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Community Interactions and Water as Drivers of Soil Microbial Communities

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COMMUNITY INTERACTIONS AND WATER AS DRIVERS OF SOIL MICROBIAL COMMUNITIES

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MICROBIAL COMMUNITIES

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Understanding the response of soil microbial communities to various environmental stresses is of current interest, because of their pivotal role in nutrient cycling, soil organic matter mineralization and influence on plant growth. Determining the affect of several biotic and abiotic factors on soil microbial communities is the overall objective of the study. The specific goals are to determine 1) the response of microbial communities to water deficit in soil and 2) how the presence of a rich biotic community determines the direction of microbial community development in cultures. Both goals are novel and unique contributions to understanding microbial ecology in soil.

Dynamics in water potentials due to drying and rewetting of soil impose significant physiological challenges to soil microorganisms. To cope with these fluctuations, many microorganisms alter the chemistry and concentration of their cytoplasmic contents. The aim of this research is to understand how the microbial biomass and their cytoplasm change in response to water potential deficits under in situ soil conditions. To address this objective we characterized intracellular and extracellular metabolites in moist, dry and salt stressed soils. Our results provided the first direct

evidence that microbial communities in soil in situ utilize sugars and sugar alcohols to cope with low water potential.

While the cultivation and isolation of microorganisms is essential to completely explore their physiology and ecology, 99% of soil microbes resist growing in cultures. Presence of very unnatural conditions in the culture plates was considered as main reason for low cultivability. Thus, a culture-based study was conducted whereby microorganisms were grown in association with their native habitat with an objective of mimicking native conditions to promote the growth of previously uncultivated microorganisms. Moreover, the importance of biotic communities (microbe-microbe) and abiotic soil effects were assessed on bacterial growth. Our results strongly indicate that the presence of living microbial community in the vicinity of the target culture resulted in the cultivation of novel members of rare bacterial taxa from phyla *Verrucomicrobia, Bacteroidetes, Proteobacteria,* and *Planctomycetes*. These results emphasize the need to develop new culturing methods to tap the hidden microbial potential for emerging anthropogenic needs.

Key Words: water stress, water deficit, compatible solutes, cultivation, 16S rRNA gene, biotic effects, PLFA, phospholipid, microbial diversity.

DEDICATION

This work is dedicated to my family, without whose love and moral support I would have never achieved my goal.

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LB Medium - Luria Bertani Medium

K_2SO_4 - Potassium Sulfate

TMS - Tri Methyl Silyl

Mol % - Mole Percent

RCF - Regenerated Cellulose Filters

CCRA - Cellulose Congo Red Agar

PLFA - Phospholipid Fatty acids

FAME - Fatty Acid Methyl Esters

NMS - Non-metric Multidimensional Scaling

OTU - Operational Taxonomic Unit

MPa - Mega Pascal

KPa - Kilo Pascal

nmol - nano moles

PSA - Phenol Sulfuric acid Analysis

NRN - Ninhydrin Reactive Nitrogen

DGGE -Denaturing Gradient Gel Electrophoresis

ANOVA - Analysis of Variance

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Microorganisms in soil ecosystems

Soils are inhabited by vast diversity of microorganisms. One gram of soil is believed to have more than 6000-38,000 bacterial species (Curtis et al. 2002), 10^9 microbial cells (Torsvik et al. 2002) and up to 200 m fungal hyphae (Leake et al. 2004). With such diversity and richness, microbes are known to occupy every niche of the soil ecosystem. Soil microbial communities are comprised of viruses, bacteria, fungi, algae and protozoa present as a mixture of actively growing and resting states in varying densities. Some species are represented by a few individuals whereas other populations dominate the community. All these different groups live together in consortia, interacting with each other and with other parts of the soil biota (Price 1988, Torsvik et al. 2002). However, soils are very heterogeneous systems with wide range of physiochemical gradients and discontinuous microhabitats. The environmental, chemical, physical and biological characteristics of these microhabitats differ in both time and space influencing the existing microbial community composition (Nannipieri et al. 2003).

Importance of soil microorganisms in biogeochemical cycles

Soil microorganisms play a vital role in maintaining and/or enhancing soil quality by regulating organic matter decomposition, cycling of nutrients, enhancing nutrient availability and macro aggregate formation (Bossio et al. 1998, Batjes et al. 1999, Øvreås 2000). Soil microorganisms are the driving force behind soil organic matter

transformations such as mineralization and immobilization of organic constituents (Smith et al. 1992). Microbes are also important in bioremediation of pollutants in the natural environments and can regulate plant access to nutrients (Daubaras and Chakrabarty 1992, Lovley and Coates 1997, Salanitro et al. 1997, Wardle et al. 2004) thus acting as both a source and sink of soil nutrients.

Factors influencing the soil microbial communities

The microbial communities in soil ecosystems are constantly exposed to different environmental factors. These include carbon and energy sources, mineral nutrients, available water, temperature, air composition, pH, redox potential and surfaces chemistry. All these can affect the ecology, activity and population dynamics of microorganisms in soil (Tate 1995). Along with these, biotic factors like competition and interaction with other organisms, and anthropogenic activities like soil tillage, use of pesticides and pollution, all affect soil microbial communities (Zhang and Dong 2004). As the microorganisms have intimate relations with their surroundings due to their high surface to volume ratio, they respond quickly (changes in microbial populations or activity) to environmental stress compared to higher organisms (Pankhurst et al. 1995). Thus, the changes in the microbial communities resulting from environmental and anthropogenic factors act as an indicator of soil health and simultaneously have profound impacts on ecosystem dynamics (Bossio and Scow 1995).

Of all the factors, availability of water could be considered the most vital, for determining the activities and functions of microbial populations and communities. However, very little is known regarding the physiological and structural changes to microbial communities that occur under limited water availability.

Effects of water availability on the soil microorganisms

Water is an important component of microbial cells and is also a primary participant in a variety of cell process. Optimum water is essential for nutrient diffusion, microbial motility and gaseous exchange in the soil. Total microbial activity varies from nearly nonexistent levels at low water availability to a maximum level under optimal soil moisture levels. But, drying and rewetting of the soils, which is a common phenomenon in terrestrial ecosystems, results in fluctuations in available water content. Even though, the fluctuations are common in any majority of soil ecosystems but more drastic in semi arid climates. As the soil water is known to be a major determining factor in organic matter turn over, the variations in water content greatly affects the nutrient cycling. The fluctuations in water availability are often discussed as terms of soil water potentials.

Soil water potential

Soil water potential is measure of gravitational, matric and osmotic potentials. In soil ecosystems, microbes often face water potential deficits by matric and osmotic components. Drought or drying of the soil results in matric potential deficits. As the soil gets drier, matric potential decreases, the continuity of the water film gets disconnected and microbes become substrate–limited as substrate and nutrient diffusion is restricted (Ilstedt et al. 2000). Matric stress not only reduces the matric potential but also increases the solute concentration in the surrounding soil solution so the water availability is comprised of both matric and osmotic factors of the water potential (Papendick and Campbell 1980).

Conversely, microbes under moisture saturated environments often face osmotic potential deficits. Presence of high concentration of solutes or salts in soil solution lowers the water activity and the microbes face limitation in water availability which is often

referred to as osmotic or solute potential. Matric stress is a common scenario in most of the terrestrial ecosystems, but the intensity vary in semi arid ecosystems, whereas soil salinity is an increasing problem in irrigated agriculture and arid ecosystems. Rewetting of dry soils and dilution of salts in the environment either by precipitation or irrigation results in sudden increase in the soil water potential. The microorganisms living in the soil habitats need to adapt to the rapid changes in the water potential to survive.

When the dry soils are rewetted, a flush of nutrient availability and soil respiration was reported previously (Franzluebbers et al. 2000, Mikha et al. 2005). The pulse in C and N mineralization could be attributed to both biotic and abiotic factors. The release of labile substrates from microbial cell lysis, release of intracellular osmolytes into the environment (Lund and Goksøyr 1980, Magid et al. 1999) or by increasing the substrate availability by disruption of soil aggregates (Lundquist et al. 1999, Xiang et al. 2008) which in turn would be mineralized by surviving soil microorganisms upon rewetting (Bottner 1985, Van Gestel et al. 1991, Appel 1998) are the probable mechanisms resulting in C and N flush.

Microbial cellular response to water potential fluctuations

Soil microorganisms are small and live in intimate contact with the soil water. The internal cell turgor of microorganisms generally equilibrates with external water potential because of their semi permeable membranes (Schimel et al. 2007). Under reduced soil water availability, the microorganisms cannot hold the water in the cytoplasm i.e. drying triggers the efflux of water from the bacterial cytoplasm resulting in plasmolysis of the cells. Since microorganisms lack well developed regulatory systems to

maintain cellular water, they retain cell turgor by adjusting/increasing the concentration of solutes in the cytoplasm (Kempf and Bremer 1998).

On rewetting of dry soil, water potential of the soil increases instantaneously and microbial cells that adjust to the dry conditions must readjust their internal matric potential rapidly to the new situation to avoid cell lysis (Mikha et al. 2005). Microbial cells that do not survive to desiccation or rapid fluctuations in water potential are considered to be a part of soil organic matter (Marumoto et al. 1977). Thus, both the extremes in water potentials pose a challenge to microbial survival and force them to develop acclimatization strategies (Figure 1.1). Many theories have emerged on how the microbes acclimatize to the water potential fluctuations and osmolyte accumulation hypothesis is one of them. In our present study we focused on how the microbes respond to the reduced water availability emphasizing the osmolyte accumulation hypothesis.

Adaptation of microorganisms to low water potentials

Microorganisms show several morphological adaptations to their habitat. A low surface to volume ratio, thick cell walls, mucilaginous layers plus formation of cell aggregates protects the cells from rapid water loss (Tate 1995, Neidhardt et al. 1990). Pure culture studies revealed that microbes also exhibit biochemical strategies such as accumulation of osmolytes to maintain cellular homeostasis under desiccation stress. The osmolytes include K^+ ions and a group of organic compounds like glutamate, proline (amino acids) sucrose, trehalose (sugars), peptides, N-acetylated amino acids (amino acid derivatives), glycine betaine, carnitine (Quaternary amines) and tetrahydropyramidines like ectoine (Csonka 1989, Poolman and Glaasker 1998) which are rich in carbon and

nitrogen. The organic solutes are either directly taken from the surrounding environment or synthesized by the microbes from the precursor molecules.

Organic osmolytes are typically low-molecular weight organic compounds, soluble at high concentrations in water, and either uncharged or zwitterionic at physiological pH values. In contrast to inorganic ions like K^+ and Na^+, the organic solutes can safely be up or down regulated with little impact on cellular functions and protein stability (Yancey 1994) (Kempf and Bremer 1998, Record Jr et al. 1998) and hence often referred as compatible solutes. The accumulation of these organic solutes not only helps in regulating the cell turgor of the organisms, but also supports their tolerance to other environmental stresses. The secondary benefits of the organic osmolytes, and their compatibility with macromolecular structure and functions, might have played a very important role in the evolution of this adaptive strategy to overcome the environmental stress by different microorganisms (Welsh 2000).

Effect of drying and rewetting on nutrient cycles

Earlier studies on the effect of dynamics in water content on soil biogeochemical processes have reported a sudden flush in C and N mineralization in 1-4 days following the rewetting of dry soil (Birch 1958, Sorensen 1974, Schimel et al. 1999, Franzluebbers et al. 2000, Mikha et al. 2005, Miller et al. 2005, Williams and Xia 2009). The size of the flushes seem to have direct relation with soil type, size of the organic pool, organic matter quality and size of soil biota (Van Gestel et al. 1993) and particularly to the size of microbial biomass (Sparling et al. 1985, Turner et al. 2003). The specific cost in terms of lost osmolyte C from a single drying/rewetting event is modest, but when repeated multiple times, as is common in many ecosystems, has significant effect global carbon

and nitrogen cycles. Based on a rough estimate, the total C cost of producing osmolytes in a single drought period can easily consume 3–6% or more of total ecosystem annual net primary production (NPP) in a grassland ecosystem and the total N contained in osmolytes would be 10–40% of annual net N mineralization (Burke et al. 1997, Schimel et al. 2007). While the osmolyte regulation process seem to have huge impact on ecological scales very little work was done to understand and measure the response of in situ soil microbes to varying intensities of water stress which is one of the major goal of my research.

Culturing the microorganisms

Microbial communities consist of a complex assemblage of species, with different metabolic characteristics, physiological requirements and ecological attributes, each species driving at least one of the multiple reactions in soil processes. A major challenge is, identifying the microbial populations that are involved at different levels of nutrient cycling (Bastian et al. 2009). However, culturing of the soil microorganisms is one of the important strategies in establishing the link between metabolic properties and potential of these diverse organisms (Kaeberlein et al. 2002).

Over decades the presence, abundance, diversity and phylogenetic traits of soil microorganisms were studied using traditional culture-based techniques. However, recent development of cultivation-independent molecular techniques has illuminated the immense diversity of soil microbes (Chandler et al. 1997). It is now known that less than 1% of the microorganisms present in the soil grow in the culture plates as most of them resist growing in the laboratory media (Torsvik et al. 2002). Of the 40 known bacterial phyla nearly half of the phyla do not have cultural representatives, reflecting the

complexity of growing soil microbes (Hugenholtz et al. 1998). The absence of pure cultures makes it difficult to ascertain the roles of specific microbes in soil environments (Zengler et al. 2002). The intrinsic selectivity of any given medium and incubation condition imposes limits on the nature, number, and diversity of microbes recovered from natural samples (Stevenson et al. 2004).

Traditional culture methods

Conventional culture-based methods used high nutrient media which often selects the opportunistic fast growing organisms, severely under representing members of certain taxa (Hugenholtz et al. 1998). In nutrient-rich artificial media, the community members with 'r'-strategy or fast-growers, often overgrow and out-compete the naturally abundant 'K'-strategists (Watve et al. 2000). Consequently, these conventional culture-dependent approaches do not reflect the actual microbial communities (Amann et al. 1995).

Improving the cultivation methods

To gain access to the uncultured microbes, many modifications have been done to culturing media and methods by numerous scientists. For example, relatively low nutrients media was used to increase the cultivability and to improve the recovery of prokaryotes from different types of natural samples (Janssen et al. 1997, Watve et al. 2000, Connon and Giovannoni 2002, Sangwan et al. 2005) whereas increasing the incubation periods have allowed for the development of strains from rarely isolated taxa (Sait et al. 2002, Stevenson et al. 2004, Davis et al. 2005, Sangwan et al. 2005, Stott et al. 2008). Culturing of novel microbes with the addition of electron transporters, inhibitors of undesired organisms was also reported before (Leadbetter 2003, Stevenson et al. 2004).

However, in natural environment the microorganism live as a part of a community in which distinct cells work in concert and communicate either by trading metabolites, by exchanging dedicated signaling molecules, or by competition for limited resources (West et al. 2007, Nadell et al. 2009). For example, bacteria are known to communicate using an interspecies quorum-sensing factor [autoinducer 2 (AI-2)] that induces synthesis of proteins (enzymes or toxins) that are useful for a community rather than a single cell (Williams et al. 2007). The addition of signaling compounds like homo serene lactones into the media have aided in the development of novel groups of bacteria (Bruns et al. 2003). These modifications often resulted in isolation of previously uncultured bacteria, but subsequent work by (Kaeberlein et al. 2002) utilized a simple but revolutionary idea to increase the presence of "uncultivable" by cultivating microbes in simulated natural environments.

In situ cultivation of microorganisms

One of the main reasons for the low cultivability of soil microorganisms is that the failure of laboratory conditions to mimic the natural environmental conditions. Therefore, strategies aimed at simulating natural conditions or culturing in situ has been proven efficient. (Kaeberlein et al. 2002) has reported the isolation of number of novel marine bacteria after growing them under in situ conditions in apparatus called diffusion chambers. Diffusion chambers are apparatuses equipped with filter membranes, which restrict the movement of cells in the chamber but allows the exchange of nutrients and chemicals between the chamber and the environment, thereby making high-density cultivation possible (Karine and Joel 2009).

Different types of membrane-based systems were used to grow microbial communities directly in their natural habitats and reported success in isolating hard to culture slow-growing organisms (Ferrari et al. 2005, Bollmann et al. 2007, Nichols et al. 2008). This led to the hypothesis, that in situ cultivation of environmental prokaryotes in association with their native habitats will enrich the strains sufficiently for their subsequent isolation onto classical solid media for their further characterization (Bollmann et al. 2007). Microbes also need syntrophy and symbiotic relationship with some neighboring species to grow in petri plates (Kennedy et al. 2008, McInerney et al. 2008).

Relevance to my research

While the recovery of bacteria from natural environmental communities using traditional cultivation methods has resulted in domestication of less than 1% of the estimated diversity (Keller and Zengler 2004) the in situ cultivation of bacteria has been shown to recover up to 40% of the bacteria found in an environment (Kaeberlein et al. 2002). Most of the in situ cultivation experiments so far were done for isolating the marine bacteria (Kaeberlein et al. 2002, Rappé and Giovannoni 2003, Bollmann et al. 2007). Also, Ferrari et al. (2005) grew the rarely cultured members of phyla TM7 using soil slurry as media on soil substrate membrane system. So far no such study has cultured microbes under aerobic conditions. With increasing attention on in situ cultivation we planned a study to culture soil bacteria close to their native habitat while maintaining the aerobic conditions. While our primary objective was to check if in situ (Tate 1995) cultivation method promotes the growth of novel bacteria in aerobic conditions, we also

wanted to understand the biotic and abiotic (substrate) effects on the growing bacterial community.

Two approaches to study soil microorganisms are 1) measuring the response of soil microbes to different factors under insitu soil conditions and 2) culturing the microbes in Petri plates for studying the specific effect of certain growth factors. We adapted both the approaches for understanding different abiotic and biotic factors on the diversity, physiology and structural composition of soil microbial communities in our study.

Aims of the research

1. To evaluate the physiological and structural response of soil microbial communities to the reduced water potential caused by matric and osmotic stress under in situ soil conditions. (Chapter 3).
2. To determine the effect of biotic and abiotic factors on selection of microbial community using a novel method of cultivation (Chapter 4).

Each goal will be discussed in detail as individual chapters.

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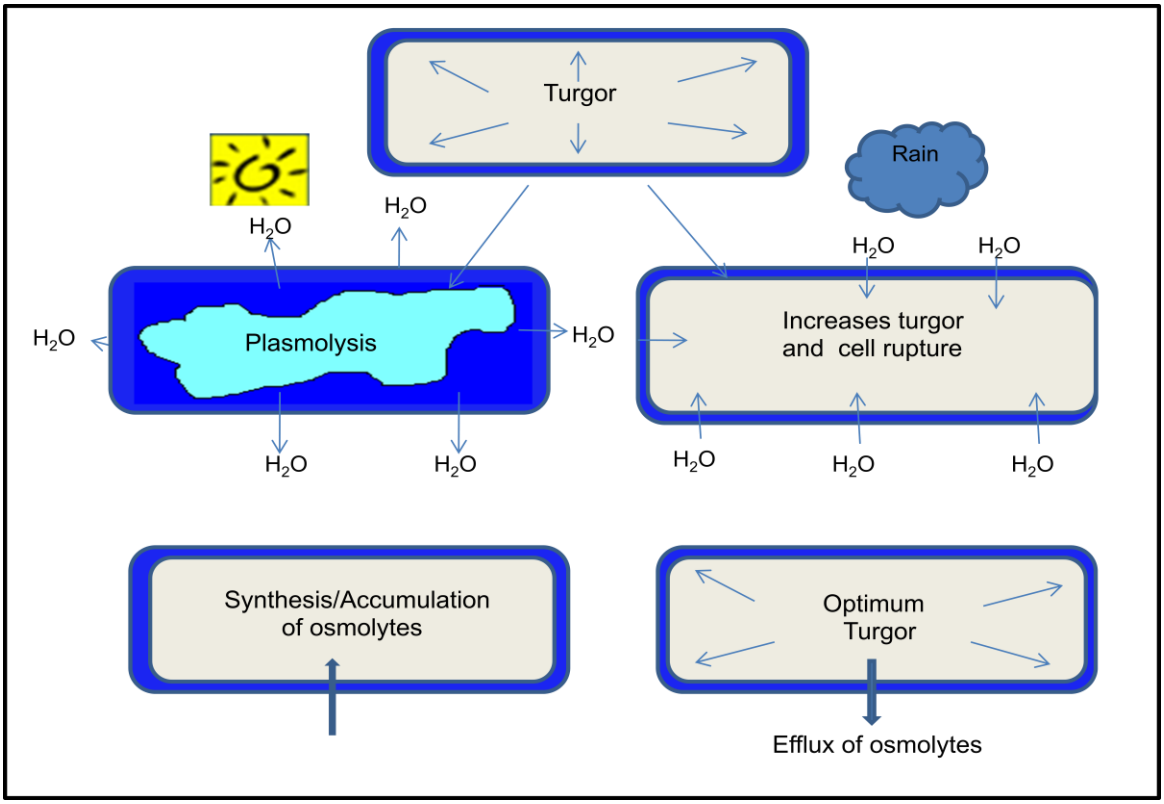

Figure 1.1 Microbial cellular responses to water availability

CHAPTER II

GENERAL EXPERIMENTAL PROCEDURES

Experimental methods that were used in this research are outlined here. Specific methods or modifications will be discussed in detail in the respective section of the dissertation.

