Kit. Once the DNA extraction was completed, PCR, was performed.

The Polymerase Chain Reaction (PCR) amplifies a specific DNA region, and this segment. The protocol includes appropriate volumes of Master Mix (provided), 16S rRNA IDT ReadyMade forward Primer® (5' AGA GTT TGA TCC TGG CTC AG 3'), and 16S rRNA IDT ReadyMade reverse Primer® (5' ACG GT ACC TTG TTA CGA CTT 3'), a DNA template, and water. A 20 μL aliquot of the PCR solution was added to an epitube containing 5 μL of an individual sample. The epitubes were then placed in the thermocycler using the following Takeuchi (Takeuchi, 1996) protocol.

The 16S rDNA Takeuchi PCR protocol, used in the BIO RAD My Cycler thermocycler, was as follows (Figure 4):

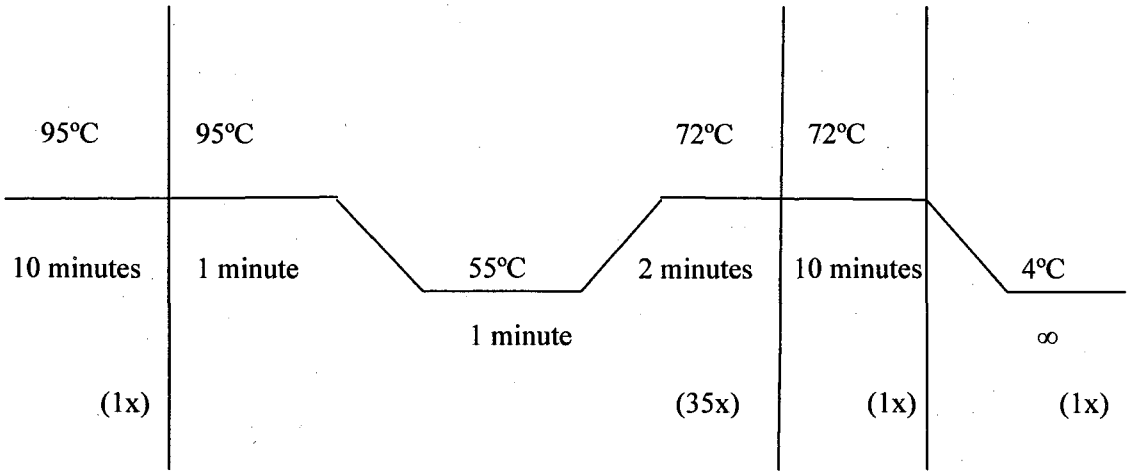


Figure 4: 16S rDNA Takeuchi PCR protocol

PCR products were used for Amplified Ribosomal-DNA Restriction Analysis (ARDRA). The DNA region under amplification was the 16S rRNA gene. The samples were run on a 0.8% gel and observed with a UV illuminator. PCR products migrated through the gel and stopped between 1000 and 1600 base pairs (bp). This measurement was obtained by loading a 1 kb ruler, also provided by BIO RAD, alongside the samples. The 16S region was amplified. Characterization of prokaryotes can be based on sequencing this region (Ercolini, 2004).

For each restriction enzyme tested, protocols and supplies were provided by Fisher BioReagents (Fisher Scientific). Similar to the PCR protocol, ARDRA has a "master solution" that must be made using the provided restriction enzyme 10X buffer, restriction enzyme, and water. These were combined using the recommended quantities.

This solution was then placed in individual tubes containing a DNA sample (PCR product). The tubes were incubated for 4 hours at 37◦C to complete the digestion process. The following restriction enzymes were used/tested: *Eco*R V, *Sal* I, *Rsa* I, *Hae* III, and *Alu* I (Appendix B).

Using gel electrophoresis, the pattern of DNA bands that was produced was used to distinguish different strains of bacteria in the different successional communities. For this study, ARDRA was used to note and characterize the differences within and between sites. The community level diversity of bacteria was determined after viewing the gels under UV trans-illumination.

CHAPTER III

RESULTS

Soil Analysis

There was no variation among sites with regards to the concentration of Aluminum, Calcium, Manganese, Nitrogen or Sulfate. Nitrate, Magnesium, Phosphorus, and Potassium, did show variation among the sites. Each site demonstrated that as these sites age, the presence or concentration is not affected. Ammonia and Iron, showed a negative relationship in that as the sites increased in age (4 Month, 41 Month, 221 Month, Control), were present in lower concentrations at older successional stages. The trends of the abiotic factors can be observed in Table 1.

Bacterial Densities

There was no significant difference between the community density among most sites when grown on OA ($F_{3,16}$=0.0585 and P=0.0501). Figure 5 shows that only the 221 Month site showed a significant difference in density, opposed to the other two revegetated sites and the Control, which were not different from each other.

Factors	4 Month Site	41 Month Site	221 Month Site	Control
Nitrate	10-40	10	10-40	10-20
Phosphorus	180-220	150-200	150-220	400
Potassium	135-350	150-390	170=300	180-300
Magnesium	Medium-High	Very low-Medium	Medium-High	Very low-High
Ammonia	Medium	Low	Low	Low
Iron	50	50	5	15
Calcium	1400	1400	1400	1400
Sulfate	<2000	<2000	<2000	<2000
Aluminum	Very low-Low	Very low-Low	Very low-Low	Very low-Low
Nitrogen	10	10	10	10
Manganese	Low	Low	Low	Low

**All factors measured in pounds per acre*

Table 1: Abiotic Factors

This table shows range of elements present in the soil. Variability and trends can be observed. In addition, there are elements present that seemingly are not impacted by succession (eg. Ca, SO_4^{2-}, Al, N, Mn).

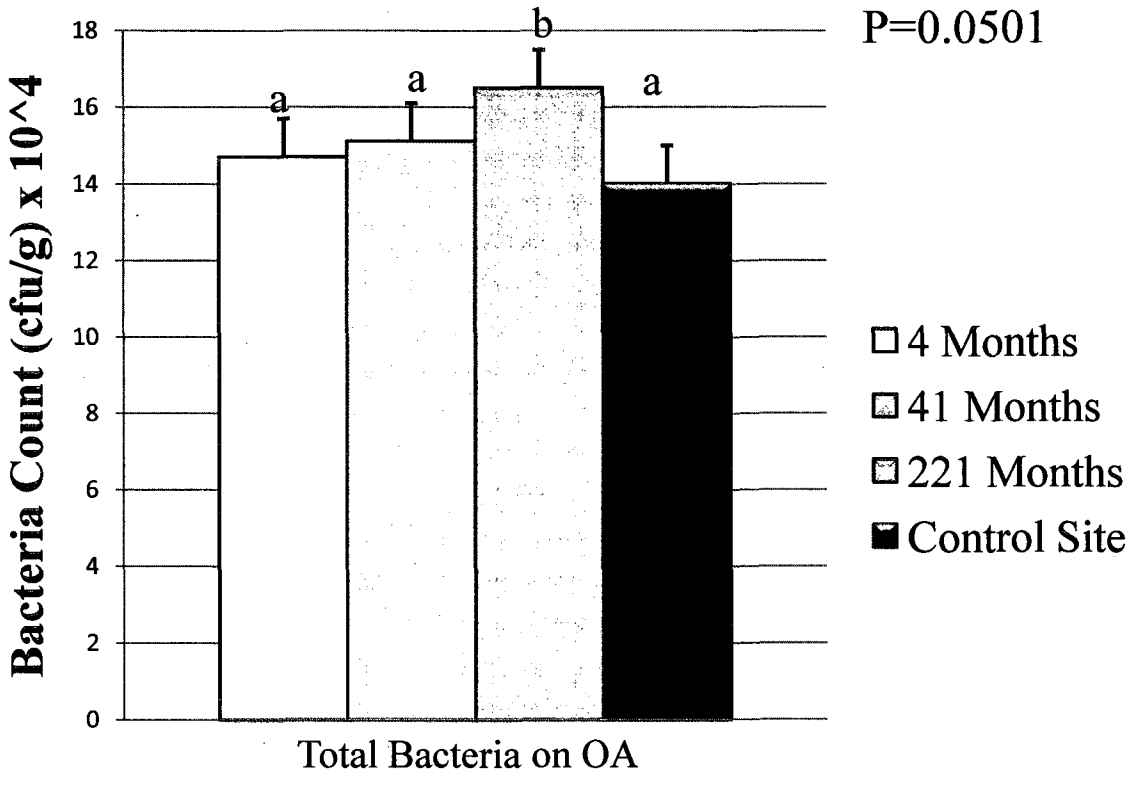


Figure 5: Bacterial Density at Different Successional Stages
The bacterial densities were not significantly different in the 4 Month, 41 Month, and Control sites, but was significantly different in the 221 Month site.