Sterilization

All the glassware and other equipment utilized in the experiments was thoroughly washed and sterilized before use. Sterile deionized distilled water was used in all the experiments unless otherwise mentioned. Cellulose filters along with syringe filter holders were also sterilized and cooled before inoculation.

Preparation of soil extracts

Soil extract was prepared as per the method suggested by James (1958). For this, ~250 g of Marietta (fine-loamy, siliceous, active, thermic Fluvaquentic Eutrudepts) soil along with ~400 ml of distilled water was autoclaved at 121 ^{o}C and 15 psi for 1 hour. After autoclaving the solution was allowed to settle overnight. The supernant was transferred into separate tubes and centrifuged for 10 minutes at rate of 4000xg. The centrifuged solution was filtered through Whatman no. 1 filter paper and the volume of 250 ml was restored by adding distilled water. The soil extract solution was dispensed into small containers and autoclaved twice, and stored in freezer until utilized.

Preparation of media plates

Various media including LB agar medium, cellulose-Congo red agar medium and soil extract agar medium were used in different experiments. The cellulose-Congo red agar medium was prepared as per Hendricks et al (1995). The LB agar medium was prepared using general lab protocol and soil extract medium was prepared as per Hamaki et al (2005). The media was autoclaved at 121 ^{o}C and 15 psi for 20 minutes and cooled to ~50 ^{o}C before dispensing into Petri plates. Approximately 25 ml of media was transferred into sterile Petri plates under flow hood and cooled until media solidifies. The plates were then carefully packed into plastic bags and refrigerated until used.

Spread plate technique

The spread plate technique was used to inoculate the plates. For this 0.1 ml aliquot of the appropriate dilution (inoculum) was evenly placed on the agar surface in 4-5 spots. The inoculum was then spread evenly all over the agar surface using a sterile glass spreader. The plates were then closed and sealed with parafilm before placing them in an incubator at desired temperatures.

Measuring the water potential of the soils

The water potential of all the soils was measured using WP4 dewpoint potentiameter from Decagon devices. Water retention curves for two soils, Marietta and Sumter, were attached in Appendix A.

Measuring the water content of the soils

The soil samples were saturated with 0.01 M K_2SO_4 solution and the excess water was removed using a suction pump at pressure of ~-30 KPa. The soil was then collected into small aluminum tins, weighed and dried for 24 hours at 105 ^{o}C. The dry weight of

the soil was then measured and the water content was determined using the following equation.

$$\text{Percent Moisture content} = \frac{\text{Wet weight of soil - Dry weight of soil}}{\text{Dry weight of soil}} \times 100 \quad \text{(Eq. 2.1)}$$

Soil pH

Soil was shaken with 0.01 M $CaCl_2$ solution in 1:2 ratio for 30 minutes and settled for a few minutes. The pH was measured with Mettler-Toledo Inlab Science electrode and multiple readings were taken until a constant reading was obtained. The pH was measured with three replicates per soil.

Total C and N

Total C and N of the finely grounded soil samples were measured using a LECO C/N analyzer.

Colorimetric analysis of soil extracts

The soil extracts were analyzed for total carbohydrates and amino acids by the phenol sulfuric acid analysis and Ninhydrin analysis (Stevenson 1982) respectively. Glucose and Leucine at various concentrations was used as standards for standard curve. The color development was measure on U.V spectrometer at 490 and 570 nm respectively.

Fatty acid analysis

Fatty acids are the primary components of the cell membranes of the microorganisms and they form a specific proportion of the microbial biomass. Thus analyzing the fatty acids gives the measure of microbial biomass. Certain groups of

microorganisms possess specific fatty acids which acts as biomarkers and the relative proportions of these PLFA biomarkers provide a fingerprint of the functional groups and variations gives overall response of the microbial community to a particular treatment (White, 1979). We used two types of fatty acid analysis in our research. One is Fatty Acid Methyl Ester (FAME) analysis and the other is Phospholipid Fatty Acid (PLFA) analysis and each of these will be discussed in detail in the respective chapters.

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CHAPTER III

PHYSIOLOGICAL AND STRUCTURAL RESPONSE OF SOIL MICROBIAL COMMUNITIES AND THEIR METABOLITES TO VARYING MAGNITUDES OF OSMOTIC AND MATRIC STRESS

Abstract

Numerous studies have undertaken the challenge to understand how soil microorganisms respond to various forms of water stress; however, there have been only few attempts to assess their physiological and functional responses in soil. An experiment was conducted to study the physiological and structural responses of in situ soil microorganisms to the increasing levels of osmotic and matric stress. Water potential was manipulated in two soils, Marietta (fine-loamy, siliceous, active, thermic Fluvaquentic Eutrudepts) and Sumter (fine-silty, carbonatic, thermic Rendollic Eutrudepts) with different water regimes. The soils were exposed to matric stress by air drying over several days to -1.5, -4.5, -10, -20 and -40 MPa and to similar osmotic water potentials (-1.5, -4.5, -10 MPa) by the gradual stepwise addition of NaCl to the soil. We hypothesized that amounts of sugars and amino acids would increase along the increasing water stress gradient and that the two soils would respond differently to water stress. The physiological response was measured by extracting the metabolites from the soil at different water potentials and analyzing them for sugars and amino acids. Structural changes to the microbial community were analyzed by extracting PLFAs. A 15-25% increase in sugar concentration was observed with drying of soil (~300 μg g-1 soil)

compared to continuously moist soils (~240 μg g-1 soil). The concentration of polyols (glucitol, inositol and xylitol), in particular, increased by ~10- 30% as a result of water stress in Sumter but not observed in Marietta soil. Multivariate NMS analysis indicated that microbial communities changed both physiologically and structurally, but at similar levels of water potentials, change caused by osmotic stress was greater compared to that in matric stressed soils. These results provide some of the first direct evidence that microbial communities in soil in situ do utilize sugars and sugar alcohols to cope with water potential deficits.

Key Words: Osmolytes, Compatible solutes, Matric stress

Introduction

Drying and rewetting of the soil is a common phenomenon in majority of the terrestrial ecosystems, altering the amount of available water in the soil microbial habitats (Miller et al. 2005). Although this is true in most terrestrial biomes, it is especially relevant in seasonally dry climates where there is often a great variability in precipitation. For instance, in the Mediterranean and temperate climates where there is a marked seasonality with hot and dry summers (low precipitation coupled to high atmospheric evaporative demand) and moist and cold winters, alters the moisture availability to soil microorganisms. Particularly in summer, prolonged warm and dry periods, interspersed with sudden rains, changes the soil water potential rapidly. These fluctuations cause physiological stress. on existing microbial population, resulting in physiological and structural changes in the microbial community (Harris 1981, Balser and Firestone 2005).

Earlier studies on the effects of water dynamics on soil biogeochemical processes have reported a sudden flush in C and N mineralization in 1-4 days following the

rewetting of dry soil (Birch 1958, Sorensen 1974, Schimel et al. 1999, Franzluebbers et al. 2000, Williams and Xia 2009).The burst in C and N mineralization has been attributed to the release of labile substrates due to microbial cell lysis, the release of intracellular osmoregulatory substances (Kieft 1987, Magid et al. 1999), or the physical disruption of soil aggregates that release protected organic matter (Lundquist et al. 1999, Xiang et al. 2008). All resulting in catabolism of released organic molecules by surviving soil microorganisms upon rewetting (Bottner 1985, Van Gestel et al. 1991, Van Gestel et al. 1993b, Appel 1998). Isotopic studies have revealed that at least part of the carbon released during the short-term pulse is microbial and thought to be either from microbial cell lysis caused by osmotic upshock (Van Gestel et al. 1992) or release of intracellular solutes from the microbes (Halverson et al. 2000). However, reference of the exact microbial response to rewetting of dry soil is still unknown.

Maintenance of cell turgor, which is vital in cell growth and survival, is highly affected by the extra cellular water dynamics (Bremer and Krämer 2000, Schimel et al. 2007). Numerous hypotheses have emerged about the adaptation strategies of microorganisms to cope with low water potentials and the accumulation of osmoregulatory substances to maintain cell turgor is a common theme among them (Harris 1981, Killham and Firestone 1984, Halverson et al. 2000). Pure culture studies on microbes under water deficit conditions, have reported the accumulation of osmolytes like K^+ ions and/or a group of compatible organic solutes like glutamate, proline (amino acids) sucrose, trehalose (sugars), peptides, N-acetylated amino acids (amino acid derivatives), glycine betaine, carnitine (Quaternary amines) and tetrahydropyramidines like ectoines (Killham and Firestone 1984b, Csonka 1989, Poolman and Glaasker 1998) which are rich in carbon and nitrogen. In contrast to inorganic ions like K^+ and Na^+, the

organic solutes can be safely up and down regulated with little impact on cellular functions and protein stability (Yancey 1994, Kempf and Bremer 1998, Record Jr et al. 1998).

With the recovery of conditions where water availability is adequate, the microbes have to release the accumulated solutes outside the cell in order to maintain equilibrium. When compatible solutes compounds are released into the environment, they act as significant carbon and energy source in the microbial systems. The release of these energy rich compounds could be partially correlated to the sudden flush in C and N mineralization when a dry soil is rewetted.

Previous research showed that accumulation of osmolytes is energy expensive and demands huge amounts of resources for their synthesis (Koujima et al. 1978). When the soil becomes dry, substrate diffusion becomes limited and microbes may experience greater resource limitation (Stark and Firestone 1995). In this context, very little is known about how the microbial communities physiologically adapt under in situ conditions to the matric stress, in oligotrophic environments like soil.

Overwhelmingly, majority of the studies that have analyzed the microbial solute accumulation under water deficit stress were done by exposing the microbes in cultures to high salt concentrations (Yancey 1994, Kempf and Bremer 1998, Poolman and Glaasker 1998). A few studies have done similar experiments by isolating the soil microorganisms and exposing them to salt and desiccation stress (Killham and Firestone 1984, Killham 1985, Schimel et al. 1989, Roberson and Firestone 1992). However, no studies so far have tried to measure the microbial response to varying intensities of matric stress under in situ soil conditions.

As reduced water availability affects the nutrient transport, microbial motility and various other factors in the microhabitats, it is very likely that these changes will reshape the microbial community composition even temporarily. Different microbial groups in the soil may respond distinctly to the stress. For example the gram positive bacteria which have thick cell walls may adapt well to the water potential fluctuations than the sensitive groups like gram negative bacteria (Williams 2007). Many researchers have previously studied the microbial community changes to water stress by fatty acid analysis (Wilkinson et al. 2002, Uhlí ová et al. 2005, Gordon et al. 2008), CLPP (Williams and Rice 2007) and DGGE (Griffiths et al. 2003) and isotopic analysis (Williams 2007). Most of these studies have looked at changes in the microbial structure after rewetting the dried soil and we in our study are focusing the microbial changes that occur as soils are dried to low water potential. As shifts in the microbial community composition will have significant impact on the long term ecosystem responses. It is very essential to understand the physiological and the concomitant structural changes to link to the functional changes that the drought causes in the soil ecosystems.

Over decades numerous scientists have been working on soil drying and rewetting process and its impacts on soil microbial community and yet many questions remained unanswered which shows the complexity of the process. Many researchers have conducted experiments on different soils and measured the soil respiration and microbial biomass changes that occur due to drying and rewetting events (Kieft 1987, West et al. 1992, Schimel et al. 1999, Mikha et al. 2005, Waldrop and Firestone 2006, Williams and Rice 2007) and reported that response varies with the soil type as each soil significantly differs with one another in biotic and abiotic factors. So, it has widely been hypothesized that soils that naturally experience climatic conditions that promote high degree of

variability in water availability may be more adapted to water stress and may thus show a more limited response to drying and rewetting induced water stress. For instance , a drought-prone grassland soil responded much less to drying and rewetting than an oak-dominated soil that tended to naturally have more moderate variability in water potential (Fierer et al. 2003). Other studies with samples taken from soils with naturally contrasting soil moisture regimes have shown, anecdotically, that this hypothesis may have widespread merit (Van Gestel et al. 1993a, Lundquist et al. 1999, Franzluebbers et al. 2000). Consequently, we were planning to measure the response of *in situ* microbial communities in two soils that are present in close proximity in Mississippi but still differ in their drying history.

Based on the discussion above, it is evident that moisture stress will have marked effect on physiology and structure of soil microbial community, there by affecting the soil processes and fertility. Thus, we were planning a study to understand how the soil microbial communities in two different soils with contrasting drying history respond physiologically and structurally to the low water potentials caused by air drying of soil. As bulk of the research conducted on physiological adaptation strategies of microorganisms to water stress was based on salt added/induced studies, we have included salt stress treatments in our experiment for comparison. The four major objectives of the experiments are as follows:

Objectives and hypothesis of the study:

1. To assess the difference between the microbial communities present in two soils with different drying characteristics and to compare the physiological response of the two communities to water stress. We hypothesized that the microbial communities in soils that tend to naturally experience drought

frequently will accumulate more osmolytes if OAH is true, while in soil that is less prone to drought cell death may occur more.

2. To evaluate the physiological response of soil microbial communities to increasing levels of matric stress by characterizing the chemical composition of soil microbial extracts. We hypothesized that there will be a positive relationship between the degree of drying and amount of extractable metabolites like sugars and amino acids from microbial cytoplasm. And also the microbes may accumulate simple organic solutes like glycerol and proline at lower degree of drying and may accumulate different kinds (mannitol, sorbitol, and trehalose) of osmolytes with further decrease in water potential to resists the efflux of water from the cytoplasm.
3. To determine the effect of matric and salt stress on the soil microbial communities across a gradient of possible soil water potential changes. We hypothesized that at any given water potential the microbes may respond similarly for both kinds of stressors, i.e. the metabolites accumulated by microbes at same water potential in both the kinds of stress will be similar.
4. To evaluate the effects of matric and osmotic stress on microbial activity, biomass and community composition in the two soils. We hypothesized that the microbial activity and biomass would be changed with the reduction in water potential in surrounding soil. The response to water potential deficit would also be reflected in the shifts in microbial community composition as the sensitive microbial groups may undergo dormancy and favoring resistant groups to water stress. We also hypothesized that at given water potential the

community composition would be the same for both matric and osmotic stress treatments.

Materials and methods

Site description

The experiment was conducted on two soils, the Marietta and Sumter series located near Mississippi State University, Mississippi, USA (33° 28' N and 088° 47' W). The Marietta soils are (fine-loamy, siliceous, active, thermic Fluvaquentic Eutrudepts) deep alluvial soils in the Blackland Prairie region of Mississippi. They are moderately well drained soils with slow run off and occasionally subjected to flooding. The water table is within a depth of 1.5 to 2.0 feet of the surface during periods of high rainfall. The site was forested with >50-y old deciduous vegetation dominated by pecans (*Carya illinoinensis)*. The C: N content of the Marietta soils is 2.35 and 0.17 respectively with pH of 6.2. The Sumter soils (fine-silty, carbonatic, thermic Rendollic Eutrudepts) were silty clays, with medium granular structure, moderately deep, well drained soils that are formed in Marly clays and chalk of the blackland prairies. The water table is deep and the permeability of the soil is slow. The pH of the soil is 6.5 with C: N content of 2.56% and 0.15% respectively. The soils were collected from the top 10 cm depth using a shovel and had soil water content of 34-36 % when collected. The collected soils were passed through 4 mm mesh sieve and thoroughly cleaned off all the big plant materials and rocks and were stored at -20^{o}C until used. Based on the soil type, vegetation, and drainage properties the two soils were expected to have diverse microbial communities with different susceptibilities to water stress. Total soil organic carbon (C) and nitrogen (N) contents were measured on a CE Elantech Model NC2100 elemental analyzer

(ThermoQuest Italia, Milan, Italy) with combustion at 625^{o}C and 900^{o}C respectively. Soil pH was measured after shaking a soil 0.01 M $CaCl_2$ (1:2, mass: volume) suspension for 30 minutes.

Experimental setup

A laboratory experiment was conducted to study the physiological and structural response of soil microbial communities to the water potential deficits caused by air drying the soil (matric) and addition of salts (osmotic) to the soil. An experiment with a total of 18 treatments comprising two soils and 9 water stress (6 matric stress and 3 osmotic stresses) treatments was designed. The treatments were replicated thrice and each replication had 5 subsets to carry out all the analysis. Approximately 10 g (dry weight) of well homogenized soil was weighed into 150 ml volume specimen cups. The water content of all the soil samples was adjusted to their respective field capacities (-0.03 MPa) by adding sterile distilled water. All the soil samples were pre-incubated at room temperature (22^{o}C) for five days to eliminate the disturbances occurred during sieving and storage. The water potential of the pre-incubated soils was lowered to various intensities either by air drying (matric stress) or adding NaCl (osmotic stress) to the soil.

For matric stress treatments, the pre-incubated soil samples at field capacity (-0.03 MPa), were slowly air dried to five different water potentials of -1.5, -4.5, -10, -20, -40 MPa over a period of 3 days at room temperature. The soils were dried for approximately 6-10 hrs per day for 3 days until the soils reached the desired water potential. The soils took approximately 16, 22, 29, 32 and 34 drying hours (drying hours are exact number of hours where the lids of the containers were kept open to let the soils dry) to reach the water potentials of -1.5 MPa, -4.5 MPa,-10 MPa, -20 MPa, -40 MPa

respectively. The soils were not disturbed during drying process. The relation between the soil water content and water potential was analyzed prior to the experiment by filter paper method as suggested by Mc Innes et al (1994). The water potentials of the soils were constantly monitored using WP4 dewpoint potentiameter by (Decagon devices inc). Continuously moist, no salt added treatment maintained at -0.03 MPa throughout the incubation period was treated as control.

For osmotic stress treatments a similar experiment was conducted on another set of soil samples, but the water potentials of the soils were brought down by adding NaCl to the soil. The NaCl was added to the soil gradually over a period of three days until the soils reached their water potentials of -1.5 MPa, - 4.5 MPa and -10 MPa, respectively. An amount of approximately 58.5 mg, 117 mg and 234 mg per 10 g of soil was added to Marietta and approximately 67.2 mg, 131.6 mg and 257.2 mg per 10 g to Sumter soil to get water potentials of -1.5 MPa, - 4.5 MPa and -10 MPa respectively. The soil samples were incubated at respective water potentials for 24 hours before further analysis was done. One set of samples were extracted for biomass carbon or metabolite analysis, one for soluble carbon, one set for NMR analysis, the fourth set for measuring soil respiration and the other for PLFA analysis. The samples for PLFA analysis were immediately stored at -80 ^{o}C. For soil respiration measurements, dried soils were transferred to the sealed serum bottles for CO_2 measurement for 24 hours.

Extraction of metabolites from the soil

The soil samples at reduced water potentials were extracted for soluble and microbial metabolites/carbon. Chloroform derivable solutes/metabolites from soil were extracted using mixture of chloroform and 0.01 M K_2SO_4 solutions and soluble

carbon/metabolites were extracted using 0.01 M K_2SO_4 solution. The principle behind this method is that chloroform lyse the microbial cells and makes the intracellular material more extractable with 0.01 M K_2SO_4. We had adapted the chloroform slurry method for extraction/lyse of microbial biomass for two reasons. One is that the fumigation of dried soils gave erratic results and were in poor agreement with biomass C estimated by the SIR (Substrate Induced Respiration) method was reported by Sparling (1984). Second being the possible activity of hydrolytic enzymes on proteins and polysaccharides in soils during fumigation which we will exaggerate our results of measurements of sugars and amino acids.