Nutrient Utilization

BIOLOG EcoPlates™ allowed for potential quantitative and qualitative comparisons between physiological activities of micro-organisms from different soils. The plates had 96 wells containing 31 different nutrient sources, each in triplicate. The remaining 3 wells were water controls. Observing a colour change in the well, if any, was the first indication of whether or not the nutrient source had been used. If the well remained clear, the substrate had not been used by the sample. However, when shades of yellow, purple, or brown were observed, the nutrient source had been utilized by the samples. The functional similarity was recorded (Figure 6) and the means of the nutrient sources used by individual samples (Figure 7) and by site (Figure 8) were recorded. Intensity/activity was recorded for both individual samples (Figure 9) and by site (Figure 10).

Figure 6 shows that there was much variation in substrate utilization within the Control site and the 4 Month site. The subsequent dendrogram, a treelike diagram depicting relationships based on shared characteristics and phenotypic divergences, was produced by Dr. Anita L. Davelos Baines using the resulting activity on day three of incubation. Samples from the 4 Month site (15 on the dendrogram) and the 41 Month site (124 on the dendrogram) showed that there was clustering within and among those two site. The 41 Month Site had one sample, 221 Month-1 (102-1 on dendrogram) that had similar activity to sample Control-3 (c3) and sample Control-2 (c2) (Figure 6). The 4 Month site (the youngest site) and the oldest site (Control) had the most variability within their sites. However, sample c1 and sample 15-1 used the substrates in the exact same way (Figure 6).

Mean number of sources utilized by sample showed that results were consistent with expectations that the younger sites used more nutrient sources. The expectation was that younger communities would be more diverse, therefore, needing a variety of sources (Figure 7, 8). Older samples used less nutrient sources possibly due to a more homogenous microbial population (Figure 7). When looking at the nutrient use as per site (Figure 8), the expectation was confirmed. The P=0.012 validates that there is a significant difference between the number of nutrient sources used by the two younger sites (4 Month (15s), 41 Month (124)) and the older sites, (221 Month (102), and Control).

Measuring activity by sample was a product of the value produced when calculating the intensity and did not constitute a unit. Results show that individually there seems to be the same trend observed in that the younger sites were more active than the older sites. The exception was sample 221 Month-4 (Figure 9). The distance from the average activity was also recorded (Figure 11) and was consistent with the findings observed in Figure 9. However, when taking into account the activity per site, a P=0.401 showed that there was no significant difference between the sites in regard to activity/intensity (Figure 10).

In processing the summation of the BIOLOG EcoPlate data, it was found that there were 4 carbon sources that were used by all of the sites: Tween 80 (also known as T80), Xylose, Hydroxybutyric Acid, and Galacturonic Acid. Arginine was the only one carbon source that was not utilized by any of the sites. In addition, Phenylalanine was only used by the 221 Month and Control sites. In this experiment, the 2 younger sites showed evidence that support the idea that they are not representative of a climax

community as they are less selective with the nutrient sources they utilized. The older sites seem to be much more selective, demonstrating that the population in these communities are closer to being stable and possibly have a more homogenous make up. This would confirm that they are close to, if not already, a climax community.

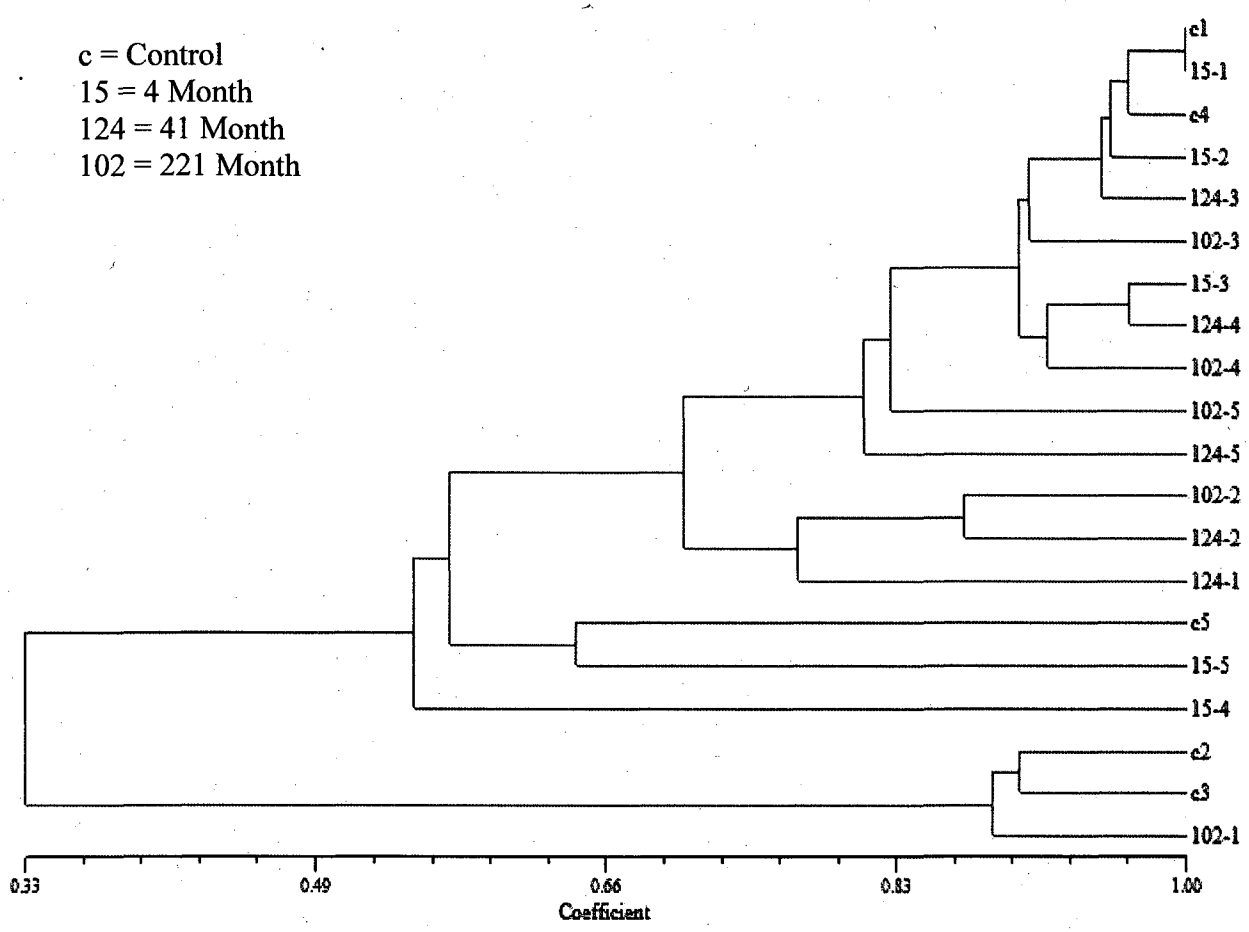


Figure 6: Dendrogram Similarities in Nutrient Use

This figure illustrates the similarities between and among sites. There is variation within the Control site (c1-5) as well as the 4 Month site (15s 1-5). However there is some evidence that the two sites have samples that function similarly in terms of nutrient use. There is some clustering between and among the samples from the 41 Month (124), and the 221 Month (102). Figure 6 provided by Dr. Anita L. Davelos Baines.

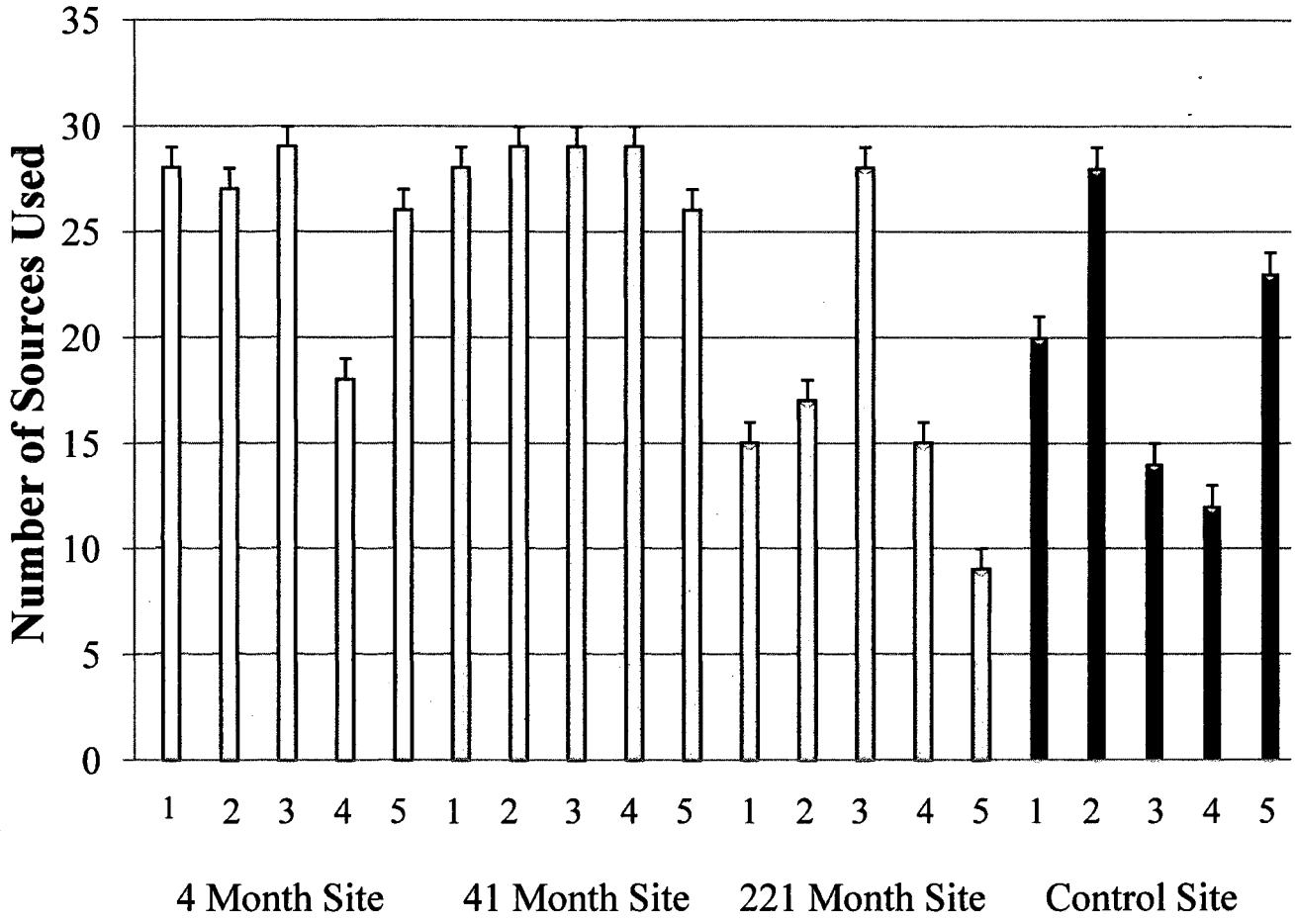


Figure 7: Mean Sources Utilized by Sample

This graph/figure demonstrates the mean for each sample in regard to number of sources used with BIOLOG EcoPlate™. The error bars indicate the standard error of the mean (each sample was done in triplicate). The numbers 1-5 correspond to the number of the individual sample as it pertains to the site. Five samples were collected from each site. The younger sites (4 Months, 41 Months) were less discrete in the number of sources used.

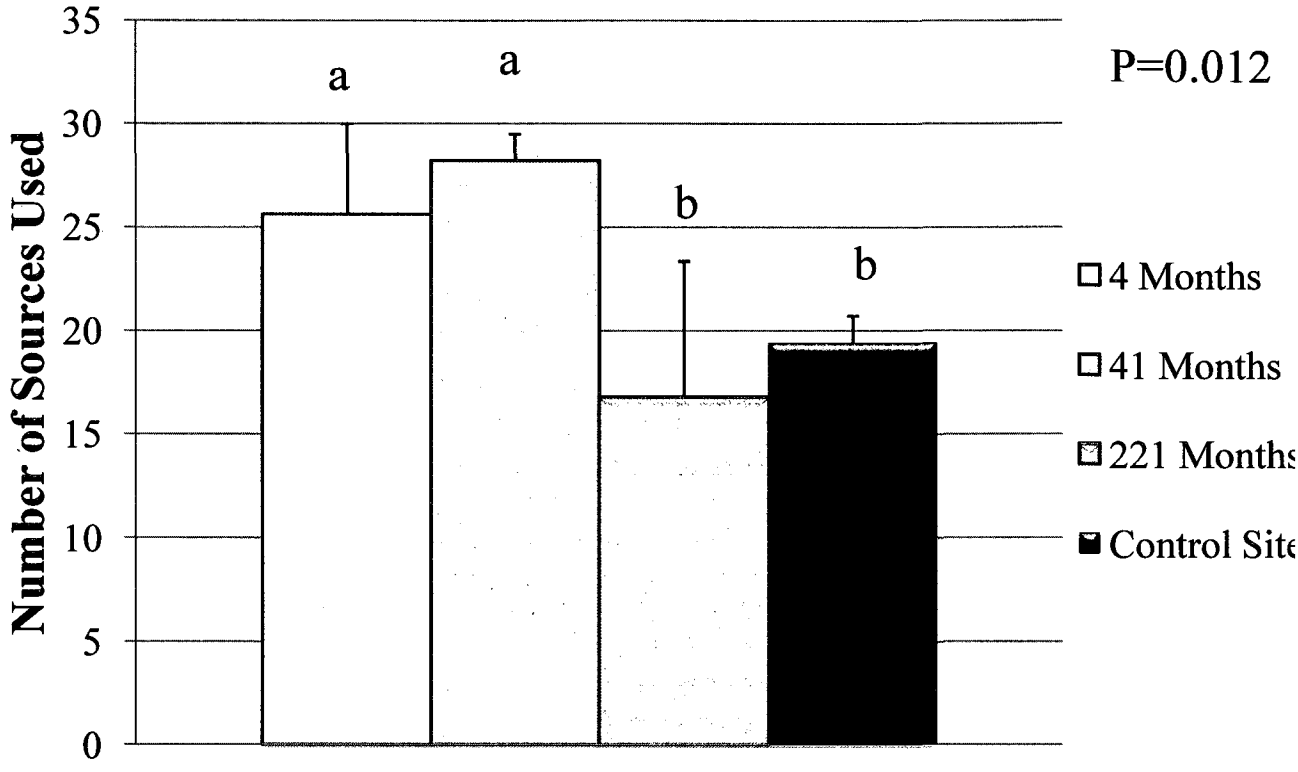


Figure 8: Mean Sources Utilized by Site
This figure shows the mean number of nutrient sources used per site. The 4 and 41 Month site used a significantly higher number of nutrient sources than the 221 Month and the Control site.