For microbial metabolite extraction, approximately 10 g (dry weight) of soil samples at their respective water potentials were transferred to 160 ml serum bottles and added with 10 ml chloroform. After a minute, 40 ml 0.01M K_2SO_4 was added to each bottle and shake on for 2 hours at 250 rpm on an orbital shaker. Another set of similar samples were extracted with 40 ml 0.01 M K_2SO_4 solution without chloroform for estimation of soluble carbon. Serum bottles were centrifuged at 1500 rpm on ITC centrifuge for 10 minutes for separation of chloroform and aqueous phase. The aqueous supernant was pipette out and filtered through Whatman 1 filter paper and the solution was lyophilized and stored at -80 ^{o}C until further analysis. The dried residues were redissolved in 1 ml of sterile distilled before analysis. These redissolved samples were used for colorimetric and GC-MS analysis of sugars and amino acids.

Analysis of soil extracts by colorimetric methods

Reducing sugars in the soil extracts were analyzed by phenol sulfuric acid method. The phenol sulfuric acid method is a simple and rapid colorimetric method to

determine total carbohydrates in a sample. Phenol reagent helps in developing color in the presence of reducing sugars. The method detects virtually all classes of carbohydrates, including mono-, di-, oligo-, and polysaccharides (Martens and Frankenberger 1993). Briefly, a small quantity of soil extract was added with 50 µl of 80% phenol solution followed by 5 ml concentrated H_2SO_4 (~18 M/l) solution. The mixture was allowed to stand at room temperature for 45 minutes. The absorbance was measured at 490 nm on UV spectrometer. A standard curve was made by measuring the absorbance at different concentrations of glucose solution.

Amino acids and α-amino-N content was determined using Ninhydrin reagent. Ninhydrin decarboxylates and deaminates α-amino groups and forms purple complex with the α-amino N containing molecules. The color is formed not only with amino acids but also with peptides, proteins, ammonium and other compounds with free α-amino groups (Jorgenson and Brooks 1990). The soil extracts along with 0.5 ml of citric acid and 2 ml of ninhydrin reagent were incubated at 100 ^{o}C for 25 minutes. The solution was cooled down and added with 5 ml of 50 % ethanol and the absorbance was measured at 570 nm on UV spectrometer. A standard curve was made by measuring the absorbance at different concentrations of L-Leucine-N.

Analysis of extractable metabolites by gas chromatography-mass spectroscopy

The sugars and amino acids in the soil extracts were characterized by GC-MS. The extracts were derivatized to increase the volatility of the substances before analyzing on gas chromatograph. All the reactions of derivatization were done in silylated vials. The surface of the laboratory glass ware was deactivated by treating the glassware with

5% solution of Dimethyldichlorosilane (DMDCS) followed by rinsing with toluene and methanol respectively.

For characterizing the sugars, the soil extracts were derivatized by N, O-bis-(trimethylsilyl) trifuroacetamide (BSTFA) solution. Approximately 250 µl of the aliquots of soil extract were taken in silylated reaction vials and dried down completely using nitrogen. The extracts were then converted to their trimethylsilyl (TMS) derivatives by adding BSTFA containing 1% trimethylchlorosilane (TMCS) and pyridine in 2:1 ratio and incubating them for 3 hours at 70 ^{o}C (Medeiros et al. 2006). The samples were allowed to stay overnight at room temperature and then were completely dried under pure nitrogen. The derivatized extracts were redissolved in 110 µl of hexane and collected into sample vials for GC analysis.

Derivatization of amino acids was done as per the method given by Fan et al (1996). The pH of the 500 µl of soil extracts was lowered to 2 by adding equal volumes 1 M HCl in reaction vial and the solution was dried completely under pure nitrogen as mentioned above. Dried extracts were sonicated with 1:1 mixture of MTBSTFA (N-Methyl-N- (Tert-Butyldimethylsilyl) trifluoroacetamide and acetonitrile for 2 hr at 60 ^{o}C. The solution was left at room temperature overnight and dried under nitrogen. The derivatized extracts were redissolved in 110 µl of hexane and collected into sample vials for GC analysis.

The samples were analyzed on Varian CP-3800 Gas Chromatograph coupled to a Varian Saturn 2000 MS/MS. The GC was equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm, with film thickness of 0.25 mm) operated using the following conditions: injector temperature, 240 ºC, column temperature, 60-280 ºC at 8 ºC/min then held at 280 ºC for 5 min; carrier gas, He; injection volume, 1 mL (splitless). The MS

mass ranged from 40 to 650 m/z, filament delay of 3 minutes, target TIC of 20,000, a prescan ionization time of 100 msec, an ion trap temperature of 150 ºC, manifold temperature of 60 ºC, and a transfer line temperature of 170 ºC.

Individual sugars were identified by comparison of mass spectra with literature, library data and comparison of mass spectra and GC retention times with those authentic standards and/or interpretation of mass spectrometric fragmentation patterns. Standard solutions of glucose, trehalose, sorbitol, sucrose, proline, glutamine which are commonly expected as microbial osmolytes, were analyzed. Compounds were quantified using total ion current (TIC) peak area and converted to compound mass using calibration curves of the external standards (glucose for monosaccharides, sorbitol for sugar alcohols and sucrose for disaccharides).

Measurement of microbial respiration

The microbial respiration was measured from both dried and rewetted soil samples after the drying cycle. Both the matric and osmotic stressed soil samples at their respective water potentials were transferred into 160 ml serum bottles and closed with a rubber septum and sealed tightly with aluminum ring. The respiration rates were determined by measuring CO_2 concentration from each serum bottles at regular intervals. The head space of the serum bottles was sampled through rubber septa using a syringe and CO_2 was analyzed on a Gas Chromatograph (Varian Model 3600 with a 2 m Porapak Q column running at 100 ^oC with thermal conductivity detector). The bottles were vented after each sampling for 10 minutes under flow hood to keep head space CO_2 concentrations from exceeding 2 percent. The microbial respiration from all the dried samples was measured for 48 hrs. After 48 hours, all the replicates of the matric stress

treatments were rewetted to field capacity or -0.03 MPa and then closed back with rubber septa. The CO_2 flush in the rewetted samples was monitored after 6, 24, 48 and 72 hours after rewetting.

Phospholipid Fatty Acid analysis

Microbial biomass and community composition was estimated by extracting and analyzing the phospholipid fatty acids (PLFAs). Total lipids were extracted according to procedure of White and Ringelberg (1998) as modified by Butler et al (2003). All the glassware was soaked in phosphorous free soap, thoroughly washed with deionized water and rinsed with acetone. The glassware was autoclaved and dried at 100 ^{o}C overnight before use. Ten grams of frozen soil (dry weight) from all the treatments was thawed for ~30 min and transferred to 160 ml serum bottles. The soils were extracted overnight using a mixture of 50mM phosphate buffer (pH 7.1), chloroform and methanol (0.8:1:2). The samples were centrifuged at 1000 rpm for 5 min and filtered using Whatman No1 filter paper and the soil was washed with methanol and chloroform and filtered twice to get the most of the lipids. The filtrate was added with 3 M NaCl solution and a pinch of Na_2SO_4 salt. The mixture was shaken well and the phases were allowed to separate for about 8 hours. The chloroform phase was collected into separate glass tubes and dried completely under stream of nitrogen. The total lipids were fractionated into neutral, glycol and phospholipids using silicic acid bonded phase extraction columns (Supelco, cat.No. 505048). First the neutral lipids were eluted by chloroform followed by glycolipids using acetone respectively. The phospholipids were eluted using 6 ml of methanol into separate test tubes and the methanol was completely evaporated under a stream of nitrogen. The dried phospholipid residue was methylated under alkaline

conditions by adding methanolic KOH, toluene and methanol mixture and incubated for 15 min at 32 ^{o}C. The mixture was then neutralized with 1 ml of 1 M acetic acid. The fatty acids methyl esters were extracted twice into 1:4 chloroform and hexane mixture and transferred into fresh tubes. The mixture was completely evaporated under stream of ultra high purity nitrogen and the residue was resuspended in 500 µl of hexane for GC analysis. Fatty acid methyl esters were separated, quantified and detected by an Agilent 6890 Series gas chromatograph (Santa Clara, CA) equipped with a flame ionization detector, an Ultra-2 column (19091B-102;0.2 mm by 25 m), and controlled by a computer loaded with ChemStation and Sherlock software. Ultra high purity H_2 was the carrier gas at a column head pressure of 20 KPa, septum purge of 5 ml min^{-1}, a split ratio of 40:1, injection temperature of 300 ^{o}C, injection volume of 2 µl. The oven temperature ramps from 170 ^{o}C to 288 ^{o}C at 28 ^{o}C min^{-1} and the analysis time of each sample was 6 min. Peak identification was carried out by the Microbial Identification System (MIDI, Inc.) following calibration with a standard mixture of 17 fatty acid methyl esters (1300A calibration mix).

Bacterial biomass was estimated from the summed concentrations of the following PLFAs: i15:0, a15:0, 15:0, i16:0, a16:0, 16:1ω9, 16:1ω7t , i17:0, a17:0, 17:0, 18:1ω7 and cy19:0 (Frostegård and Bååth 1996). Actinomycetes were estimated by 10me16:0 and 10me18:0 fatty acids and protozoa by 20:4ω6 biomarkers respectively (White et al, 1997). Fungal biomass was estimated from the concentration of the biomarkers 18:1ω9 and 18:2ω6 (Frostegard and Baath, 1996). Standard nomenclature was used to describe fatty acids. Fatty acids were designated in terms of total number of carbon atoms with the number of double bonds given after a colon. The position of the double bond is defined by the symbol ω followed by the number of carbons from the

methyl end of the fatty acid molecule. The prefixes i and a refer to iso and anteiso branching respectively and cy refers to cyclo propyl fatty acids.

Statistical analysis

The differences between treatments at different degrees of water potentials were analyzed using Proc ANOVA and Proc GLM in SAS (SAS Institute, 1996). Non-metric multidimensional scaling (NMS), a nonparametric method, was used to provide graphical ordination of the mol% PLFA-C and mol% of metabolites data. The ordination and multivariate analysis of the data utilized the PC-ORD version 4 software (MJM Software, Gleneden Beach, OR) and followed the recommendations of McCune and Grace (2002).

Results

Quantification of soil microbial/extractable carbohydrates and amino acids by colorimetric analysis

Analysis of carbohydrates by phenol sulfuric acid method

The phenol sulfuric acid (PSA) analyzable carbohydrates from chloroform labile K_2SO_4 extracts from soil indicated that Marietta soils had greater amounts of sugars than the Sumter soil under continuously moist conditions (Figure 3.1a). The amount of carbohydrates in the Marietta and Sumter soils showed very different trends in response to drying. The amount of carbohydrates in the Marietta soil increased with drying by 20-30% compared to moist soil, but with the greatest amounts measured in the moderately dry soil (-4.5 and 10 MPa). In the Sumter soil, in contrast, the amounts of carbohydrates were not consistent across drying treatments (Figure 3.1b). Carbohydrates were significantly greater due to drying at -4.5MPa, however, at all other levels of drying there was no change or a decrease compared to the moist treatment.

Osmotic stress did not consistently affect the amount of carbohydrates extracted from Marietta soil, with greater amounts detected in the -4.5 but not the -1.5 and -10 MPa treatments. In the Sumter soil, in contrast, salt stress tended to result in greater amounts of extractable carbohydrate. Overall, in both treatments, salt stress resulted in equal or greater amounts of carbohydrates than moist soil, and though amounts differed by water potential, generally, the carbohydrate detected in salt stress treatments was comparable to that in the drying treatment.

Ninhydrin reactive nitrogen

Figures 3.2a and 3.2b indicates the Ninhydrin reactive-N concentration in chloroform labile soil extracts at various intensities of matric and osmotic stress in Marietta and Sumter soil respectively. The results show that the initial concentration of Ninhydrin reactive-N in Marietta soil (7.46 $\mu g\ g^{-1}$ soil) was more than in Sumter soil (4.62 $\mu g\ g^{-1}$ soils). Contrast to carbohydrates, the amount of Ninhydrin reactive-N significantly decreased (9-30%) with drying of soil compared to continuously moist treatment in Marietta soil. Whereas in Sumter the Ninhydrin concentration increased (10-30%) at moderate drying treatments (-1.5 MPa and -4.5 MPa) and decreased significantly there after compared to moist treatment.

The treatmental effect was not consistent in osmotic stress samples, in terms of Ninhydrin reactive-N concentration. In Marietta soil, Ninhydrin reactive -N increased at -1.5 MPa and -10 MPa, but a significantly decreased at -4.5 MPa. In the Sumter soil, the osmotic stress has resulted in the significant increase (27 - 50%) in the Ninhydrin reactive-N concentration in all the three treatments compared to moist control treatments.

Since Ninhydrin analysis detects the amino acids and NH_4^+ present in the soil, much of it could be from soil and not of microbial origin.

Characterizing the soil extracts

The GC-MS analysis of Trimethylsilyl (TMS) derivatives of chloroform labile K_2SO_4 derivable soil extracts showed the presence of wide range of sugars (monosaccharides and disaccharides) and sugar alcohols in the samples. The compounds that were detected include glucose, fructose, galactose, trihydroxy butyric acid, arabinose, glycerol, glucitol, xylitol, inositol, myo-inositol, turanose and sucrose. The total sugars detected in GC-MS varied from approximately 20 $\mu g\ g^{-1}$soil to 120 $\mu g\ g^{-1}$ soil. However, glucose was the most abundant monosaccharide found in all the treatments varying approximately 45-60% of total amount of sugars.

The composition and the relative abundance of the saccharides and other compounds vary with the soil type. The variation in the relative proportions of the metabolites found in the matric and osmotic stress was more prominent than at different intensities of water potential caused by similar kind of stress. Compounds like glycerol, galactose, glucose, glucitol, myo-inositol and turanose were found in all the treatments. However, presence of certain compounds was not consistent along the moisture regime in both the soils. For example, inositol was found only in Marietta soil but was not detected in Sumter. Similarly, sugars like arabinose, fructose and polyols like xylitol were found in detectable limits only in some replications of the of the stress prone soils.

A relative increase in the glucitol and decrease in inositol peaks were observed in Marietta soil along water stress gradient whereas a gradual increase in glucitol with increase in the intensity of drying was noticed in Sumter soil. The total amount of sugars

that were detected in the GC-MS was far less (7-30%) than the amount of sugars that was detected in PSA analysis (Table 3.1). The reproducibility of the analytical procedure was assessed through the relative standard deviation of the replicate measurements and values ranged from approximately 2-20%. Figure 3.3 shows the changes in the relative abundance of 6 important sugars that were found in all the treatments. The two soils Marietta and Sumter clustered separately after multivariate analysis elucidating the fact that the relative abundance and composition of the metabolites in the two soils were different. In both the soils, metabolites in continuously moist treatment (unstressed soils) were very closely clustered to the matric stress treatments except -20 MPa in Marietta soil. Overall, the chemical composition of the chloroform labile K_2SO_4 derivable soil extracts indicates some degree of similarity between the matric stress treatments and the variation seems to be more with salt addition. The changes in the relative abundance of glucose, sugar alcohols and other saccharide concentrations with introduction of matric stress and osmotic stress in Marietta and Sumter are shown in figures 3.4a, 3.4b and 3.5a, 3.5b, respectively. The increase in polyols concentration and decrease in the glucose concentration with stress was observed in osmotic stress treatments and the trend is consistent in both the soils. Whereas, with matric stress the polyols concentration increased consistently across the stress gradient in Sumter soil but not in Marietta soil. At certain water potentials, the concentrations of other saccharides, like turanose, xylitol and fructose, increased significantly.

Amino acids, the other important group of osmolytes was found in continuously moist and osmotic stress treatments but we failed to detect them in dry soils. We identified amino acids, like alanine, valine, proline, leucine, isoleucine, glutamine, glutamic acids, and some fatty acids in the moist and osmotic stress treatments. However,

no definite pattern was observed in the quantities of amino acids detected in stress prone soils compared to moist controls, but we observed that some peaks were dying off with increase in osmotic stress. We failed to quantify the concentrations of amino acids in the samples as the recovery was very inconsistent and the variation between the replications is too large. Absence of any significant peaks in the matric stress treatments may indicate the low and undetectable amounts of amino acids present in the soil samples.

Microbial respiration

The microbial activity was measured as soil respiration. The CO_2 samples of the matric stress treatments were collected 24 hours before (dry soils) and 24 hours after rewetting of the soil samples (Figures 3.6a,& b). Our results showed that the microbial activity in dry soils (matric stress) is highly correlated with the water potential of the soil. Soil respiration gradually decreased with increase in the intensity of water stress in both the soils. In Marietta, the respiration rates at -40, -20, -10, -4.5 and -1.5 MPa were 2, 13, 10, 17 and 35 % of rates measured in moist soils respectively, whereas in Sumter soil the respiration rates were 5, 11, 13, 20 and 58 % to the moist controls respectively. Under matric stress, the respiration dropped up to 65% in Marietta and 50% in Sumter soil at -1.5 MPa and steeply dropped thereafter. In the driest soils (-40 MPa) the respiration rates had almost reached to no activity or control treatments.

When the matric stressed soils were rewetted to -0.03 MPa, a huge pulse in the soil respiration was noticed within few hours after rewetting. Following an initial flush of microbial activity that was dominant during the first 2 days following rewetting of dried soil, the respiration gradually declined to a basal soil respiration rate which is similar to moist control soils. The soils at low intensities of drying (-1.5 MPa, -4.5 MPa

and -10 Mpa) returned to their basal respiration faster than the driest soils (-20 MPa and -40 MPa) and this was true in both the soils.

Decrease in soil respiration was also observed by the addition of NaCl to the soils compared to moist control treatments (Figures 3.7a,& b). At any given water potential the microbial respiration decreased more with matric stress than with the osmotic stress. The decrease in rate of respiration (~50%) at -1.5 MPa was similar in matric and osmotic stressed treatments in both the soils. However, the activity at later water potentials (-4.5 and -10 MPa) remained higher in osmotic stress samples compared to matric stress treatments.

Total PLFA concentration

Changes in the bulk soil PLFA at different water potentials in two soils Marietta and Sumter are shown in Figures 3.8 a & b, respectively. The PLFA concentration present in continuously moist treatments in Marietta soil (~600 ng g-1 soil) was higher than the Sumter soil (~350 ng g-1 soil). With the increase in the water stress the two soils acted completely differently. In Marietta soil, both matric and osmotic stress has resulted in the decrease of the total PLFA concentration. However the significant decrease was noticed at -4.5 MPa and -10 MPa of osmotic stress. Whereas the PLFA concentration increased significantly with water stress compared to continuously moist treatment in Sumter soil and it holds true with both matric and osmotic stress.

Changes in microbial community composition

The multivariate (NMS) analysis of the community composition at different treatments in both soils was presented in Figure 3.9. The communities in the two soils

clustered separately and remained separate in all the treatments. It was very obvious that the microbial communities harboring in the two soils were very different (Figure 3.10).

However, the detailed analysis of the changes in the community composition was done by looking into absolute and relative abundances of 30 biomarker fatty acids (Tables 3.2 and 3.3) and the change (percentage) that observed in the mole percent distribution of different microbial groups (Figure 3.11) in the two soils. In Marietta soil, the absolute concentrations of bacterial fatty acids (gram positive, gram negative and actinomycetes) significantly decreased with stress compared to moist control soil. The decrease was significant in both matric and salt stress treatments however it was more prominent in osmotic stress treatments. A decrease in the fungal biomarker fatty acids was also noticed with moisture stress. Surprisingly, no significant change in the relative abundance of the fatty acids was observed with the stress.

In Sumter, an increase in the abundance of all the five groups which includes gram positive, gram negative, actinomycetes, fungi and protozoa was noticed. Approximately 25 % increase in the mole percent of the fungal PLFA biomarkers (18:2ω 6, 9 and 18:1ω9c) in stressed soils was noticed compared to situations where water availability was kept more or less constant. But the relative abundances of the fatty acids attributed to gram negative bacteria and fungi increased by 10 %, while a 4 % decrease in the gram positive bacteria was recorded. But in both soils, a drop in all the biomarker fatty acids was observed at -4.5 MPa under matric stress treatment which again increased at -10 MPa which is to be noticed. Mole percent of the protozoa (20:4ω6) biomarkers varied with water stress and was 5 and 20 % greater in the dry soils of Marietta and Sumter respectively than the moist soils. While the differences appeared to be small

compared to the other groups, they could be highly correlated to various other prey predator relations and motility of cells and substrate diffusion.