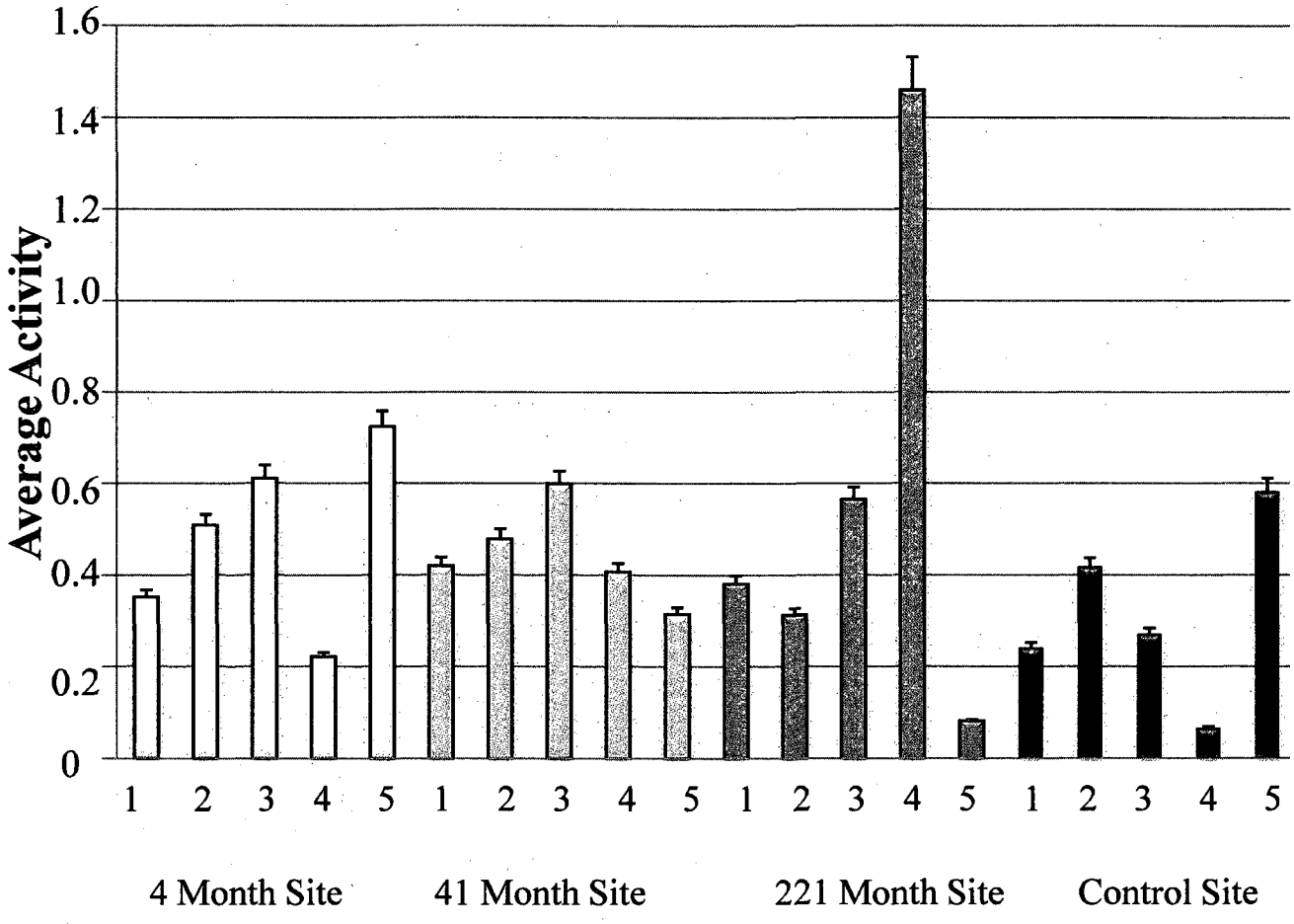


Figure 9: Mean Activity per Sample

This figure demonstrates the mean activity as per individual site sample. The numbers 1-5 correspond to the number of the sample as it pertains to the site. Five samples were collected from each site. The younger sites were more consistently intense/active.

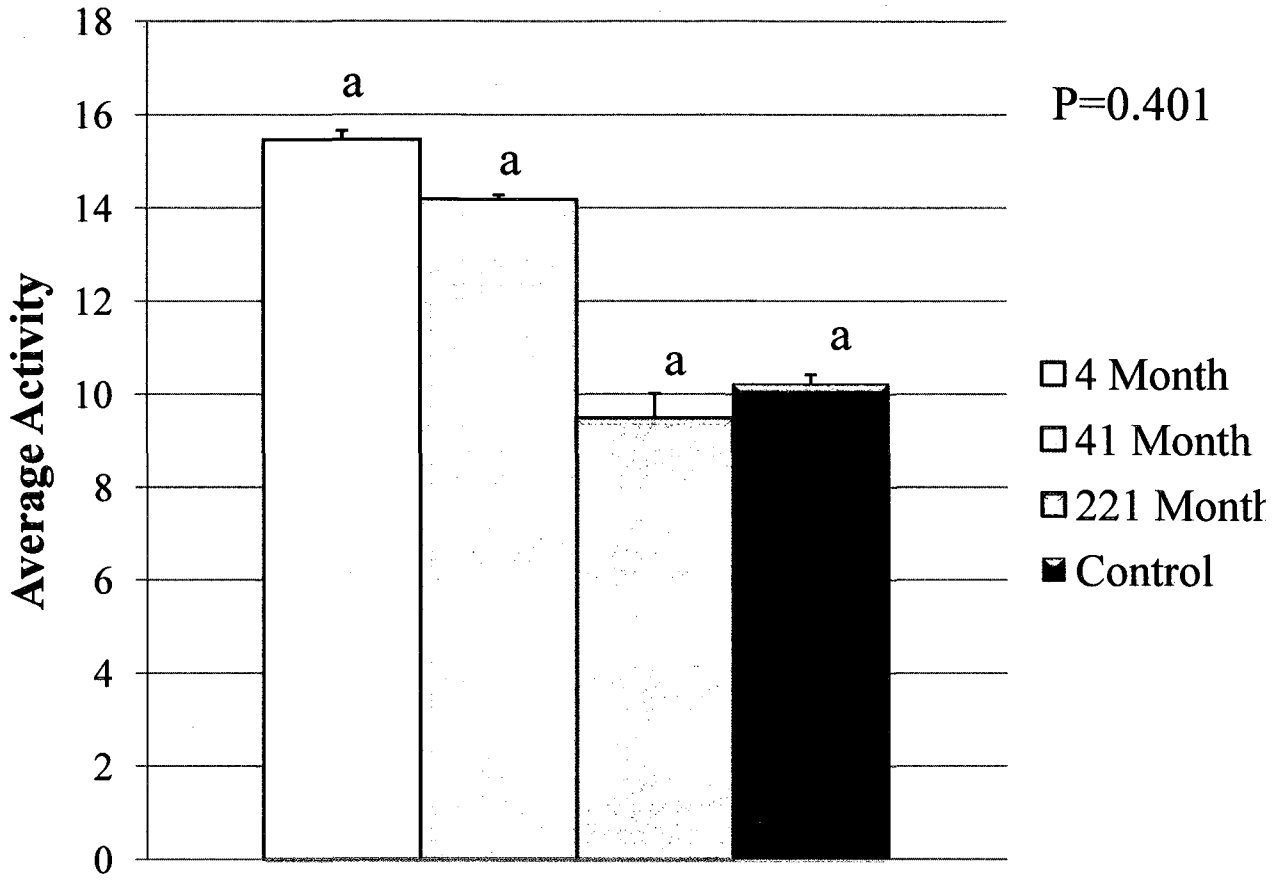


Figure 10: Mean Activity per Site

This figure shows the mean activity per site. There was no significant difference in activity when looking at activity per site opposed to individual samples.

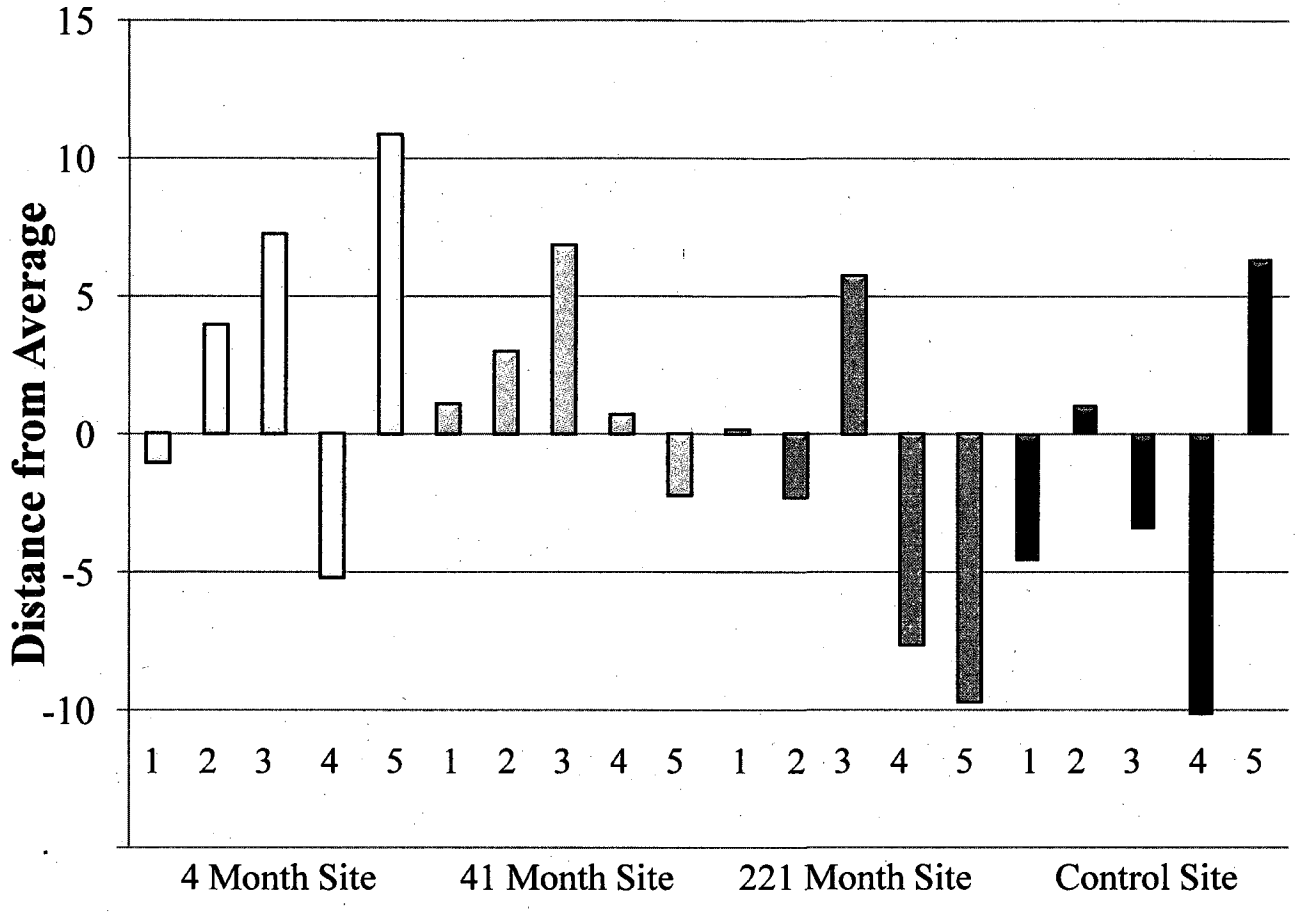


Figure 11: Distance from Mean of Total Activity per Site

This figure shows the distance, as per individual sample, from the total mean activity for all twenty samples. It illustrates which sample were below and above average activity. The 4 and 41 Month site had more samples above average activity. The 221 Month and Control site had a greater number of samples with a lower rate of activity.

Amplified Ribosomal-DNA Restriction Analysis

The reproducible patterns of restriction cut DNA can be used to profile communities of bacteria. For this study ARDRA was used to note and characterize community structure differences within and between sites. The community level diversity of bacteria was recorded and photographed (Figure 12-21).

The samples from the Control and 221 Month site showed similar band patterns within and among the two sites. These results are consistent for all of restriction enzymes tested (Figures 13, 15, 17, 19, 21). The Control Site and the 221 Month Site did not exhibit banding patterns similar to those from the 4 or 41 Month Site. There were similar banding patterns within samples from the 4 Month site. There were also similar banding patterns within samples from the 41 Month site (Figures 12, 14, 16, 18, 20). Neither had samples that showed band patterns similar to the 221 Month site or the Control site. In addition, the 4 Month Site and the 41 Month site had some samples with similar banding patterns. This further supports the expectation that microbial communities change as the ecosystem undergo succession.

Figure 12: ***Eco*R V**

4 Month Site 41 Month Site

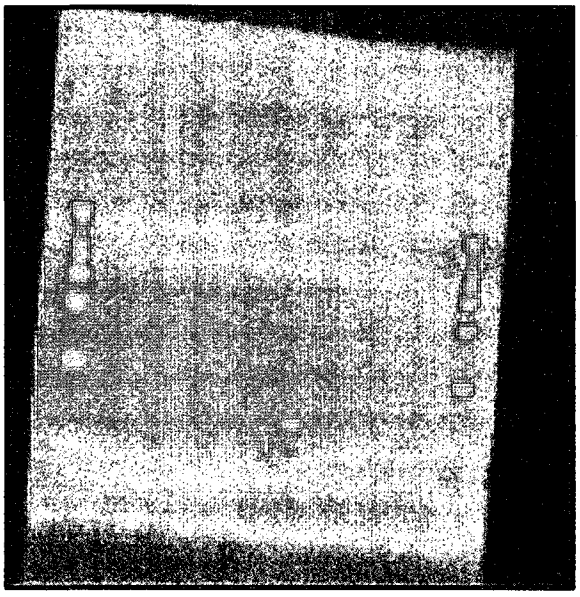


Figure 13: ***Eco*R V**

221 Month Site Control Site

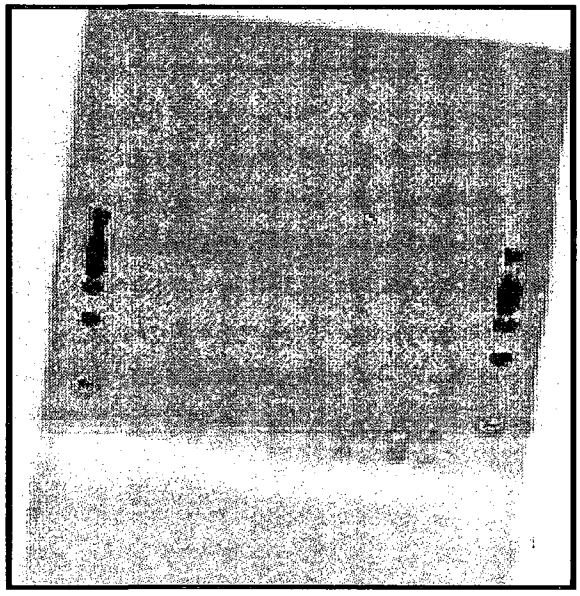


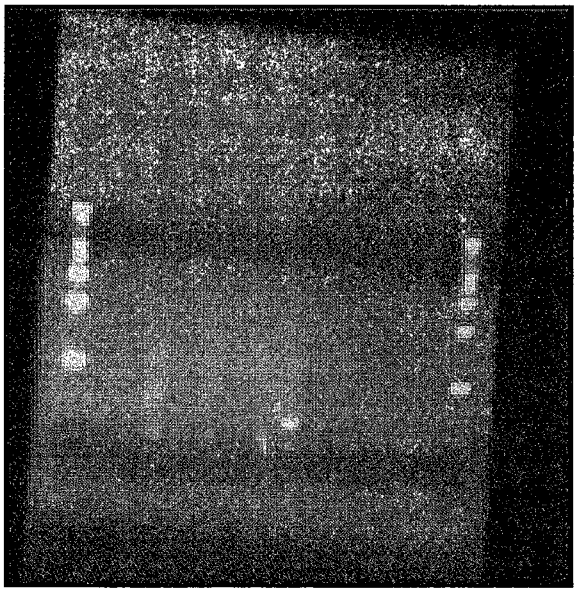
Figure 12: Demonstrates that the 4 and 41 Month site shows patterns within their sites.

Figure 13: Demonstrates that Control site and the 221 Month site show similar patterns among the two sites.

Figure 14: ***Sal* I**

Figure 15: ***Sal* I**

4 Month Site 41 Month Site



221 Month Site Control Site

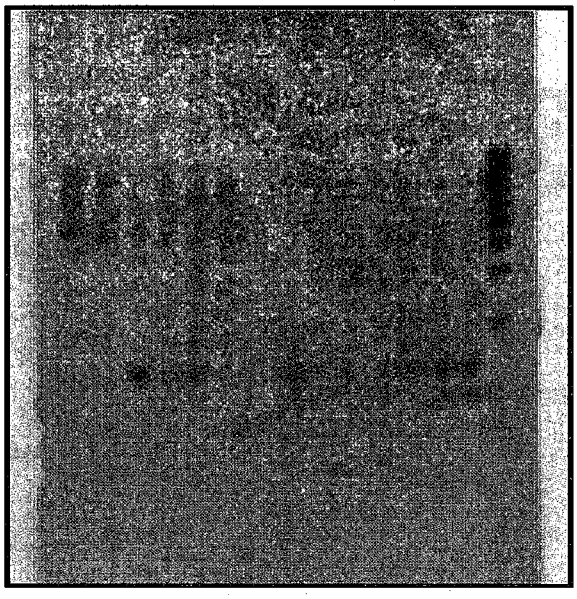


Figure 14: Demonstrates that the 4 and 41 Month site shows patterns within their sites.

Figure 15: Demonstrates that Control site and the 221 Month site show similar patterns among the two sites.

Figure 16: ***Rsa* I**

4 Month Site 41 Month Site

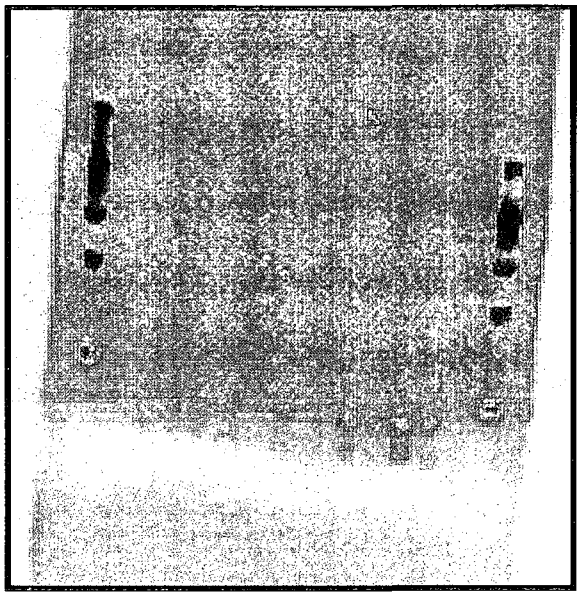


Figure 17: ***Rsa* I**

221 Month Site Control Site

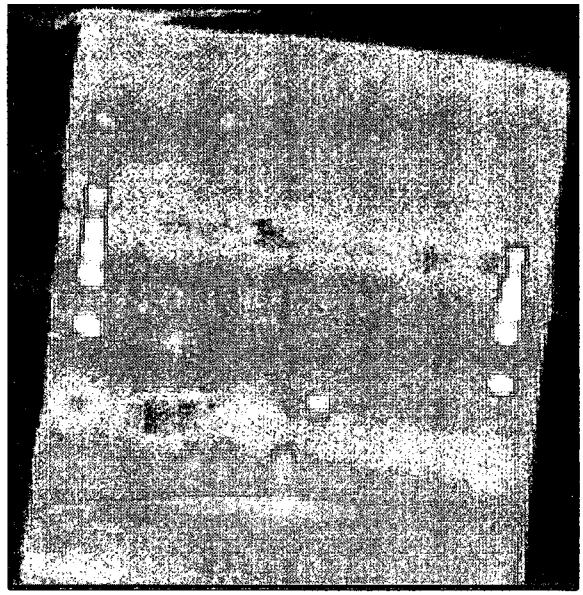


Figure 16: Demonstrates that the 4 and 41 Month site shows patterns within their sites.