Fungal to bacterial ratio of the two soils at different water potentials under matric (Figure 3.12a) and osmotic stress (Figure 3.12b) was calculated based on relative abundance data. No significant affect of water potential deficit was noticed on fungal to bacterial ratio in Marietta soil under both osmotic and matric stress, where as a significant increase was noticed in the Sumter soil. The increase was highly correlated to the increase in the fungal biomass with stress. An increase in the stress biomarkers by 10 and 25 % in the driest treatments compared to moist controls was observed in two soils (Figure 3.13).

Discussion

A lab experiment was conducted to determine the physiological and structural response of soil microbial communities to different intensities of matric stress. Two soils, Marietta and Sumter, with different histories of drying were selected for our study to understand if the soil history of water availability and tendency to dry plays key role in the response of microbial communities to lab induced drying. The pre-incubated moist soils at ~-0.03 MPa were air-dried to varying levels of matric potential of -1.5 MPa, -4.5 MPa, -10 MPa, -20 MPa and -40 MPa respectively. The microbial response to osmotic stress was also determined at -1.5 MPa, -4.5 MPa and -10 MPa where the water potential of the soil was lowered by adding NaCl salt. All the soils were extracted for metabolites and PLFAs for understanding the physiological and structural response of soil microbial communities.

The key findings of our study were:

1. An increase in the amount of extractable sugars was observed in matric and osmotic stress treatments (up to -10 MPa) compared to the moist controls (-0.03 MPa) but decreased with further drying of soil. Detectable amounts of amino acids were absent in matric stress treatments. Thus, our results are in partial agreement with osmolyte accumulation hypothesis (OAH).
2. Increase in concentration of sugar alcohols like glycerol, glucitol and inositol in dried soils is consistent with OAH, especially fungal responses to water stress in culture-based studies. However, we were unable to detect amino acids which were considered as another important osmolyte group used by microorganisms under low water potentials.
3. The increase in the accumulation of osmolytes was not linear in matric stress treatments under in situ soil conditions unlike osmotic stress in culture based studies. The results from the salt induced culture based studies may not entirely relate to matric stress response of soil microorganisms in oligotrophic environments like soils.
4. Inconsistency in the osmolyte accumulation pattern and difference in the structural changes with water stress in the two soils shows that the soil type or stress history of the soil might be one of the influential factors in the microbial response to water stress along with nutrient availability.

Microbial communities in Marietta and Sumter soils

While the primary objective of this experiment was to determine the effects of intensity of water stress (drought) on physiology and structure of microbial communities,

we also wanted to assess the discrimination between the microbial communities of the two soils and how these differences may have influenced the stress responses. Our results showed that the total number of fatty acids and their relative proportions was approximately the same in the two soil types. However, the multivariate analysis of the total PLFA's demonstrated that the microbial communities inhabiting the two soils, Marietta and Sumter, were distinct from one another (Figure 3.9). When the scores of the NMS ordination were plotted, the samples clustered together by soil type with no overlap. Similar results were obtained with principal component analysis of the 30 biomarker fatty acids. There are few possible explanations why the two soils may harbor distinct bacterial communities. Differences in soil abiotic conditions, such as soil moisture (Bossio and Scow 1995, Schimel et al. 1999), distinct above ground plant communities associated with the two soils and differences in the pH, C and N content might have contributed to the differences in microbial community composition (Van Gestel et al. 1993a, Bossio et al. 1998, Griffiths et al. 1998).

Physiological response

The culture based studies on microbial adaptation to water potential fluctuations have reported an increase in cytoplasmic sugar and amino acids concentrations at low water potentials (Galinski and Truper., 1994; Kempf and Bremer., 1998; Poolman and Glassker., 1998). Few researchers have attempted to check the response of soil microorganism to low water potential by introducing one or two microbial isolates to desiccation and osmotic stress in soil matrix (Killham and Firestone, 1984; Firestone 1985; Schimel et al., 1989; Halverson et al., 2000). As far as our knowledge goes, no

study has attempted to measure the physiological response (osmolyte accumulation) of in situ soil microbial communities to low water potentials.

The physiological response of soil microbial communities to different levels of drying was determined by quantifying and characterizing the metabolite pool extracted from dry soils using chloroform and 0.01 M K_2SO_4. We hypothesized that the soil microbial communities accumulate organic solutes/osmolytes in the cytoplasm to cope up with low water potentials and the nature and composition of the osmolytes would vary with intensity of drying.

Metabolite concentration at different drying intensities

The Marietta and the Sumter soils responded to the gradient of drying in many dissimilar ways. For instance, we observed an increase in the PSA analyzable sugar concentration along drying gradient compared to moist controls in Marietta soil (Figure 3.1a) whereas the response was not very consistent in Sumter soil (Figure 3.1b). Sumter is mesic soil exposed to more drying and rewetting cycles, so the microbial communities in the Sumter soil might be more adapted to the water potential fluctuations and perhaps have limited response in drying.

A significant increase in sugar concentration of ~ 25-50 µg g-1 soil was noticed at -4.5 MPa and -10 MPa drying treatments in Sumter and Marietta. When compared to the moist controls, it is ~10-25 % increase in C concentration in the microbial metabolites. These results corroborate with the previous studies that reported an increase in cytoplasmic C content ranging from 10 to 40% (Koujima et al. 1978, Schimel et al. 1989) and N content from 11 to 60 % (Killham and Firestone 1984b, Schimel et al. 1989) at low water potentials in culture-based studies.

Under extreme dry conditions (-20 MPa and -40 MPa) a slight to significant decrease in sugar concentrations was observed in the two soils. Even though it is difficult to explain all of the differences in sugar content within the microbial community in response to drying, the variable results could be a consequence of the knowledge about the ways in which soil microbial communities respond to drying. For instance, the soils at water potentials of -20 MPa and -40 MPa are so extremely dry and apparently the soil microorganisms in climates like Mississippi experience such extreme conditions so rarely i.e. may twice or thrice in a decade. So, under such conditions the microorganisms may utilize other types of osmolytes or some other strategy to cope with water stress. For example, the extraordinarily low soil water potentials may induce cells to undergo transition into dormancy rather than adaptation as accumulation of osmolytes is energy expensive process. And also under extremely dry conditions the water in the soil gets so thin and discontinuous resulting in the decrease in substrate diffusion and nutrient availability to microorganism. Under such conditions the limitations in nutrient and energy availability, could lower the capacity for an organism to produce appropriate concentrations of osmolytes to counterbalance cellular water loss (Stark and Firestone 1995). This latter scenario could also ultimately result in organism transition into some state of inactivity or dormancy.

Ninhydrin reactive Nitrogen (NRN) concentrations, a measure of amino acids, peptides, proteins and NH_4^+, was in the range of ~7 μg g^{-1} in Marietta soil and ~4.6 μg g^{-1} in Sumter soil when soils were maintained under moist (-0.03 MPa; Figures 3.2a & b). These concentrations of NRN are low and in the absence of extensive nitrification could be accounted for soil ammonium. While, amino acids are considered important osmolytes accumulated in many types of bacteria during water deficit (Csonka 1989), and yet a

decreasing trend in the NRN occurred during drying in the Marietta soil. However, the general trend was different for the Sumter soil, whereby NRN increased up to -4.5 MPa but later decreased significantly. Nevertheless, the ~1 μg response of NRN could account for only a very tiny proportion of microbial adaptation under water stress. Because amino acids, peptides and proteins in soils are attracted to the numerous reactive sites in soils, the results may also be more indicative of the soils chemical rather than biological properties (Jones et al. 2003). However, we assume that most of the NRN which we find could be from NH_4^+ ions present in soil and may not be from amino acids.

It is interesting to note that Roberson and Firestone (1992) reported an increase in Extracellular Polysaccharide (EPS) concentrations and decreased in protein concentration when *Pseudomonas* sps was subjected to desiccation in sand matrix. Following rewetting, an increase in protein concentration and decrease in EPS were observed, indicating that proteins and possibly other cellular carbon components were used for polysaccharide production in response to desiccation which may be related to our observations.

Another major factor determining the microbial response to matric stress in soils is the nutrient limitation. The PSA analyzable C and the NRN are in a ~20:1 ratio, which shows the low N concentration in the soil. Soils are oligotrophic and soil microorganisms are frequently known to be limited by either C or N. This could be exacerbated in soils with low matric potentials, as thin water films can limit nutrient diffusion (Schimel et al. 1989). The costs of osmolyte production for a single drought have been calculated to consume up to 5% of net annual productivity in certain grassland systems (Schimel et al. 2007). Because of low substrate and nutrient availability during periods of drying, microbial responses to drying under typical oligotrophic and N-poor situations in soil may differ from those of studies that occur in nutrient and energy rich cultivation

systems. It is also possible that different mechanisms exist for coping with drying in soil than in non-soil microbial cultures (Tschichholz and Trüper 1990, Schleyer et al. 1993). Counter to our hypothesis, accumulation nitrogen based osmolytes may not be a common theme in oligotrophic environments like soil. The contrasting results in the two soils shows that microbial community surviving in the soil and nutrient availability may also play an important role the nature of osmolytes accumulate by the microbes.

Microbial community response to matric and osmotic stress

Comparing how microbial communities in soil respond to osmotic and matric stress provide the opportunity to assess whether research using salts to lower water potential provide comparable results to those utilizing matric potential deficit. The majority of previous studies on osmolyte accumulation were conducted under controlled laboratory conditions whereby salts were used to induce water potential deficit. Our results on osmolyte accumulation under osmotic stress were partially comparable to the matric stress treatments. However, across the water potential gradients the sugar and Ninhydrin concentration of the osmotic stress treatments was greater than the respective matric stress treatments. Even though the exact reason for higher concentrations of sugars and ninhydrin reactive N were not known, there are few a possible explanations. Salt stress is a completely different phenomenon compared to the matric stress. Microorganisms under matric stress are subjected to desiccation and complete deprivation of water. In contrast, microbes under osmotic stress persist in a liquid environment with access to water, albeit one of diminished water activity (Potts 1999).

Under osmotic stress the microbial motility and substrate diffusion is not hindered unlike matric stress. So, there is a possibility for microbial growth and/or accumulation of

more osmolytes in the cytoplasm to survive under low water salt stress. Moreover, the high concentrations of sugars and amino acids detected in the PSA and ninhydrin analysis could partially be related to the mineralization of polysaccharides and proteins in the presence of enzyme activity and water. It has been previously shown that the two forms of stress (matric and osmotic) affect the physiological performance of heterotrophic bacteria (Chang et al. 2007) and fungi (Ramirez et al. 2004) differently.

Overall, our results were in partial agreement with the osmolyte accumulation hypothesis. Most of the studies often compare the microbial response to matric stress in complex soil systems, to the response of microorganisms to osmotic stress in cultures. But our results suggest, that the matric stress can be comparable to salt induced culture based studies up to moderate drying intensities (-4.5 and -10 MPa) but later we find lot inconsistencies in the osmolyte accumulation. It was little hard to explain the reasons for discrepancy at extreme dry conditions in soils, as majority of the studies conducted so far on microbial adaptations to water stress was tested below -10 MPa, and so we are one of the first to test the microbial response to such wide range of water potential deficits.

Metabolite composition at low water potentials

The GC-MS analysis of chloroform labile K_2SO_4 derivable soil extracts showed the presence of wide range of saccharides in both the soils. Among all the samples (from both Marietta and Sumter soils), approximately 11 different saccharides including, glucose, fructose, galactose, glycerol, arabinose, glucitol, inositol, myo-inositol and sucrose, were identified. While the diversity in metabolites show limited differences between the two soils, they had sufficient resolution to separate the microbial communities of the two soils on the basis relative abundances of metabolites. The plot of

NMS ordination based on metabolite composition shows that the samples clustered together by soil type with no overlap. The metabolite composition in air-dried samples was much closer to moist controls suggesting that the salt addition may have a completely different effect on the metabolite composition.

Although the saccharides found in the two soils were relatively similar, the relative proportions of each sugar were different in the two soils (Figure 3.3). Glucose was the dominant monosaccharide found in both the soils followed by glycerol, myo-inositol and sorbitol in Marietta soil and sorbitol, galactose, glycerol in Sumter soil respectively. These are some of the compounds that were reported previously to be used as compatible solutes by microbes to hold the cell turgor supports our hypothesis of osmolyte accumulation by microbes under in situ conditions to survive the matric stress.

One significant trend that was noticed in both the soils was an increase in the relative proportions of polyols concentration with the stress. The results indicate that the variations in the sugar concentrations in the Sumter soil are closely related to the fungal fatty acid signatures. The increase of intracellular polyols concentration with increasing osmotic stress in numerous fungal isolates strongly supports the function of this polyols as organic osmolytes under low water potentials (Wethered et al. 1985).

The commonly reported polyols in fungi like glycerol (Hocking 1986, Al-Hamdani and Cooke 1987), sorbitol(Shen et al. 1999) and mannitol (Kelly and Budd 1991, Shen et al. 1997) were found in our samples suggesting the possible accumulation of compatible solutes by in situ soil microorganisms. Brown (1972) reported the accumulation of glycerol in yeast (*Saccharomyces rouxii*) under salt stress conditions. Accumulation of glycerol, erythritol, mannitol in two filamentous fungi, *Aspergillus*

niger and *Pencillium chrysogenum* as a response to increased salinity in the growth media was reported by Alder et al.1982.

We have noticed a concomitant decrease in sugar glucose and increase in polyols concentration in stress prone soils compared to moist controls. Similar results were reported by Pascual et al. 2000 when *Pencillium frequentans* was grown under reduced water availability. However, no clear patterns of accumulation of many compounds like xylitol, arabitol was noticed in stressed soils. While trehalose is another known reserve carbohydrate in microorganisms, primarily fungi (Martin et al. 1988, Sillje et al. 1999) we could not detect traces of it in our samples. Part of our hypothesis was that the nature of osmolytes varies with intensity of stress. Contrary to our hypothesis we did not find any novel or new osmolytes in the soil with increasing levels of drying but have seen a change in the proportion of the (mole percentage) of certain groups of compounds with drying compared to unstressed soils.

Instead of finding one or two compounds in higher concentrations we found a cocktail of metabolites whose concentrations changes with the intensity of stress. Unlike culture studies, soil is habitat for different microbial groups that responds differently to the water stress and there is possibility that different groups accumulate different kinds of osmolytes making the extracts cocktail of metabolites or osmolyte accumulation could even be decided by carbon supply and demand in the microhabitats (Schimel et al. 2007, Williams and Xia 2009). Gustav et al (2010) reported that mixtures may reduce the toxicity associated with high concentrations of a single osmolyte, and obviate feedback mechanisms that down regulate metabolic pathways in the presence of high concentrations of product. These factors probably underlie the complex patterns of osmolyte accumulation that have evolved among the microorganisms.

Our effort to characterize the amino acids from dry soils was much of a vain. We could not detect any amino acid peaks in dry soils which relate that much of the ninhydrin reactive nitrogen we find in dry treatments could be $NH4^+$ in soil and not amino acids. Contrary to matric stress treatments, we have detected a range of amino acids like alanine, valine, proline, glutamine, glutamate, leucine, isoleucine in moist controls. We were able to detect some of these amino acids in salt stress soils but we have noticed a decrease in an order magnitude in the concentration of salt stress treatments and noticed that some peaks were dying away.

The non-metric multidimensional analysis of metabolites shows the clustering of matric and osmotic stress treatments separately in the two soils. We found ~20% increase in the accumulation of turanose in Marietta soil at -20 MPa and ~10-15% fructose in Sumter soil in osmotic stress treatments (-4.5 MPa and -10 MPa) compared to moist controls. This shows that the microorganism may opt for certain compounds to adapt under certain water potentials.

Glucose, fructose and sucrose were previously reported to be extracted from soils but were mostly tied to the plant origin (van Hees et al. 2005). Increase in amino acids, sugars and sugar alcohols in soils in summer months compared to samples in winter months was previously documented (Medeiros et al. 2006). Though none of the studies have discriminated the sugars from microbial or plant origin, some studies have related the increase in sugar concentration with increase in biomass (Medeiros et al. 2006). They also reported an increase in the mannitol concentration in the dry months (May to August) compared to milder months. Since our extracts are from bulk soil we are not denying the fact that part of the metabolites could be of plant origin. But, when the soluble extracts from similarly treated soils were analyzed on GC-MS we failed to detect

any peaks, suggesting that the peaks which we found from chloroform labile soil extracts could be of microbial origin. Soluble extract did not show huge amounts of sugars in either colorimetric analysis or GC-MS analysis which is agreement with Williams and Xia (2009).

Soil respiration during water stress

Soil respiration rates have shown correlation with soil water potential. As the soil water potential decreases either by matric or salt stress, a decrease in soil respiration was noticed and the decrease was obvious in matric stress in driest treatments. Our results were in agreement with many previous studies (Schimel et al. 1999, Williams and Xia 2009). Decrease in microbial activity can be attributed to two simultaneous occurring mechanisms –osmotic regulation and limited diffusive transport (Voroney 2007). Microorganisms may invest substrates into acclimatization, become inactive or even die due to severe living conditions during periods of drought (Bottner 1985, De Nobili et al. 2006) and microbes may become metabolically inactive decreasing the C and N mineralization under dry conditions. This decrease could be further enhanced by diffusive limitations. Stark and Firestone (1995) reported that the substrate limitation is the dominating factor when osmotic potentials are greater than -0.6 MPa, whereas cell dehydration is the major inhibiting factor when osmotic potentials are below -0.6MPa.

In salt stress treatments the decrease in soil respiration was similar to matric stress up to -1.5 MPa but further decrease in the water potential did not affect the soil respiration significantly. Under salt stress, microbes are bathed in water of diminished activity but that with a matric stress, bacteria are dehydrated due to low water contents and the availability of the water is reduced through its interaction with the matrix. So the

availability of substrate and extracellular enzyme diffusion during salt stress may support the activity of some resistant microbial groups which accounts for the respiration that was detected.

Shifts in microbial community composition

The fourth objective of our experiment was to determine the changes in the microbial community composition to the increasing magnitude of matric stress in two soils. The total PLFA concentration which is an indicator of microbial biomass showed contrasting results with stress in the two soils (Figures 3.8a and 3.8b). We observed a marginal decrease in the total PLFA concentration in Marietta soil along the water stress gradient. As the actively growing microbes are more susceptible to desiccation than the slow growing community (Van Gestel et al. 1993b) there is possibility for the marginal decrease in the microbial biomass in Marietta soil. Similar results related to the changes in the biomass with water potential fluctuations were reported previously by many researchers (West and Sparling 1986, Wilkinson et al. 2002, Williams and Rice 2007).

Whereas, the total PLFA concentration increased significantly with stress in Sumter soil compared to moist controls. The microbial communities in arid and semiarid regions are usually considered to be better adapted to drought than communities in aquentic environments (Sparling et al. 1987, West et al. 1988, Schimel et al. 2007). Apparently the microbial community in the Sumter soil must be more adapted to drought and thus the microbial biomass was not negatively affected by drying. As Bottner (1985) posited the existence of two functionally distinct communities, one that was resistant to the stress and other that decline quickly during the dry period. Perhaps with the lysis of susceptible microbial groups, the dry conditions may favor the resistant microbial

community composition that have the ability to metabolize substrates that are not used by the members of the microbial community at lower water potentials (Zogg et al. 1997). The substrate pool size for microbes could potentially increase which results in the increase in the microbial biomass.

The NMS analysis of the 60 PLFAs from all the treatments shows that the microbial communities harbor in the two soils was very different. It was clear that the treatmental effects on the microbial communities were smaller than the differences between the two soils (Figure 3.9). These results are consistent with other studies which show that the soil types have major influence on the structure of the microbial communities than moisture effects (Bottner 1985, Lundquist et al. 1999, McLean and Huhta 2000, Wilkinson et al. 2002, Fierer et al. 2003). The two soils harbored distinct microbial communities and remained different across the water potential gradient induced by matric and salt stress.