Figure 17: Demonstrates that Control site and the 221 Month site show similar patterns among the two sites.

Figure 18: ***Hae* III**

4 Month Site 41 Month Site

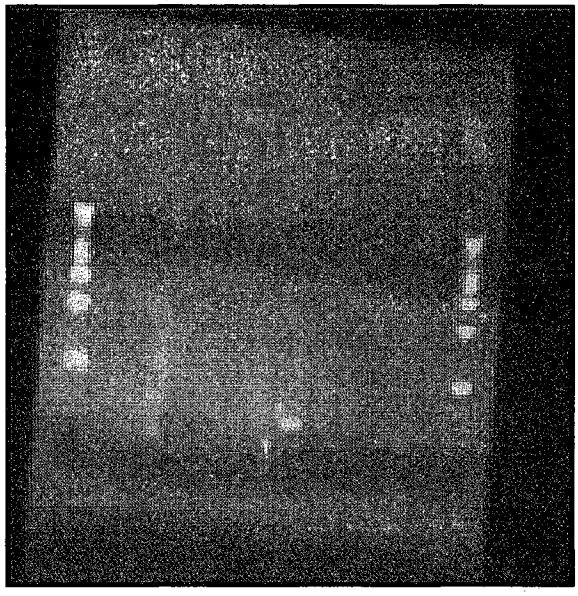


Figure 19: ***Hae* III**

221 Month Site Control Site

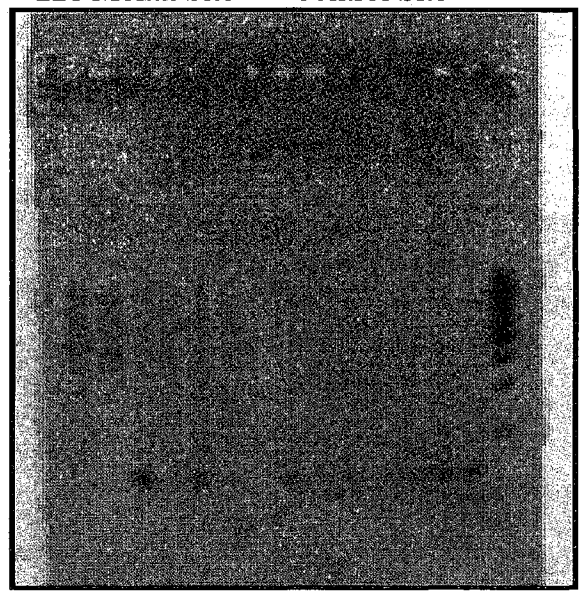


Figure 18: Demonstrates that the 4 and 41 Month site shows patterns within their sites.

Figure 19: Demonstrates that Control site and the 221 Month site show similar patterns among the two sites.

Figure 20: ***Alu* I**

Figure 21: ***Alu* I**

4 Month Site 41 Month Site

221 Month Site Control Site

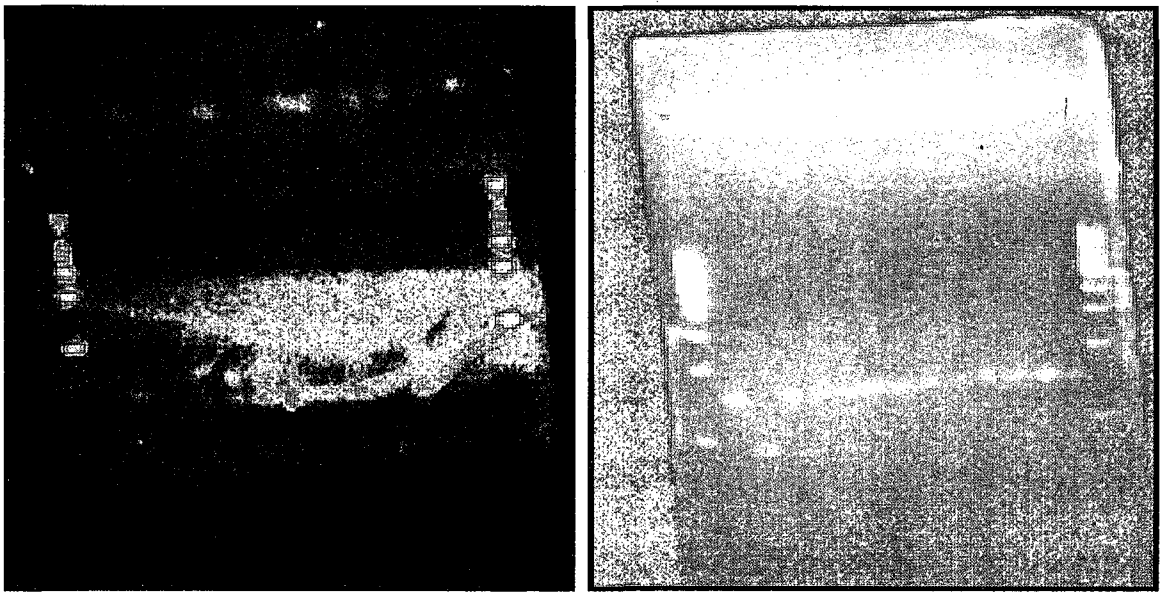


Figure 20: Demonstrates that the 4 and 41 Month site shows patterns within their sites.

Figure 21: Demonstrates that Control site and the 221 Month site show similar patterns among the two sites.

CHAPTER IV

DISCUSSION

Soil microbial community densities were opposite of what was expected. The bacterial densities not only increased, but at the 4 Month, 41 Month, and Control sites there was no significant difference in community size. The density at the 221 Month site was significantly higher than that of the other three sites (Figure 5). It is more common to expect that soil communities would show a decline in density over varying stages of succession; the community is working toward stabilizing (Stephan et al., 2000). A stable community would be representative of a climax community, and therefore, the populations within a stable community would be more homogenous. Both plant and soil communities remain relatively unchanged until disturbed (Jackson, 2003).

Given the results from this study we can say that although the density did not show differences over the varying successional stages, there was no indication that the apparent anomaly (221 Month site), could affirm a trend as a standalone test. Other studies, such as Ohntonen et al., 1999 and Pennanen et al., 1999, show that the changes over time occurring in soil communities are largely due to disturbances (discrete and severe) and the microorganisms undergo succession to achieve a climax community. Having observed Streptomycetes from the same samples (as an individual member of the bacterial community) to establish density, it reinforces that what is going on at these

revegetated sites is not according to expectations. Streptomycetes, as an individual member of the bacterial community, when grown on the same differential media as the remainder of the population, showed that there was no significant difference in density from the younger to the oldest site (Appendix A). This suggests that the revegetated sites are not close to being stable. This may be due to the microbial interactions with the seedlings (if at the younger site) or the vegetation (if at the older sites). The 221 Months site would be expected to show a decrease in numbers and have more in common with the Control site, as it is the oldest revegetated site. Given that succession is a slow process, the results for the density experiment, and trying to determine how the length of the interaction with plants affects the soil microbial community, it is difficult to conclude the trajectory of the microbial succession. If the sites involved had achieved a climax community and followed a trend toward a more homogenous community, one would expect that the results would resemble the following scenario (Figure 22):