Analysis of 30 different biomarker fatty acids representing different microbial groups from the two soils, shows that Gram-negative bacteria was the dominant group in both the soils (Figure 3.10). However, the Sumter soil which was predicted to be more stress prone harbored relatively more fungal biomass than Marietta soil, reflecting that fungi could be more dominant in the drought prone and drier climates (Harris 1981, Wilkinson et al. 2002). Although the absolute concentrations of the Gram-positive, Gram-negative and fungal biomarkers significantly decreased with increase in intensity of matric stress in Marietta soil, the proportion of the Gram-negative increased. Where as in Sumter soil the concentration of bacterial (Gram-positive and Gram-negative) and fungal biomarkers increased with matric stress. Shifts in the community composition result because the costs associated with tolerating moisture stress fall differently on

different organisms depending on their inherent resistance and acclimatization abilities (Schimel et al. 2007).

It is widely recognized that certain groups of soil microbes are well adapted to particular moisture regimes. Some studies have shown that fungi to be more sensitive to drying as bacteria are located in small pores and water is retained longer in small pores (Tisdall and Oades 1982, Williams 2007). But contrary to this, our results show an increase in proportions of fungal biomass in Sumter soil with drying of soil. Systems dominated by fungi have also been postulated to be more drought-tolerant because fungi are generally considered more resistant than bacteria, remaining active at soil water potentials down to -10 MPa (Griffin 1981, Luard and Griffin 1981, Freckman 1986). In fact, the most stress tolerant fungi could grow at near maximum rates at -20 MPa and will make at least some growth at -50 MPa (Deacon 2006). While Gram-positive bacteria are thought to be more resistant to the to the water potential fluctuations in the soils because of their resistant cell wall structure, we observed an increase in both Gram-positive and Gram-negative bacteria with increase in matric stress along with fungi. This suggests that the long term exposure to fluctuations in water availability might have resulted in the selection of resistant microbial species in Sumter soil.

Our treatments did not impact the fungal to bacterial ratio significantly in Marietta soil but the ratio significantly increased in Sumter soil. As Sumter has relatively more fungal biomass and the fungi are known to remain active in soils at very low water potentials, contrary to bacteria (Harris 1981, Shipton and Burggraaf 1982) might have promoted the fungal growth. While Sumter soil did not show much of a response in terms of sugar and amino acid accumulations to water stress, there is a possibility that the microbial community in the Sumter soil might have reallocated the resources in the cell

walls or new cell growth during stress periods as an adaptation strategy to survive under the matric stress conditions. Decreasing the water potential may increase cell wall growth relative to cytoplasmic growth, thus increasing the fungal C/N ratio was reported by Paustian (1987). Since Sumter has more fungal biomass which might have reallocated the resources in cell walls and mycelia instead of increase in the cytoplasmic concentration.

The decrease in the total PLFAs and proportion of bacterial and fungal biomarkers at -4.5 MPa and increase in the further drying in both the soils shows that drying up to certain threshold limits could affect certain susceptible microbial groups and therefore providing an extra pool of substrate (dead biomass) to other microbial groups. Competition could occur for available substrate between the microbial groups and drying and rewetting could alter the soil structure which then affect microbial populations and their sensitivity to drying and rewetting (Denef et al. 2001).

Increase in the ratio of cyclopropyl fatty acids to their precursors, also observed changing in our study. These ratios increases under a variety of conditions in pure cultures of Gram-negative bacteria including oxygen depletion decrease in pH, high temperature and low nutrient availability (Kieft 1987, Kieft et al. 1994, Lundquist et al. 1999). In our study the ratios cyclo17:16:1ω7 and cyclo19:18:1ω7 were initially low but significantly increased with drying in Marietta soil suggesting the nutrient stress in the microbial community. On the other hand stress biomarkers did not change significantly in Sumter. So, the shifts in PLFA composition following soil drying may be change in the lysis or growth of microbial biomass or physiological adjustment of living organisms.

Conclusion

Known for their complexity, understanding the mechanisms involved in global nutrients cycles, is a challenging task. And studying the role and response of soil microbial communities to/in the soil process in situ soil conditions is much more complicated. Studies on physiological response of in situ soil microbial communities to wide range of matric potentials are rare. Our study was focused on determining the physiological and structural response of soil microbial communities, to varying intensities of water stress under, in situ soil conditions. Our data is some of the first to show that microorganisms in soil, in situ, acclimate to soil drying by accumulating compatible solutes, such as sugars and alcohols. We expect that a deeper characterization of microbial & soil pools that are dynamic during soil drying will shed further light on mechanisms of microbial adaptation to water stress. The two soils which we have chosen to test the hypothesis were exposed to relatively similar climatic conditions (precipitation and temperature); but still we could see huge variations in the response between them to the water stress. Thinking of the huge diversity of microbial populations and wide range of climatic conditions on the global scale it is unimaginable to apply any one strategy of process that the soil microbial communities adapt for survival.

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Table 3.1 Amount of Sugars (µg g-1 soil) identified in GC-MS Marietta and Sumter soils at different water potentials.

	Marietta		Sumter	
Treatment	Sugars (µg g-1 soil)	% Detected†	Sugars (µg g-1 soil)	% Detected†
Moist Control	34.3 (15.9)	14.3 (6.8)	93.5 (13.8)	50.5 (9.6)
Matric Stress				
-1.5	37.8 (23.1)	15.2 (9.6)	46.2 (10.6)	26.6 (5.4)
.4.5	69.4 (16.3)	15.0 (5.1)	46.1 (6.9)	21.1 (2.61)
-10	37.7 (4.1)	12.2 (1.2)	58.0 (18.5)	30.4 (10.2)
-20	21.4 (1.1)	7.7 (2.7)	39.5 (14.8)	23.2 (8.8)
-40	21.2 (7.5)	7.7 (2.7)	27.0 (3.4)	16.4 (2.0)
Osmotic Stress				
-1.5	35.7 (22.2)	12.9 (7.6)	36.5 (4.3)	20.3 (1.6)
-4.5	67.8 (15.3)	22.3 (5.0)	76.2 (36.3)	33.7 (16.0)
-10	34.3 (1.1)	8.7 (0.6)	120.3 (4.0)	33.7 (5.3)

Values are means and the the figures in the parenthesis indicates the standard error with in the treatments.
† % Detected is amount of sugars detected in GC-MS/Sugars detected in PSA analysis *100

Table 3.2 Absolute concentration of PLFA biomarkers (nmol g-1 soil) indicative of different groups of micro biota at different matric potentials in Marietta and Sumter soil.

Treatments	Gram Positive	% Change†	Gram negative	% Change	Actinomycetes	%Change	Fungi	% Change	Protozoa	% Change
Marietta										
Moist	114.9(7.9)	0	201.3(13.6)	0	80.7(5.6)	0	47.4(3.3)	0	1.80(0.11)	0
-1.5	110.3(2.3)	-4.1	195.5(4.2)	-2.8	80.1(1.4)	-.0.9	47.2(1.1)	-0.4	1.67(0.05)	-7.1
-4.5	103.7(1.6)	-9.7	182.3(2.9)	-9.4	73.5(1.2)	-8.9	43.2(0.5)	-8.9	1.48(0.02)	-17.7
-10	105.9(2.1)	-7.8	189.9(3.9)	-5.6	75.1(1.4)	-6.9	45.1(0.9)	-5.1	1.62(0.01)	-9.9
-20	100.2(1.6)	-12.8	183.2(3.1)	8.9	70.4(1.1)	-12.7	43.0(0.9)	-9.3	1.44(0.04)	-19.7
-40	96.5(4.7)	-16	174.3(8.7)	13.4	66.2(2.8)	-17.9	40.5(1.9)	-14.5	1.33(0.04)	-25.9
Sumter										
Moist	66.8(0.4)	0	111.9(1.7)	0	33.5(0.3)	0	39.5(0.7)	0	0.34(0.02)	0
-1.5	80.7(2.6)	20.8	132.3(4.2)	18.2	38.3(1.2)	14.3	49.5(1.1)	25.1	0.26(0.02)	-24.4
-4.5	72.1(1.1)	7.8	121.1(3.6)	8.2	35.7(1.0)	6.3	49.6(2.8)	25.2	0.26(0.01)	-24.8
-10	77.3(3.6)	15.7	130.3(5.8)	16.4	37.7(1.5)	12.5	52.1(2.5)	31.7	0.24(0.02)	-30.7
-20	85.5(4.8)	27.9	151.8(7.9)	35.7	42.2(2.4)	25.9	62.6(3.9)	58.4	0.31(0.05)	-9.2
-40	82.3(0.4)	23.2	150.24(0.6)	34.2	41.4(0.1)	23.4	61.0(2.5)	54.2	0.28(0.01)	17.6

Values are means and the the figures in the parenthesis indicates the standard error with in the treatment.
† % change indicates the increase (positive values) or decrese (negative values) in the absolute concentartions of the indicative biomarkers relative to moist controls at respective water potentails.

Table 3.3 Absolute concentration of PLFA biomarkers (nmol g-1 soil) indicative of different groups of micro biota at different osmotic potentials in Marietta and Sumter soil.

Treatments	Gram Positive	% Change†	Gram negative	% Change	Actinomycetes	%Change	Fungi	% Change	Protozoa	% Change
Marietta										
Control	114.9(7.9)	0	201.3(13.6)	0	80.7(5.6)	0	47.4(3.3)	0	1.80(0.11)	0
-1.5	93.1(5.1)	-18.9	173.2(10.2)	-13.9	68.1(2.7)	-15.7	40.6(2.3)	-14.4	1.58(0.05)	-12.2
-4.5	69.9(1.1)	-39.1	133.1(3.1)	-33.9	52.1(1.1)	-35.6	31.3(0.6)	-33.9	1.29(0.03)	-28.1
-10	77.1(3.1)	-32.8	135.8(4.5)	-32.5	52.9(2.0)	-34.4	32.2(1.1)	-32.2	1.16(0.05)	-35.3
Sumter										
Control	66.8(0.4)	0	111.9(1.7)	0	33.5(0.3)	0	39.5(0.7)	0	0.34(0.02)	0
-1.5	74.3(1.9)	11.2	132.7(2.7)	18.6	38.8(0.7)	15.7	50.6(1.0)	27.9	0.46(0.23)	34.6
-4.5	81.1(3.6)	21.3	145.1(5.3)	29.6	41.7(1.2)	24.3	60.5(1.8)	53.0	0.36(0.02)	-1.2
-10	78.4(4.2)	17.3	148.2(5.7)	32.4	41.5(1.4)	23.9	65.6(2.3)	65.8	0.32(0.01)	-6.9

Values are means and the the figures in the parenthesis indicates the standard error with in the treatment.
† % change indicates the increase (positive values) or decrese (negative values) in the absolute concentartions of the indicative biomarkers relative to moist controls at respective water potentails.

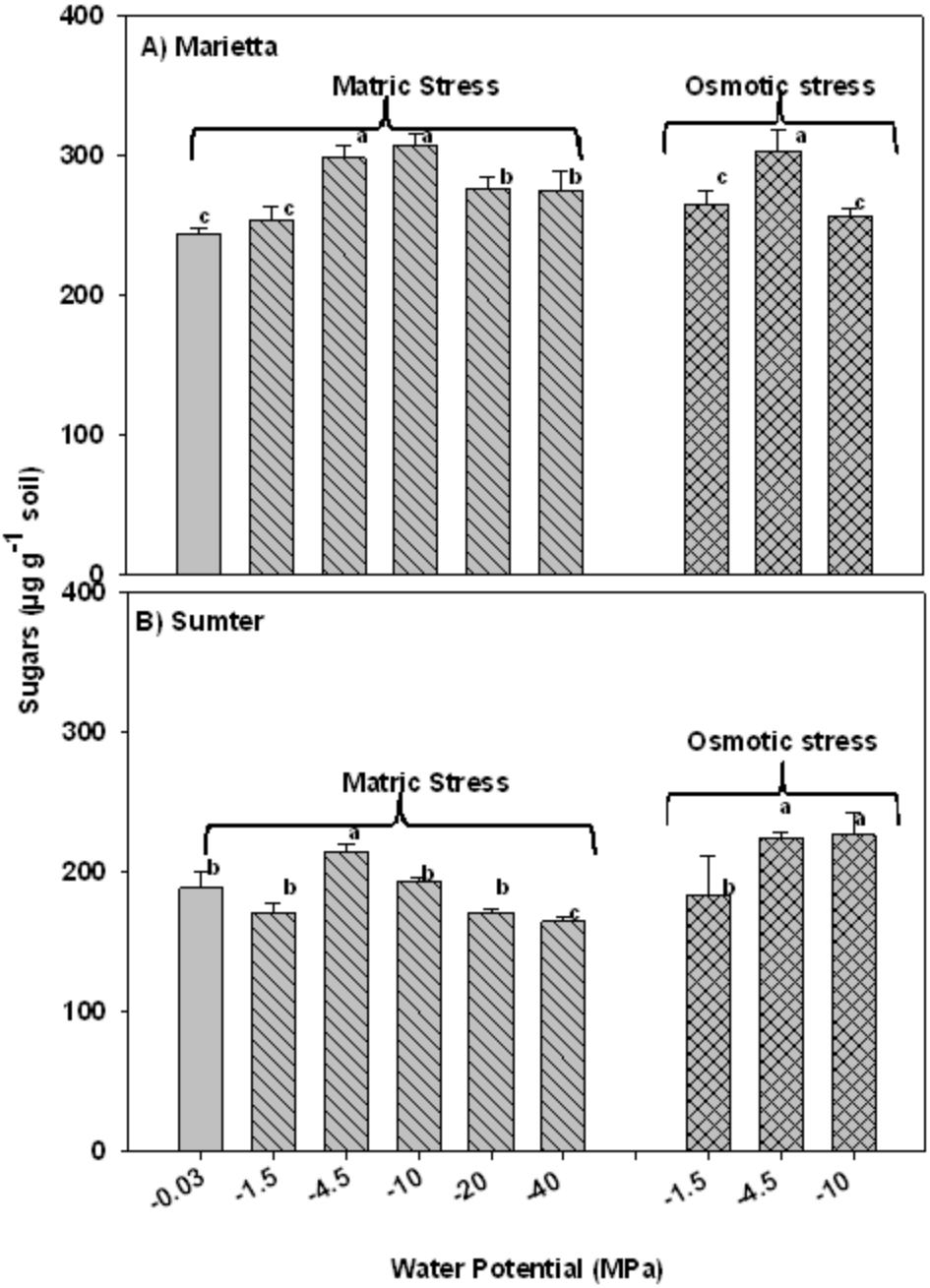

Figure 3.1 The amount of sugars (µg g-1 soil) detected (phenol sulfuric acid analysis) in chloroform-labile K_2SO_4 derived soil extracts at different water potentials induced by matric and osmotic stress in a) Marietta b) Sumter Soils (n=3).

Notes: Error bar represents the mean standard error with in the treatment.

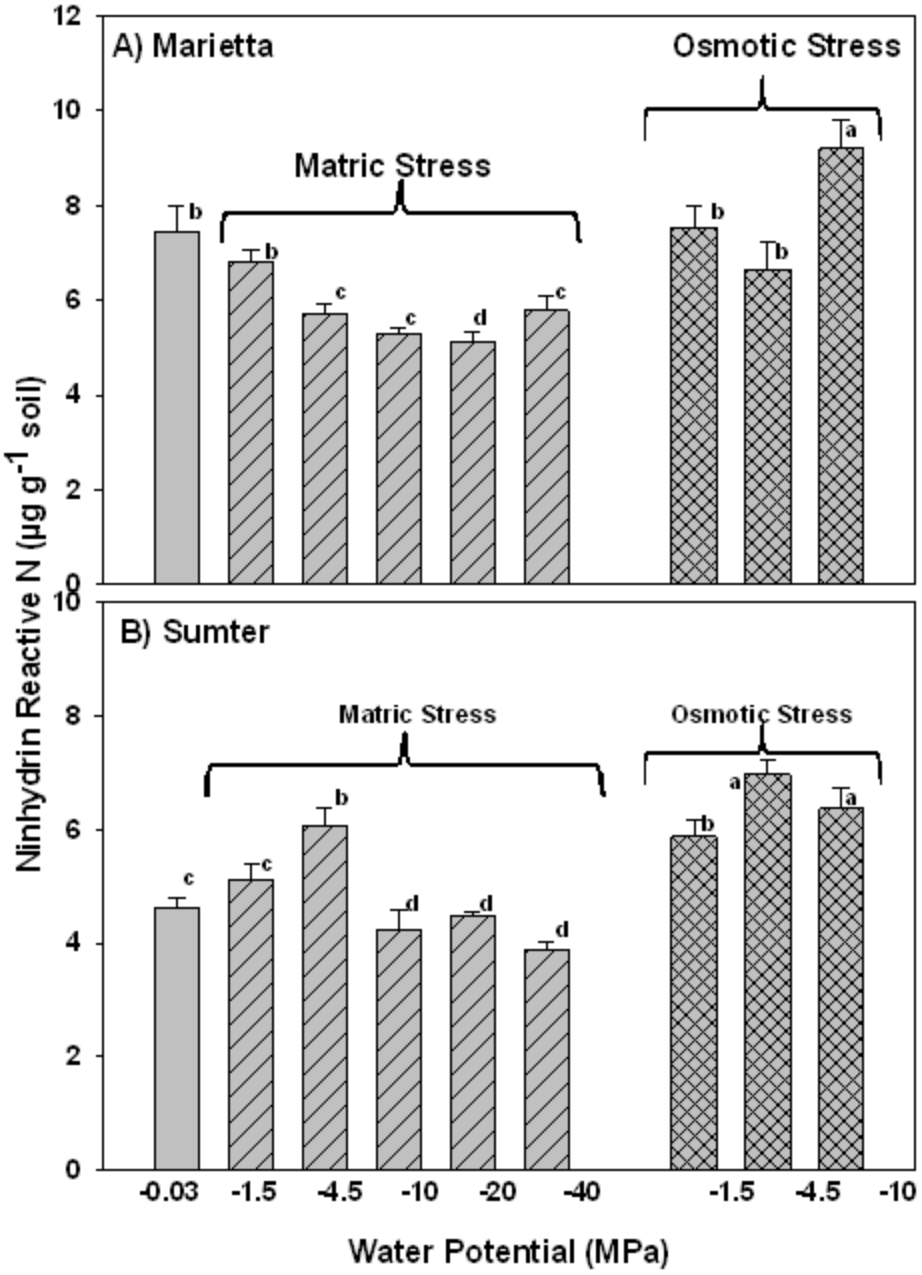

Figure 3.2 Amount of Ninhydrin reactive-N (µg g-1 soil) in chloroform-labile K_2SO_4 derived soil extracts at different water potentials induced by matric and osmotic stress in a) Marietta b) Sumter Soils (n=3).