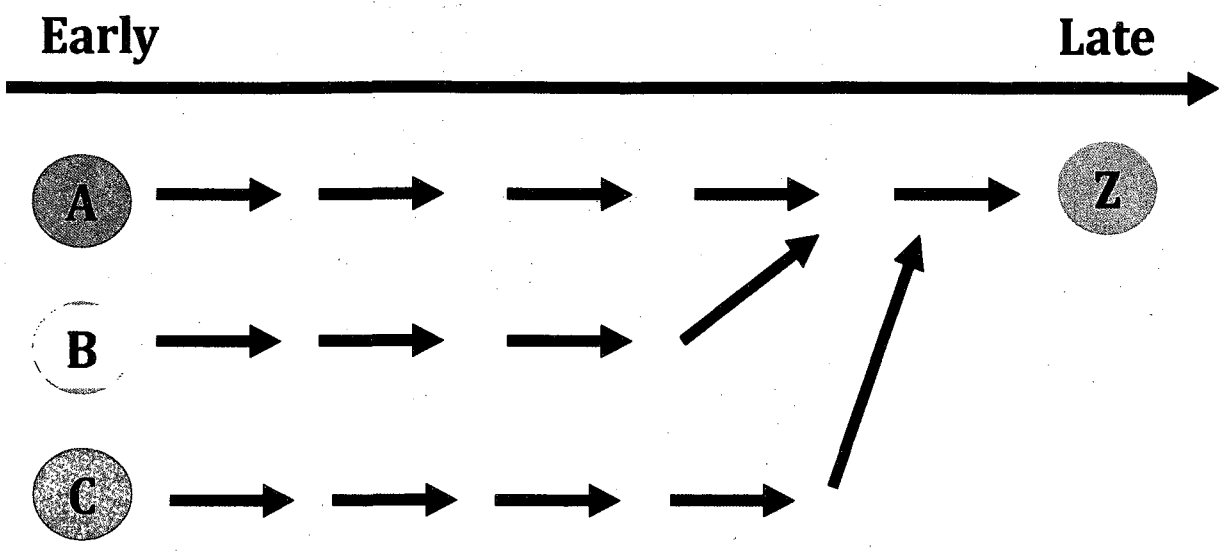


Figure 22: Succession Scenario 1
Communities A, B, and C, have different starting points, and follow different trajectories. Eventually the independent communities merge and follow a single trajectory to achieve the same end point at climax community Z.

The most recent revegetated site, 4 Month site (15s), as well as the 41 Month site (124), should have the greatest microbial diversity as they were less selective with regards to the nutrient sources they utilized (Chaubaud, 2008). This would imply that they are not representative of a climax community and these sites have heterogeneous populations. The Control site, as well as the 221 Month site (102), appear to be more selective in the nutrients that they utilized. This would be indicative of these sites having less variation in the soil microbial community, and are possibly closer to a climax community or level of stability (Konopka et al., 1998).

Given the age of the 221 Month and Control sites, one would presume that they would be nearing, if not already achieved, stability (Garland, 1994). When looking at the activity of the BIOLOG samples and how they used a varying number of nutrient

sources, the results could be interpreted in different ways. The dendrogram shows that the oldest site, Control, and the youngest revegetated 4 Month site (15s), utilized substrates similarly. These sites also showed variability within their respective sites. The 221 Month site (102) and 41 Month (124) site showed similar substrate utilization, which was demonstrated by the clustering on the dedrogram of sample profiles depicting the similarity within and among both sites. However, there was variability observed, as well. The sites tended not to show similarities in functional use of substrates between the 4 Month and Control site. When assessing the activity level, the most recently revegetated site, 4 Months site (15s), as well as the 41 Month site (124), should have the most amount of microbial diversity as they were less selective with regards to the nutrient sources they utilized. This would imply that they are not representative of a climax community, therefore still undergoing succession. The Control site, as well as the 221 Month site (102), appeared to be more selective in the number nutrients that they utilized (Fierer and Jackson, 2008; Jackson, 2003). This would indicate that these sites have less variation in their soil microbial communities (Allison, 2005) and are closer to a climax community; therefore, closer to being stable. Reaffirmation for these results can be seen when looking at the distance from the mean activity per sample. Figure 11 clearly shows a level of activity well above the mean for a number of the samples from both the 4 Month and 41 Month sites. The majority of the samples from the 221 Month and Control site were far below the mean activity. Again, this is indicative of the older sites being more selective and possibly not having use for an array of nutrients as the population is more homogenous. The anomaly, thus far, would be the dendrogram showing high variation within and between the sites, as well as the densities not showing a significant difference

over time. This could be due to the younger site having lower microbial numbers at the time of collection or due to possible error in the controlled lab experiment.

As a whole, the 221 Month site acted much like the Control site in regard to nutrient utilization. The numbers of sources used, as well as the actual substrates used were most often the same. However, this was not the case when looking at the level of activity per site. Though the 221 Month site had demonstrated earlier that its substrate use was representative or at least similar to that of the Control (already a climax community), it was unexpected to have that trend eliminated when assessing the activity per site as opposed to individually. The results generated by the dendrogram shows that there is variability within the 4 Month site and the Control site. In addition, there were samples from the Control and the 4 Month site that functioned similarly, if not the same. This was demonstrated by the clustering of samples between these two sites. Since the variability was great, if this assessment were to stand alone, one could predict that these sites have commonalities in their substrate use leading to the possibility that the youngest site is closer to a climax community than the 41 Month site and the 221 Month site. This would go against expectation (Collins, 2009). However, taking that idea further, the similarities that were present between the Control site and the 4 Month site could imply succession that is similar to Figure 23.

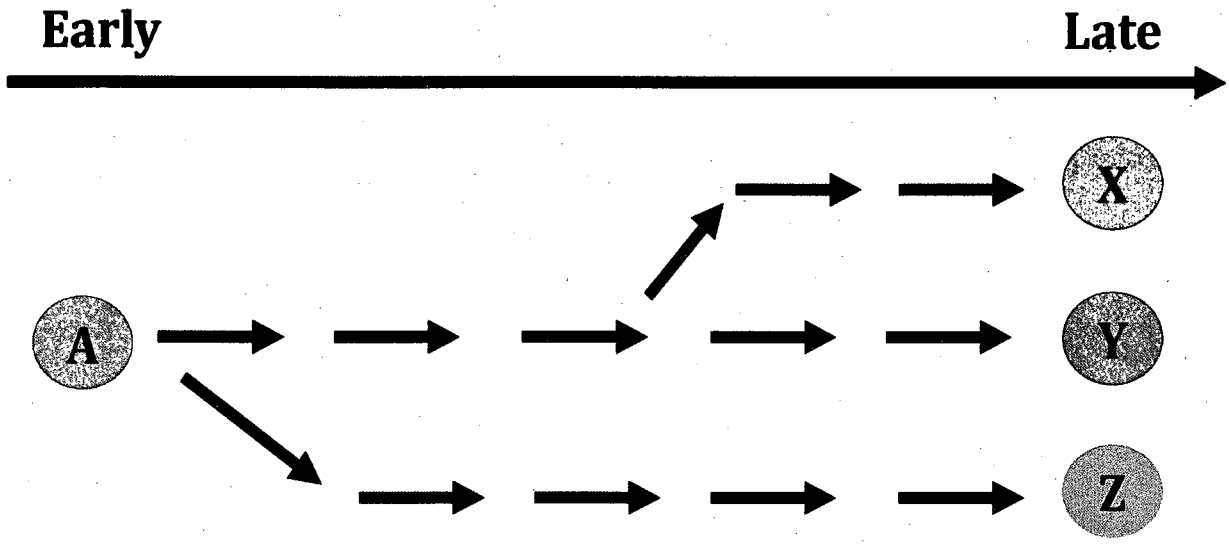


Figure 23: Succession Scenario 2
A single community A, diverges and follows separate trajectories, thus establishing separate climax communities X, Y, Z.

The resulting patterns from the restriction digests, observed on the gels after ARDRA, illustrated that there were consistent similarities within and among the 221 Month and Control sites. There were fewer similarities between the 4 Month site and the 41 Month site. They showed more commonalities within their sites than with each other. Neither the 4 Month nor the 41 Month site had anything in common with either of the older site. This is further evidence that the youngest site are in a state of flux and still undergoing succession.

ARDRA demonstrated the clearest findings of all of the experiments. It also reinforced the idea that the oldest revegetated site, 221Month site, would have the most in common with the Control site as the population was more homogenous. The younger sites, however, showed that the variability within and between the 4 Month and 41 Month site was great. It was rare that there were similar banding patterns between these sites.

In addition, it was also shown that there was variability within the sites as there were not many bands (i.e., populations) within the 4 Month site samples that were the same (in all samples). This result was identical at the 41 Month site. These results were consistent for all restriction enzymes tested. The implication in regard to succession would reflect a scenario similar to the depiction in Figure 24.