Notes: Error bar represents the mean standard error with in the treatment

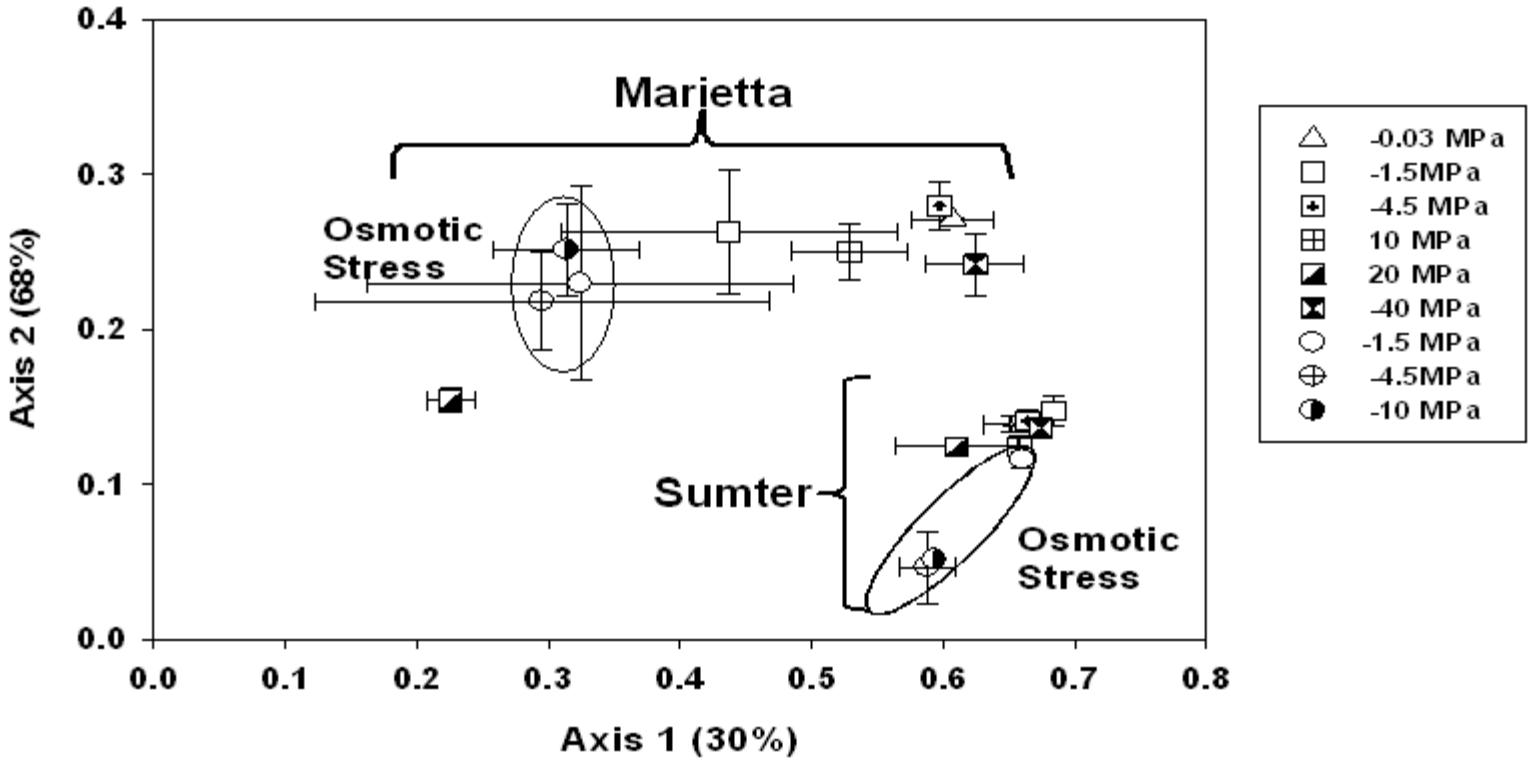

Figure 3.3 Non-metric multidimensional plot of mol% of metabolites identified at varying intensities of water potentials induced by matric and osmotic stress in Marietta soil and Sumter soil.

Notes: Percentages in the parentheses are the proportion of variance explained by each axis. Symbols and bars are indicative of the means and standard error (n=3).

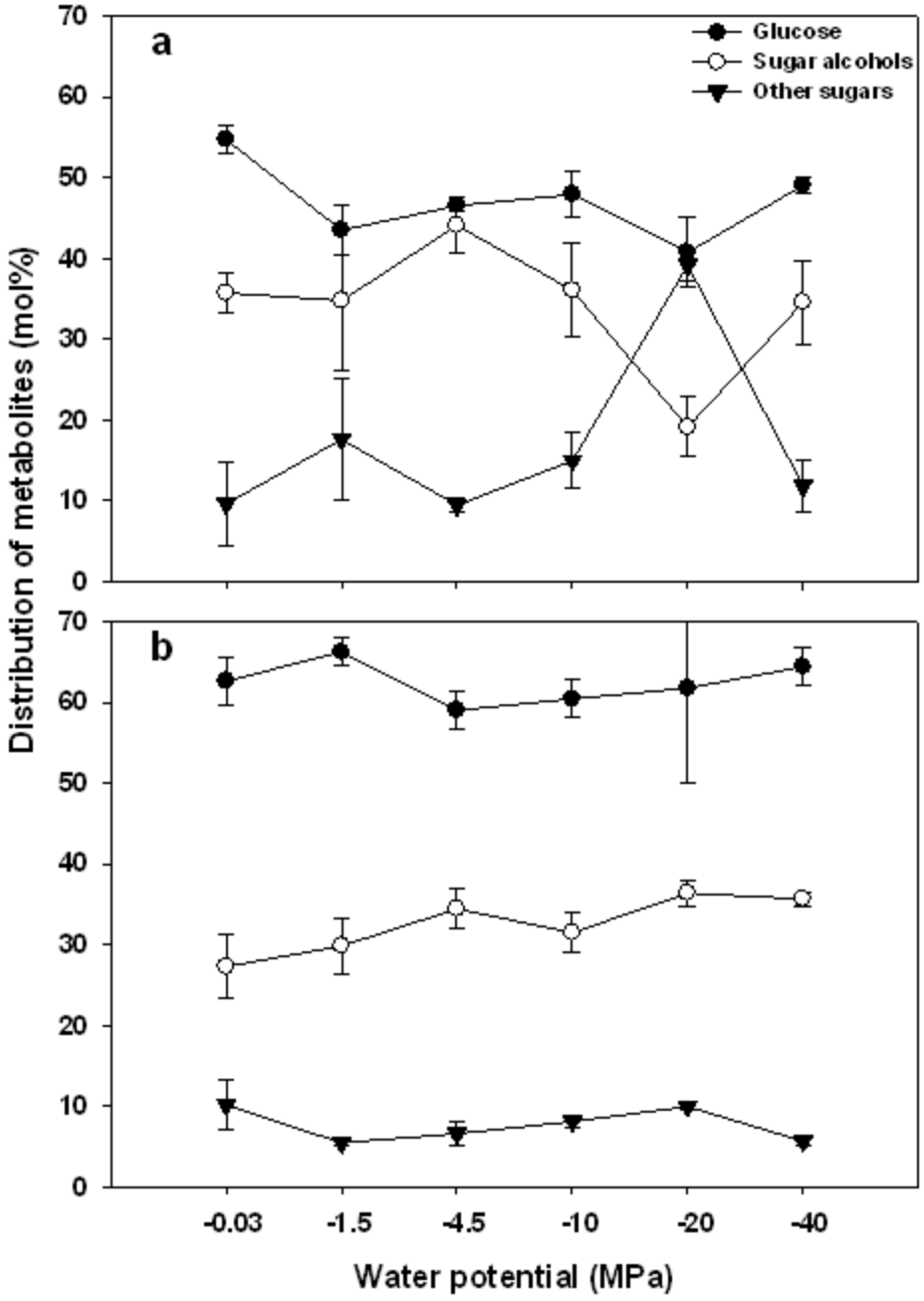

Figure 3.4 Mol% distribution of different metabolites at various intensities of matric stress treatments in a) Marietta soil b) Sumter soil (n=3).

Notes: Error bar represents the mean standard error with in the treatment.

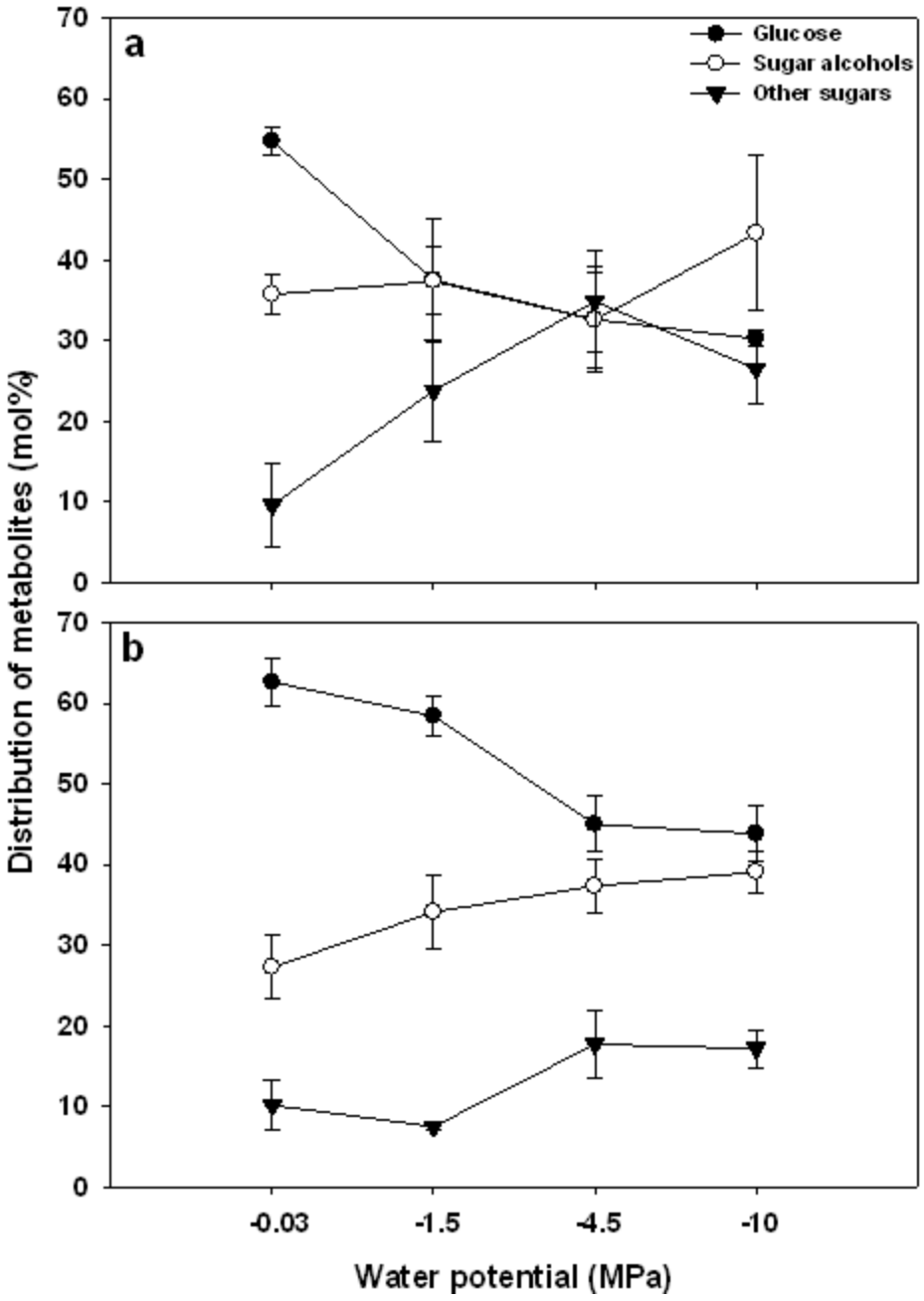

Figure 3.5 Mol% distribution of different metabolites at various intensities of osmotic stress treatments in a) Marietta soil b) Sumter soil (n=3).

Notes: Error bar represents the mean standard error with in the treatment.

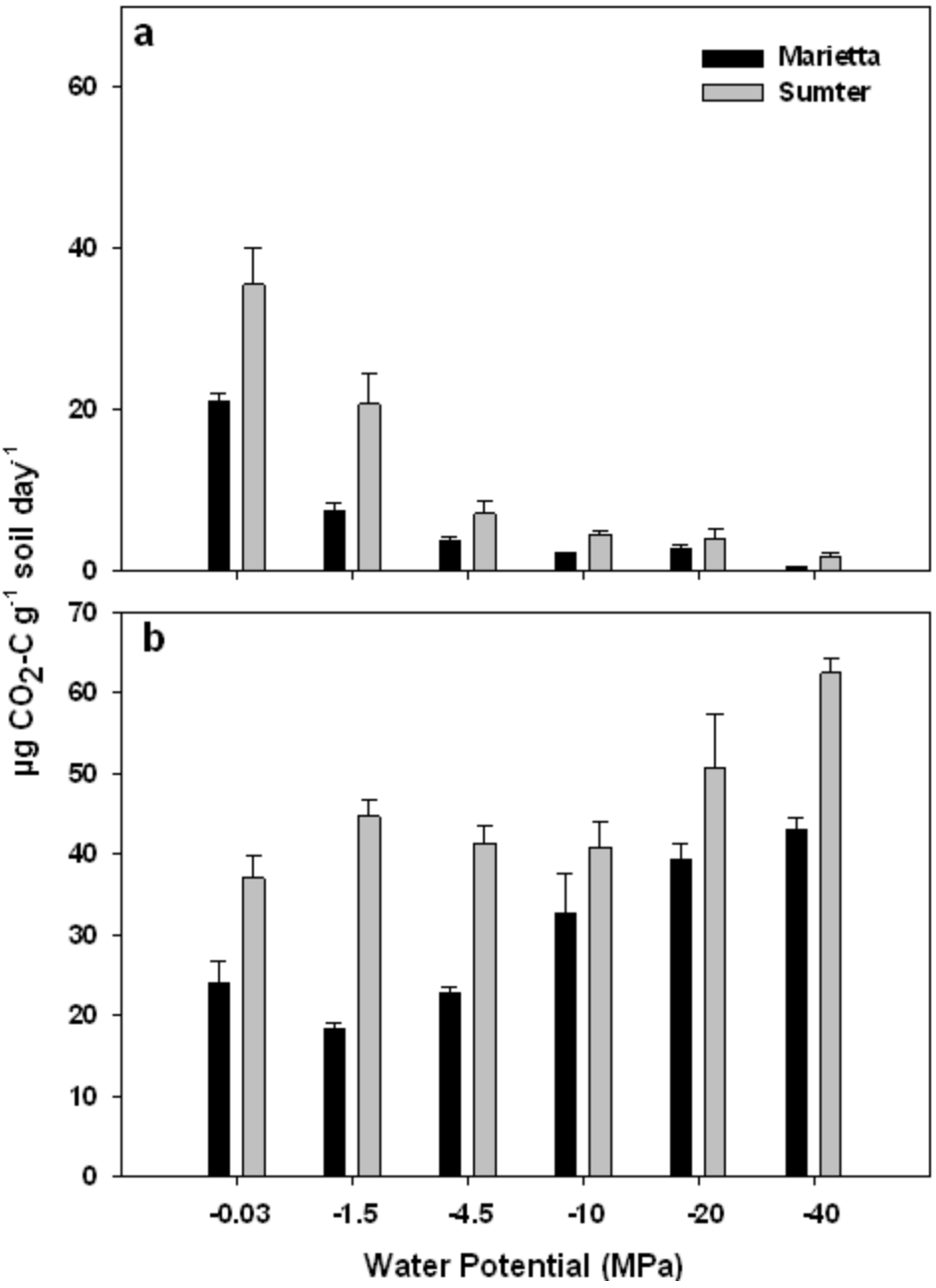

Figure 3.6 Soil respiration measured in Marietta and Sumter soils over a 24 h period in a) dried soils (Matric stress) across water stress gradient and in b) rewetted soil that were previously dried to specific water potentials and brought back to -0.03 MPa.

Notes: Error bars represent the standard error with in the treatment (n=3)

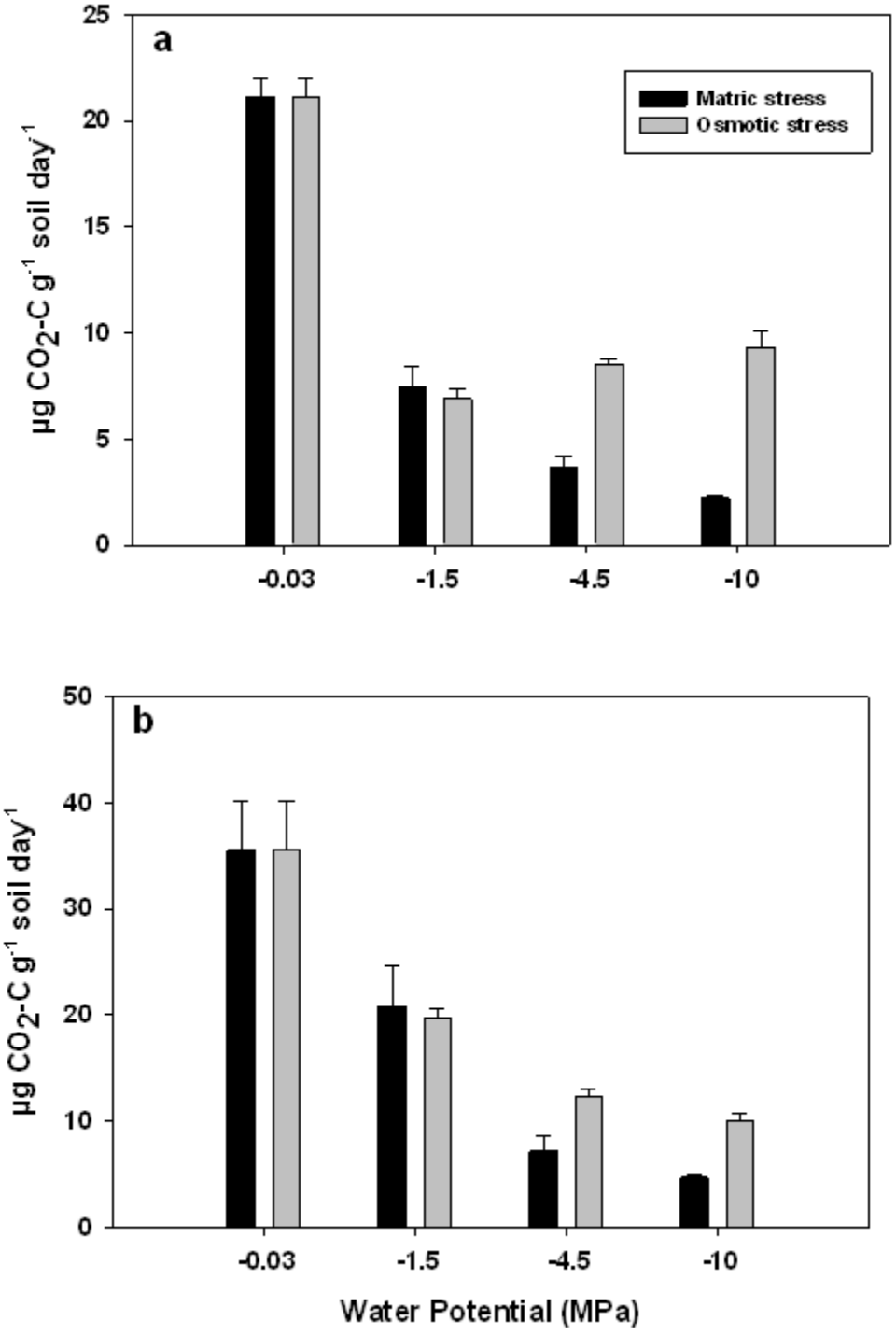

Figure 3.7 Soil respiration measured in a) Marietta and b) Sumter soil at varying intensities of water potentials induced by matric and osmotic stress.

Notes: Error bars represent the standard error with in the treatment (n=3).

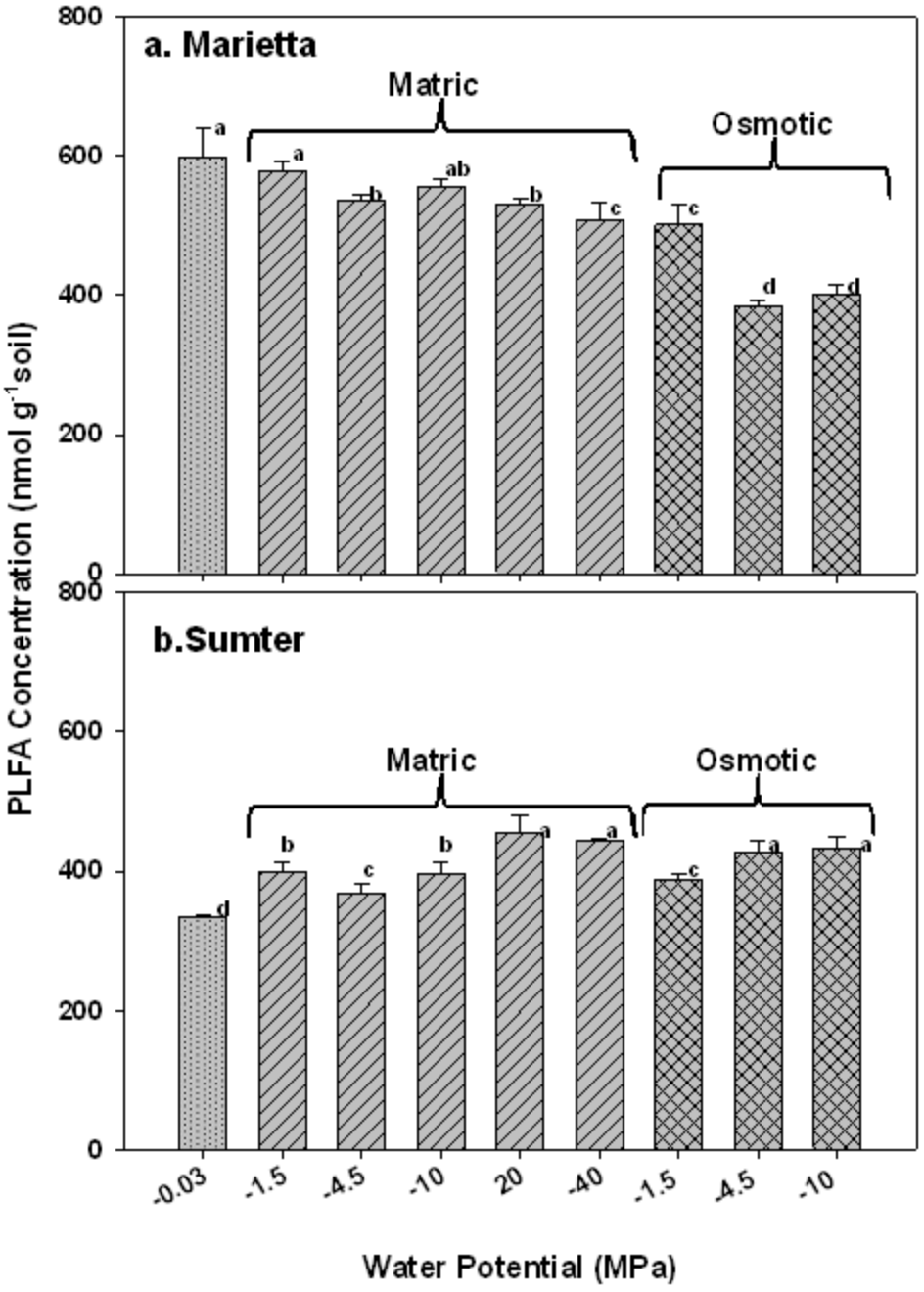

Figure 3.8 Total PLFA concentration (nmol g-1 soil) at different water potentials induced by matric and osmotic stress in a) Marietta soil b) Sumter soil.