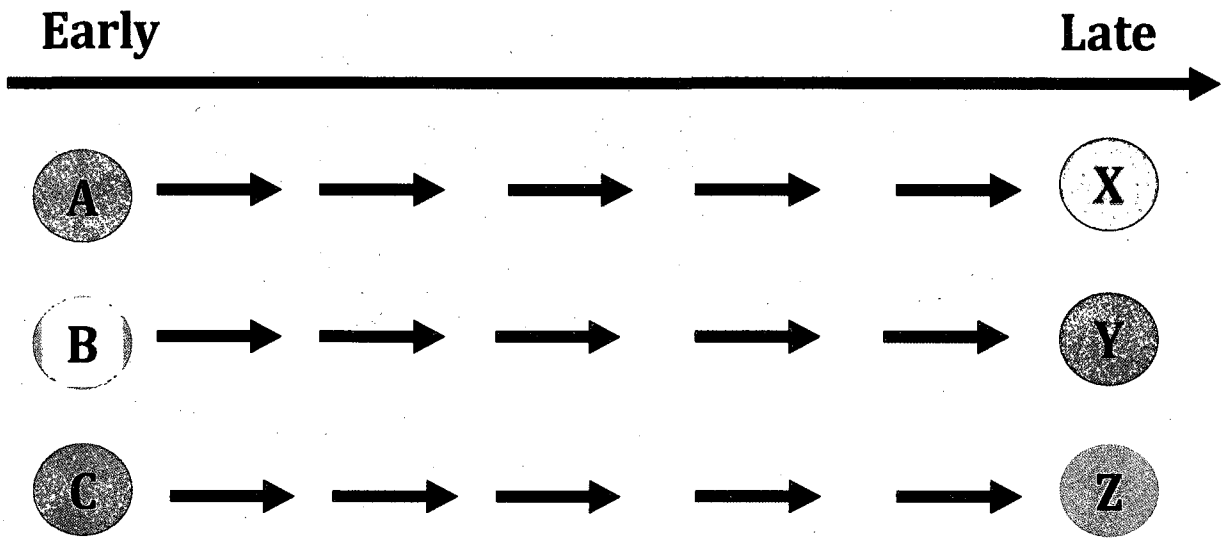


Figure 24: Succession Scenario 3
Separate communities A, B, and C, follow separate trajectories and achieve independent climax communities X, Y, and Z.

To say with all certainty that revegetation at the Ranchito Tract is successful would be a leap. However, there was vegetation at each site at the time the samples had been collected. Though the 4 Month site had sparse vegetation, this was an improvement from previous efforts. It is safe to say that this study has shown that the revegetated sites are still undergoing succession (Collins et al., 2009). Since there is vegetation present at each site during these states of flux, the goal would be to continue the land management practices that have been in effect and monitor progress, if any. There were some aspects

of the experiments, namely the nutrient utilization, where the results were seemingly inconsistent. Different assessments revealed different trends, as well as anomalies. This has laid the ground work for a follow up project. Given the data obtained with this collection, it would be logical to collect at different times of year (to determine seasonal effects), as well as visit other sites that had been revegetated between 1989 and 2003. Such studies would further support (or perhaps refute) the major finding here; that is, revegetation efforts by the SANWR are still undergoing succession, especially with regards to the soil microbial community.

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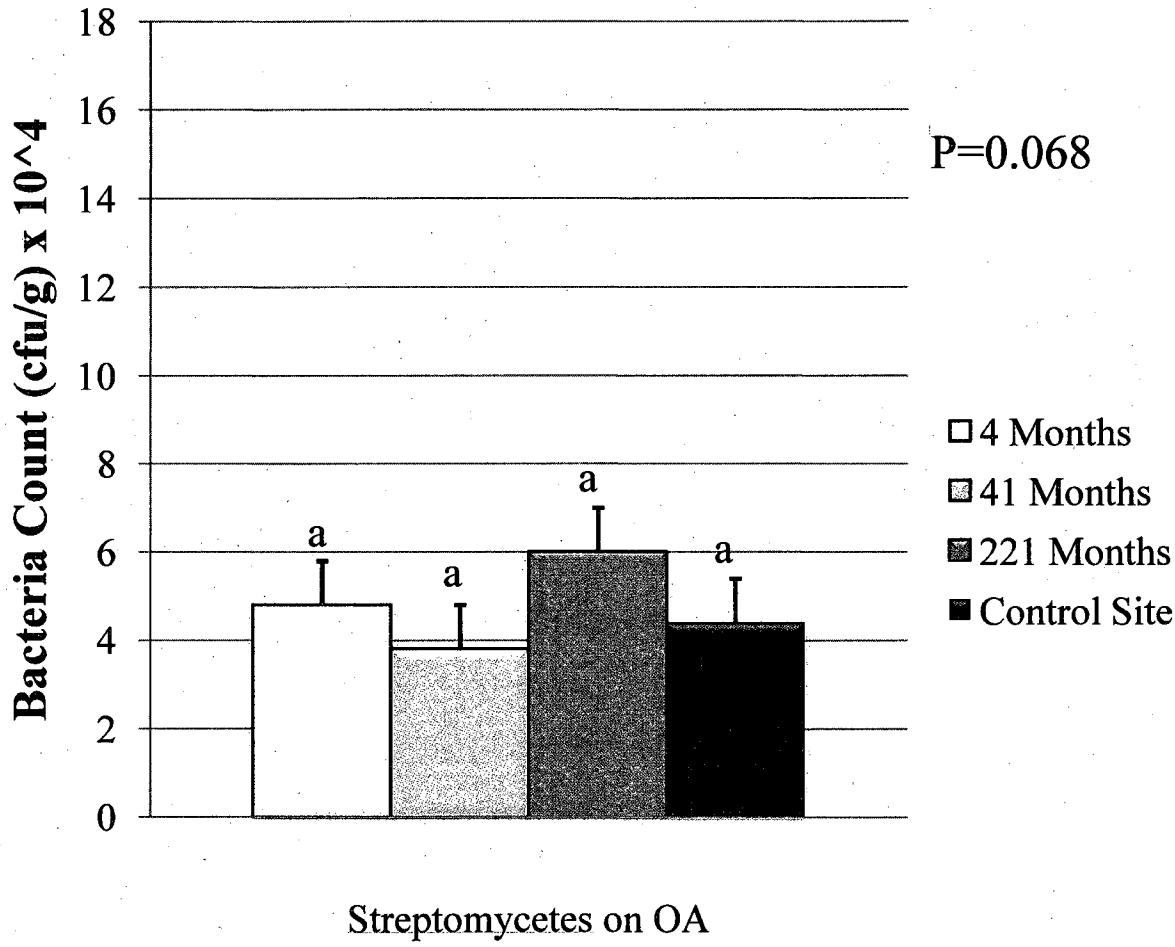
APPENDIX A

APPENDIX A

Streptomycetes at Different Successional Stages



A: There was no significant difference between the numbers of Streptomycetes grown on OA. The results were $F_{3,16}=0.948$, and P=0.068

APPENDIX B

APPENDIX B

Restriction Enzymes

Enzyme	**Source**	**Recognition Sequence**	**Cut**
*Eco*R V	*Escherichia coli*	5'GAUAUC 3'CUAUAG	5'---GAU AUC---3' 3' ---CUA UAG---5'
Sal I	*Streptomyces albus*	5'GUCGAC 3'CAGCUG	5'---G UCGAC---3' 3'CAGCU G---5'
Rsa I	*Rhodopseudomonas sphaeroides*	5'GUAC 3'CAUG	5'---CA UG---3' 3'---GU AC---3'
Hae III	*Haemophilus aegyptius*	5'GGCC 3'CCGG	5'---GG CC---3' 3'---CC GG---5'
Alu I	*Arthrobacter luteus*	5'AGCU 3'UCGA	5'---AG CU---3' 3'---UC GA---5'

B: This table depicts the restriction enzyme used and its subsequent information

BIOGRAPHICAL SKETCH

Born and raised in Toronto, Ontario, Canada, with Caribbean heritage, Rowena Hamlet grew up with a rather close knit family unit; mother, Sylma Edwards Hamlet, older brother, Raunel K. Hamlet, and younger sister, Racquel Y.C. Hamlet. Her activities ranged from the arts to athletics throughout her formative, teenage, and adult years. Hamlet attended Cawthra Park Secondary School (Mississauga, Ontario, Canada) as a music (vocal and piano) major, but graduated from Lorne Park Secondary School (Mississauga, Ontario, Canada) in December 1997. In January 2002, Hamlet earned an athletic scholarship for track and field and was student-athlete at the University of Texas-Pan American (UTPA), throughout the entirety of undergraduate studies. She earned her BSc degree in Pre-Med Biology, while she minored in Chemistry, and English. She graduated in May 2006. That summer she enrolled at UTPA to pursue graduate studies in the Biology Master's program, under the direction of Dr Anita L. Davelos Baines Dr. Kristine L. Lowe, and Dr. Jonathan H. Lieman, concentrating on soil microbes, namely bacteria. Hamlet completed MSc thesis graduate studies in May 2009. Hamlet intends to pursue an athletic career full time and eventually return to academia to pursue a Ph. D. in Microbiology.