Notes: Error bars represent the standard error with in the treatment (n=3).

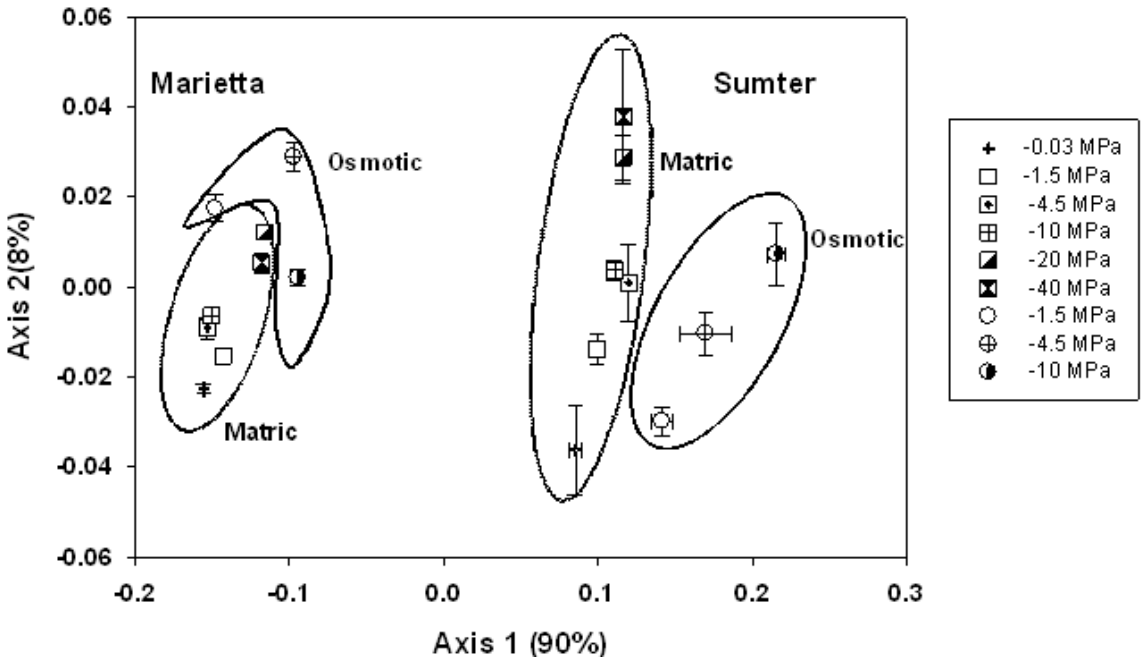

Figure 3.9 Differences in the structure of the microbial community associated with the Marietta and Sumter soils using a non-metric multidimensional scaling (NMS) analysis of the mol% PLFA.

Notes: The designations matric and osmotic in the graph refers to the two kinds of the water stress the soils are induced. Percentages denote the amount of variability associated with each axis. Values of symbols represent the means of the treatment and bars represent the standard errors of the treatments (n =3).

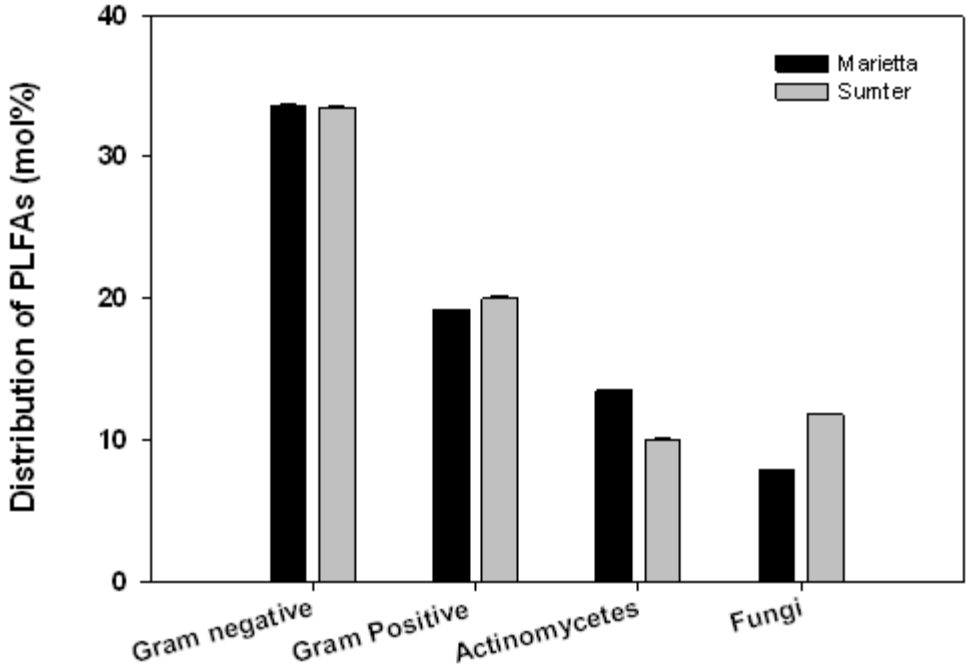

Figure 3.10 The mol% distribution of bacterial and fungal biomarker fatty acids in the moist controls (-0.03 MPa) of Marietta and Sumter soils.

Notes: Error bars represent the standard error with in the treatment (n=3).

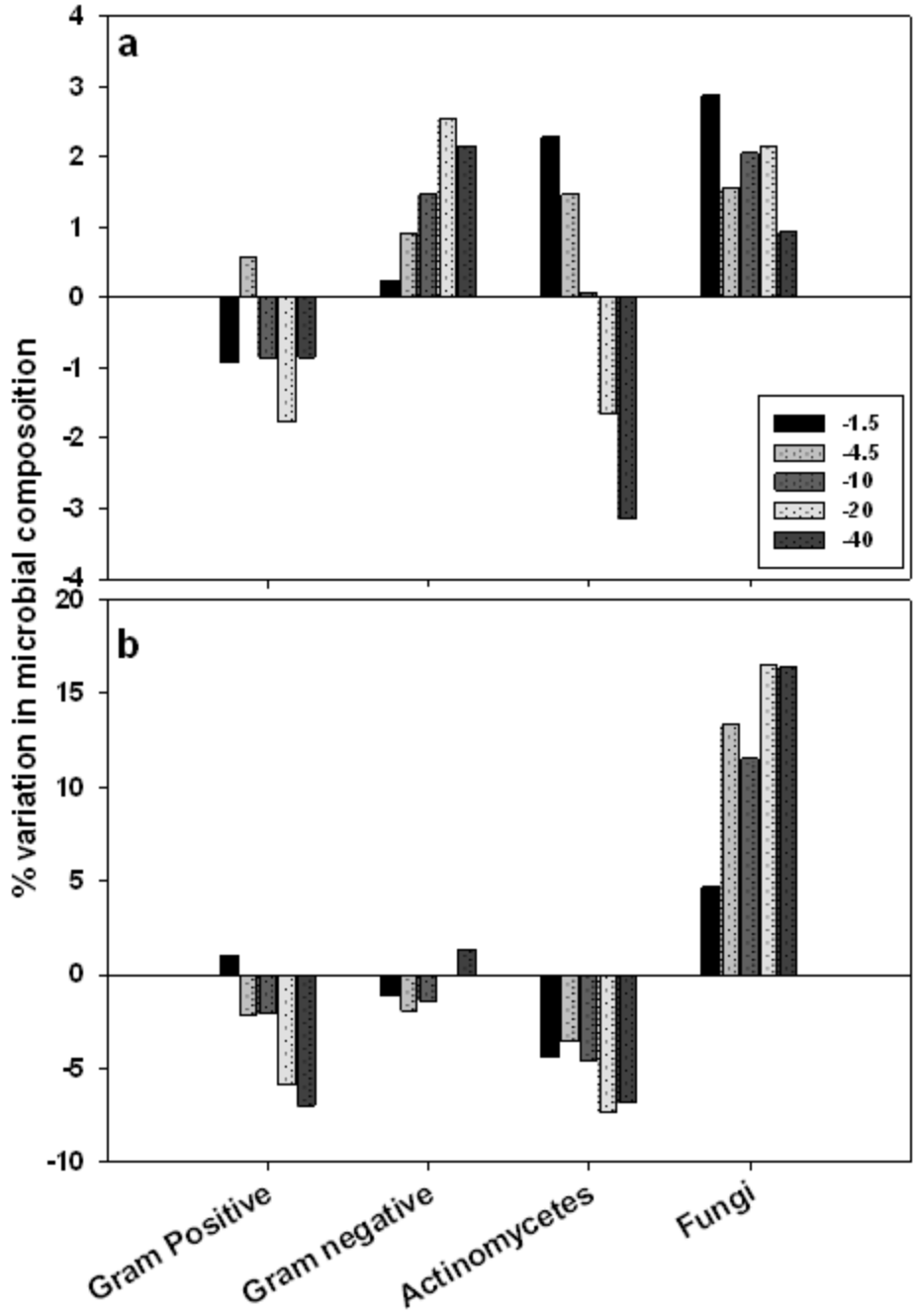

Figure 3.11 Percent variation in the mol% of PLFA biomarkers indicative of bacteria and fungi across the water stress gradient in the matric stress treatments in a) Marietta b) Sumter.

Notes: The bars below zero indicate decrease and above zero indicate the increase in the biomarkers at respective water potentials relative to moist control (-0.03 MPa).

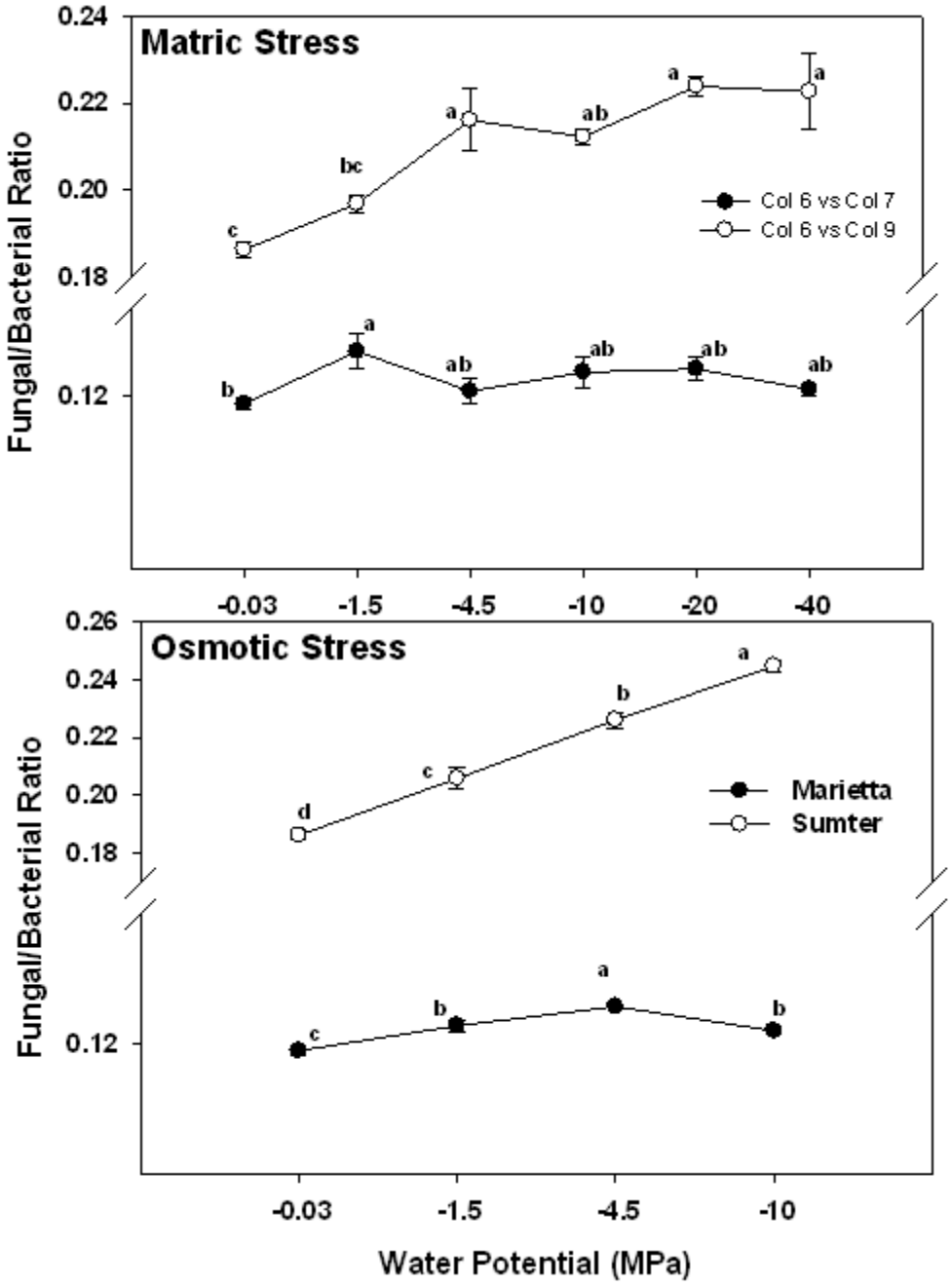

Figure 3.12 Changes in fungal to bacterial ratios at different water potentials induced by a) matric and b) osmotic Stress in Marietta and Sumter soils (n=3).

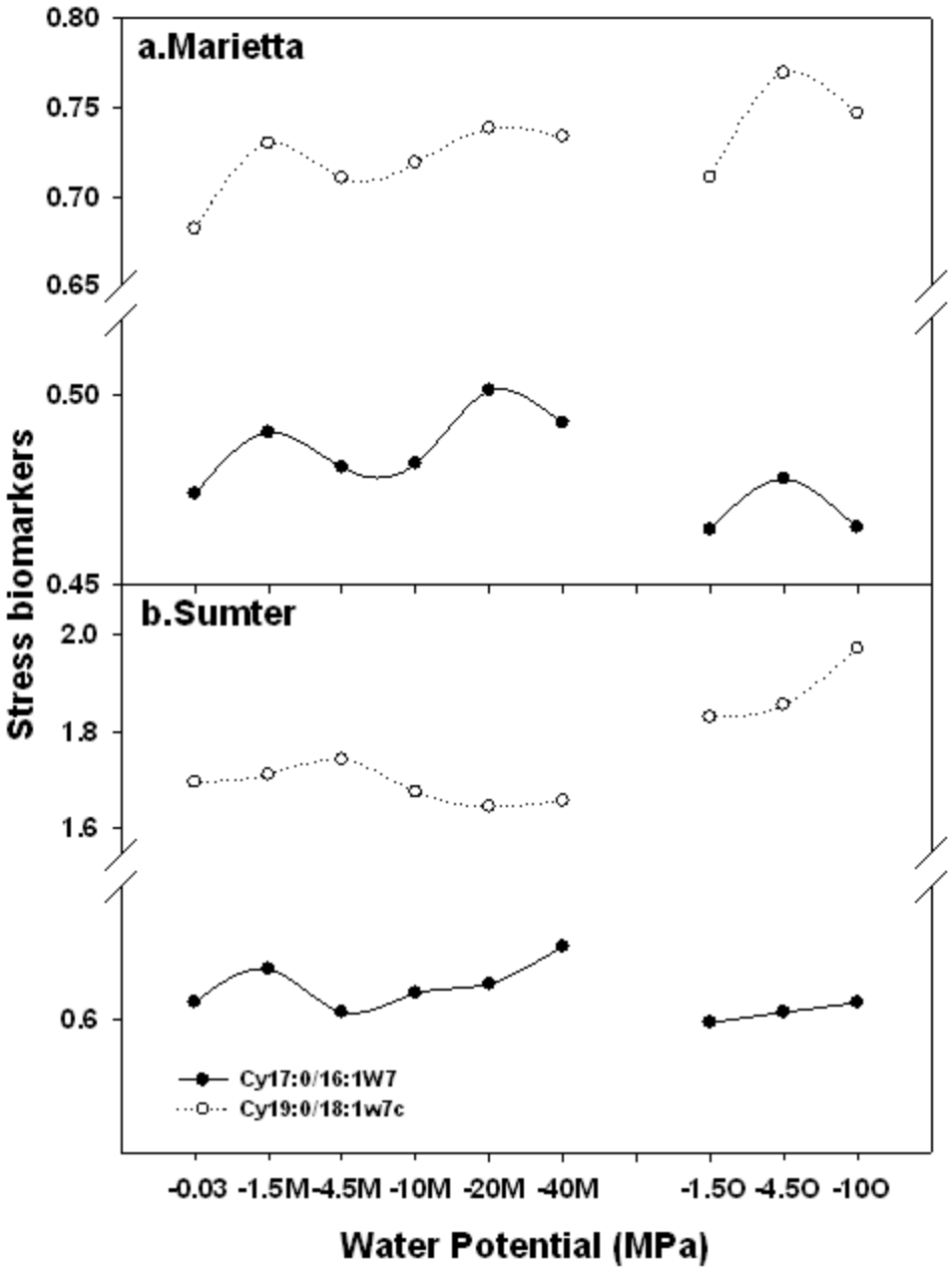

Figure 3.13 Changes in stress biomarkers (Cy17:0/16:1ω7 and Cy19:0/18:1ω7c) at different water potentials induced by matric (M) and osmotic (O) stress in a) Marietta and b) Sumter soils (n=3).

CHAPTER IV

EFFECT OF BIOTIC AND ABIOTIC SOIL ENVIRONMENTS IN THE SELECTION OF CULTIVABLE MICROBIAL COMMUNITIES

Abstract

Recent studies have demonstrated that cultivation of microorganisms in association with their native habitats promotes the growth of previously uncultured bacteria. We developed a new cultivation method where the soil bacteria were allowed to grow on regenerated cellulose filters (RCF) which in turn were tightly coupled with soil habitat. The objective of this study was thus to test if the new method supports the growth of previously uncultured bacteria and simultaneously assess the biotic (microbe-microbe) and abiotic soil effects on structure of growing microbial community. A polycarbonate membrane (pore size - 0.02 μm) was inserted between the RCF and the soil to prevent the movement of bacteria and larger organisms. Three treatments where inoculated RCF's were coupled to 1) unsterilized soil ($Biotic_{RCF}$) 2) autoclave sterilized soil with high nutrient content (Abiotic-HN_{RCF}) and 3) low nutrient soil extract amendment without soil (Abiotic-LN_{RCF}) were designed. The high and low nutrient treatments were included as alternative ways to compare the abiotic to the biotic effects. A more conventional cellulose congo red agar (CCRA) medium was also used for comparison. Following 20 days of incubation, the developing communities from all the treatments were characterized using 16S rDNA clone libraries. A total of 112 OTU's (D=0.03) were derived from approximately 341 clones. Nutrient levels had a small effect on the

cultivable communities, and as hypothesized the community growing on the living biotic soil ($Biotic_{RCF}$) was the most unique. Previously uncultured members of the phyla *Proteobacteria* and *Bacteroidetes* and few members of *Verrucomicrobia* were common on the $Biotic_{RCF}$ treatment. Diversity and richness were much greater in the $Biotic_{RCF}$ compared to Abiotic and CCRA treatments. For example, the Simpson's inverse diversity was 3 to 6 X greater in the unsterilized treatment (1/D = 33.6) compared to the other RCF and traditional plating methods (1/D ~ 5 to 10). Hence, the presence of a living and metabolizing microbial community appears to have an important impact on the cultivation of bacteria. This method shows considerable promise for cultivation and eventually the isolation of previously uncultured microorganisms and hints at the importance of microbial interactions that support bacterial community growth and development.

Keywords: 16S rRNA gene, cultivation, uncultured bacteria, biotic and abiotic effect on cultivation

Introduction

The overwhelming majority of microbial life in soil remains uncultivated (Skinner et al. 1952, Amann et al. 1995, Liesack et al. 1997, Hugenholtz et al. 1998, Torsvik et al. 2002, Leadbetter 2003). However several researchers have succeeded in cultivating novel bacterial types by modifying inoculum size, increasing cultivation time and altering nutrient status (Aagot et al. 2001, Zengler et al. 2002, Bruns et al. 2003, Davis et al. 2005, Stott et al. 2008). Low nutrient media has supported the growth of previously uncultured members in the former studies where as extended incubation periods and quorum sensing compounds in the media have supported their growth in the latter.

A few other studies have had success in cultivating and isolating novel bacteria by simulating natural environments (Kaeberlein et al. 2002, Ferrari et al. 2005). While the simulation of the biotic environment does not provide information on the specific microbial needs for growth, it does allow for an assessment of how microbial activities, including signaling and metabolite production, might be important for bacterial growth and the cultivation of soil microorganisms. Indeed, several studies recently emphasized how signaling molecules, presence of neighboring microbes, and cell-cell communication stimulate the growth of certain bacterial species (Bruns et al. 2003, Kato et al. 2005, Bollmann et al. 2007, Diggle et al. 2007, D'Onofrio et al. 2010) . The addition of a helper microbe and signaling molecules, for example, supported the growth of a previously uncultivated bacterium *Psychrobacter* sp. strain MSC33 (Nichols et al. 2008). Bruns et al., (2003) furthermore reported the growth of many previously uncultured bacteria by adding signaling compounds such as homoserine lactones and cAMP in the growth media. These studies point to the relevance of biotic habitat features for growing microorganisms.

Taking a broader view of community level microbial interactions, a study was designed to test the biotic and abiotic effects on bacterial growth during culture. Regenerated cellulose filters were selected as a supporting media to represent the most common substrate for microbial growth in soils (Mullings and Parish 1984, Ulrich and Wirth 1999). Numerous bacterial representatives have been cultured using cellulosic media, but it is expected that a large proportion of the cellulolytic community remains uncultured (Lynd et al. 2002, de Boer et al. 2005, Ulrich et al. 2008). A cellulose substrate should thus be useful for promoting the growth of previously uncultured bacteria.

A preliminary experiment was done to test the efficiency of in situ cultivation method in supporting the growth of previously uncultured bacteria. In this experiment, sterile cellulose filters were inoculated with soil inoculum and incubated for 20 days in close association with soil. Traditional cellulose Congo red agar medium was used as control. The DNA analysis of the microbial cultures from cellulose filters and CCRA plates after 20 days of incubation showed that ~50 % of the bacteria which were grown on the cellulose filters were matching to previously uncultured bacteria in RDP data base and it is less than 5% on CCRA plates (Data shown in appendix B). With the motivating results we obtained in the preliminary run, we designed an experiment with broader goal to understand the community interactions and substrate effect on the growing microbial communities.

The objective of this study was to determine whether, growing bacteria in association with their native soil habitat would increase the cultivability of previously uncultured members and diverse types of taxa. It was hypothesized that the soil provides a diverse suite of microorganisms and microbial-derived molecules that would support the growth of previously uncultured bacteria and thus greater levels of diversity and richness in bacterial community composition in the biotic than the abiotic conditions.

Materials and methods

Sample collection and residue incubation

Soil samples were collected from the A-horizon of the Marietta series located on the University farm adjacent to the Mississippi State University campus. The site was forested with >50-y old deciduous vegetation dominated by *Carya illinoinensis*. The Marietta series (Fine-loamy, siliceous, active, thermic Fluvaquentic Eutrudepts) is a deep

alluvial soil in the Blackland Prairie region of Mississippi. Organic matter content is close to 4% and the pH is neutral.

Large plant material and rocks were removed and the soil was passed through 5 mm sterile brass sieve. Approximately 2 grams of rice straw (*Oryza sativa*) was mixed thoroughly in 100 grams of soil and incubated at 25 ^{o}C for over 3 months. The water content of the soil was maintained at field capacity throughout the incubation period.

Preparation of inoculum and cultivation of microorganisms

One gram of decomposing rice straw residues along with the adhering soil particles were collected and dispersed into 100ml of 0.9% sterile NaCl solution. The aliquot was vortexed repeatedly for about 10 min and allowed to settle for 5 minutes which was considered as 10^{-2} dilution. A tenfold serial dilution (10^{-3}) was made further from 10^{-2} dilution which was used as inoculum in all the treatments. The 10^{-3} dilution was found to be ideal dilution in our preliminary tests, resulting in 50 to 200 colonies.

Regenerated cellulose filters (RCF; Sartorius, 18407-47-N) with pore size 0.2 μm were used as a support matrix and major carbon source for microbial growth. 0.l ml of the 10^{-3} soil suspension was mixed with 5 ml of sterile physiological saline solution (0.9% NaCl solution) and filtered onto regenerated cellulose filters using sterile polycarbonate filter holders (John Morris Scientific, Australia, 29550-44), There were 5 treatments with three replications: 1) Inoculated RCF on unsterile soil ($Biotic_{RCF}$), 2) Inoculated RCF on autoclave sterilized soil (Abiotic-HN_{RCF}), 3) Inoculated RCF without soil but amended with soluble soil organics (Abiotic-LN_{RCF}), 4) Uninoculated RCF (Non-$Innoc_{RCF}$) on unsterile soil and, 5) Traditional Cellulose Congo Red Agar Media (CCRA). Biotic and abiotic RCF treatments vary with one another in the presence and absence of

microbial activity respectively in the associated soil substrate (Table 1). Autoclave sterilization of soil results in the release of high concentrations of nutrients, and so to better assess the effects of the abiotic treatment and nutrients, a low nutrient treatment was also included.

Biotic$_{RCF}$ treatment: Unsterilized Marietta soil was used as the native habitat for microbial cultivation. Approximately 35 g of air dried soil was taken in a Petri plate (100 mm x 15 mm) and a small circular mound of approximately 42 mm wide and 10 mm high was raised in the center. A thin layer of soil was spread and placed in contact with the soil mound. Autoclaved water was added to the base of the soil mound to achieve a water potential of ~-0.03 MPa at the top of the mound. The soil mound was then covered with a sterile 47 mm diameter Polycarbonate membrane (PC; Sterilitech Corporation, PCT00347100) with pore size of 0.03 μm and lightly dripped with water to initiate contact with the underlying soil. The PC membrane prevented the migration of bacteria and larger organisms across the membrane but allowed for the diffusion of nutrients and other large molecules that may support microbial growth. Inoculated RCF were then placed on the sterile PC membrane and the moisture from the inoculants solution helped to initiate contact between the membranes (Figure 4.1). The cellulose in the RCF mimics the natural form of cellulose in plants and acts as a major carbon source for the growing bacteria.

Control treatment: Similar but uninoculated cellulose filters placed on the unsterile soil was used as control treatment to check for contamination through mites and other possible sources. After 20 days of incubation we could not find any conspicuous growth on the top of the cellulose filters however we find some light yellowish growth in the inter phase between PC membranes and cellulose filters. The RC filters of control

treatments were analyzed by DNA analysis and fatty acid analysis similar to other treatments.

Abiotic$_{RCF}$ treatments: Abiotic$_{RCF}$ treatments were designed to determine the effect of the microbe free soil environment on the composition of the microbial community growing on the associated RC filters. The Abiotic-HN$_{RCF}$ and Abiotic-LN$_{RCF}$ treatments vary with one another in the amount of available nutrients that support microbial growth. HN and LN represent the high nutrients and low nutrients, respectively.

The Abiotic-HN$_{RCF}$ treatment was created identically to that of Biotic$_{RCF}$ treatment but utilized sterile rather than unsterilized soil. Soil sterilization was achieved by autoclaving 3 times at 121 ^{o}C for 1 hour with 2 days of incubation between each autoclave event (Wolf and Skipper, 1994). The sterilization of soil was confirmed by plating the soil inoculum on LB plates and by measuring soil respiration over 3 days. In addition to sterilization, autoclaving of soil has resulted in a large flush of N and other nutrients into soil solution.

In order to check the inhibiting activities of the high nutrients and toxic compounds in the sterile soil a low nutrient treatment was devised (Abiotic-LN$_{RCF}$) but without soil, but amended with adding the sterile soil organics. The inoculated RCF membrane was placed in sterile Petri plates without soil but amended with 0.3µl of soluble organics from soil. Soluble organics were derived from autoclaving (121 ^{o}C for 1 h; at 100 kPa) 100-g of soil with 150 ml of tap water. The soil was allowed to settle overnight and the liquid was centrifuged at 3500x*g* for 10 minutes. The supernant was autoclaved again for 30 minutes and frozen at -20 ^{o}C until use. All the RCF treatments were maintained at optimum moisture over the incubation period of 20 days at 28 ^{o}C.

The $Biotic_{RCF}$, Abiotic-HN_{RCF} and Abiotic-LN_{RCF} treatments will be collectively called as RCF treatments in further chapters. Figure 4.2 indicates the Biotic, Abiotic and control treatments after 20 days of incubation.

CCRA: Cellulose Congo Red Agar (CCRA) medium has cellulose as the major carbon source, gelatin and Noble Agar as solidifying agents (Hendricks et al. 1995). It consists of 0.25g $MgCl_2$, 0.5g of K_2HPO_4, 1.88g of acid-washed Cellulose powder, 2g gelatin, 0.2g of Congo red, 5.0g of Nobel agar, 100 ml of soil extract and 900ml of tap water and autoclaved for 20 minutes at 121 ^oC. The plates were inoculated with same 10^{-3} dilution and incubated for 20 days at 28 ^oC.

Collection of microorganisms and DNA extraction

The microorganisms from cellulose filters were collected by method adapted from Ferrari et al., 2005 with some modifications. Briefly, the cellulose filters were carefully removed from Petri plates and cut into small pieces using sterile scissors. The pieces were placed in sterile 15 ml centrifuge tubes and added with 1.5ml of sterile physiological saline. To dislodge microorganisms from the filters the samples were vortexed vigorously for two minutes. The suspension were transferred to micro centrifuge tubes and centrifuged for 5 minutes at 15000 x g to pellet the microorganisms. The extraction and pelletizing process was repeated once again but using 1ml of sterile solution. DNA was extracted from that pellet using the MOBIO DNA isolation kit, as suggested by the supplier.

The collection of microbial colonies from the traditional cellulose agar plates was done using a plate wash technique (Stevenson et al. 2004) . The bacteria from the aggregate of colonies was obtained by flooding the surface of CCRA media with 2ml of

sterile physiological saline solution and dislodging the colonies using sterile glass spreader to get as many colonies as possible. The suspension was collected into micro centrifuge tubes and centrifuged for 5 minutes at 15000 x g to pellet the microorganisms. DNA was extracted from that pellet using the MOBIO DNA isolation kit, as suggested by the supplier. The extracted DNA was aliquoted into multiple tubes and stored at -80 ^{o}C until used for clone library preparation.

Preparation and analysis of 16S rRNA gene libraries

To obtain 16S rRNA gene products for cloning, the bulk microbial DNA recovered from the different treatments was amplified by 15-cycle PCR using the 27F forward and 1492R reverse primers. Cloning of target genes was done using the PCR2.1 vector from Invitrogen. Clones from each treatment were randomly picked and placed in separate 96 well plates i.e one library was made from each treatment. The clone libraries were then stored in freezing medium (LB agar with 10% glycerol added with 25mg/ml Ampicillin and 12.5mg/ml Kanamycin) and sent to the synthesis facility of the ARS, Stoneville MS, USA for sequencing. Prior to statistical analysis, the sequences were edited using Codon Code Aligner software and were checked for chimera using Mallard and Pintail programs. The chimera free sequences were then analyzed using LIBSHUFF and DOTUR software (Schloss and Handelsman 2005a). LIBSHUFF was used to determine whether two clone libraries were significantly different whereas assigning the sequences into different operational taxonomic units (OTU's; D=0.03) was done using DOTUR. DOTUR also calculates the values that are used to construct randomized rarefaction and collector's curves of observed OTUs, diversity indices and richness estimators (Schloss and Handelsman 2005b). The taxonomic assignment for the clones

was done using the online tools at the ribosomal Data project II at http://rdp.cme.msu.edu and at gene bank http://www.ncbi.nlm.nih.gov (GenBank accession numbers JF489234-JF489571).

Fatty acid methyl ester (FAME) analysis

The total microbial community composition of all the treatments was assessed by doing FAME analysis (Sasser 1990, Williams et al. 2010). Briefly, at the end of incubation period the filter papers were cut into small pieces and placed in 20 ml glass tube with PTFE cap. The FAME analysis was done in four steps. 1) Saponification: 1.25 ml of solution containing 3.75 M NaOH in aqueous methanol was added to the tube, vortexed and heated to 100 ^{o}C for 30 min for lysing the microbial cells and saponifying the fatty acids. 2) Methylation: fatty acid methyl esters were formed by adding 2 ml of HCL and methanol and heating to 80 ^{o}C. 3) Extraction: The fatty acid methyl esters (FAMEs) were extracted by adding 1.25 ml of MTBE and hexane (1:1) solution. 4) Base wash: The lower aqueous phase was discarded and the left over MTBE and hexane solution was washed with 3 ml of 0.3 M NaOH solution. The upper organic phase was collected in separate tubes and dried under ultra high purity nitrogen. The FAMEs were redissolved in 110 µl hexane and analyzed on Sherlock MIDI GC. Fatty acid methyl esters were separated and detected by an Agilent 6890 Series gas chromatograph (Santa Clara, CA) equipped with a flame ionization detector, an Ultra-2 column (19091B-102;0.2 mm by 25 m), and controlled by a computer loaded with ChemStation and Sherlock software. The carrier gas included UHP H_2 at a column head pressure of 20 kPa, septum purge of 5 ml min^{-1}, a split ratio of 40:1, injection temperature of 300 ^{o}C, injection volume of 2 µl, and a column temperature that ramps from 170 ^{o}C to 288 ^{o}C at

$28^{o}C\ min^{-1}$. Peak identification was carried out by the Microbial Identification System (MIDI, Inc.) following calibration with a standard mixture of 17 fatty acid methyl esters (1300A calibration mix). The upper organic phase was transferred to a test tube and evaporated under 99.999% UHP N_2 gas. Standard nomenclature is used to describe fatty acids. Microbial Biomass carbon was calculated based on total amount of FAMEs extracted from each treatment (Haack et al. 1994).

Statistical analysis

Mol % of the 47 dominant Fatty Acid Methyl Esters and the relative abundance of 38 most common OTU were analyzed using PCord software (MJM Software, Gleneden Beach, OR). Non-metric multidimensional scaling (NMS), a non-parametric method was used to provide graphical ordination of FAMEs and OTU. The fatty acids i15:0, a15:0, 15:0, i16:0, 16:0, 16:1ω7, i17:0, a17:0, cy17:0, 18:1ω7, 18:0 were chosen as bacterial fatty acid biomarkers and 18:2ω6 was used as fungal biomarker. The fungal to bacterial ratio was represented by the ratio of 18:2ω6 to bacterial PLFAs. The relative abundance of bacterial and fungal fatty acids was expressed as percentage of the total fatty acid methyl esters.

Results

Phylogenetic assignment of sequences

Bacterial communities were characterized using 16S rRNA gene analysis. The negative control known as the $Non\text{-}Innoc_{RCF}$, as expected, showed the lowest degree of richness and diversity, with ~60% of the clones most closely related to *Cellvibrio fulvus* (EF692635.1). *Rhizobium spp*. made up the remainder of the identified sequences (data not shown). Because the bacterial communities that grew on the Non-Innoc RCF were

very simple in structure and clearly different from those of the inoculated systems, the focus of the reported data in the tables and figures will be on the four inoculated systems.

Of the total 384 clones sequenced in the four treatments, 341 sequences remained following chimera check. The $Biotic_{RCF}$ and CCRA treatments were dominated by bacteria belonging to phyla *Proteobacteria* and *Bacteroidetes*, whereas members of *Firmicutes* were predominant in Abiotic-HN_{RCF} and Abiotic-LN_{RCF} (Table 3.2). However, representatives of 8 different phyla, including members of rarely cultivated groups like *Planctomycetes, Acidobacteria* and *Verrucomicrobia, OP10,* and *unclassified bacteria* were detected in RCF treatments. All the clones from the CCRA treatment were distributed among three phyla, the *Proteobacteria, Bacteroidetes*, and *Firmicutes* (Table 4.2). *Proteobacteria* (58%) was the dominant group in $Biotic_{RCF}$ and conventional CCRA plating media where as *Firmicutes* was the dominant phylum in Abiotic-HN_{RCF} and Abiotic-LN_{RCF} treatments. Interestingly, members of the rarely cultivable group *Planctomycetes* occupied up to 13% of the total clones in Abiotic-HN_{RCF} treatment. Nonetheless, the differences among the treatments were more pronounced at finer levels of taxonomic resolution. For instance the members of *Proteobacteria* in $Biotic_{RCF}$ treatment were relatively evenly distributed into 9 different orders whereas such even distribution into wide range of taxonomic groups was not obvious in the other treatments (Table 4.2).

LIBSHUFF analysis

The community composition of the bacteria growing on regenerated cellulose filters was significantly different from that of traditional plates (LIBSHUFF). A significant difference was also noticed between the microbial communities growing on

Biotic$_{RCF}$ treatment to that of microbial communities grown on Abiotic-HN$_{RCF}$ and Abiotic-LN$_{RCF}$ treatments. However, the difference between bacterial communities growing on Abiotic-HN$_{RCF}$ and Abiotic-LN$_{RCF}$ treatments was insignificant.

Diversity indices and community composition

To assess bacterial diversity, clones were placed into OTU'S with <0.03 evolutionary distance (Table 4.3) using DOTUR software. The number of OTU's obtained was greater in the RCF treatments compared to CCRA. For instance, in Biotic$_{RCF}$ treatment a total of 77 sequences were placed into 41 OTU's whereas in CCRA 91 sequences were distributed into 14 OTU's. The diversity indices showed that the bacterial community richness and evenness was considerably greater in the RCF compared to CCRA media. Among the three RCF treatments the Simpson reciprocal and Shannon index were greater in Biotic$_{RCF}$ followed by Abiotic-HN$_{RCF}$, and Abiotic-LN$_{RCF}$ respectively. It is also clear that evenness was considerably higher in the Biotic$_{RCF}$ compared to the other treatments. The rarefaction analysis supported the findings of the indices (Figure 4.3). The 5 most abundant OTU'S accounted for 72% of the clones in the CCRA treatment whereas they accounted for only 2% in Biotic$_{RCF}$ (Table 4.4). Although there was some overlap in the 95% confidence interval of the Chao1 estimator between RCF treatments, no overlap is observed between CCRA and RCF.

The compositional and structural distribution of the bacterial community cultures within the treatments reflected the results of Libshuff and diversity indices. The community composition of the Biotic$_{RCF}$ was very different from the abiotic treatments. However, the Abiotic-HN$_{RCF}$ and Abiotic-LN$_{RCF}$ treatments were structurally similar (Table 4.4; Figure 4.4a). In this regard, clones most closely related to *Bacillus*

megaterium were strongly dominant members of both the Abiotic-HN_{RCF} and Abiotic-$LN_{RCF.}$ This dominance played a huge role in the patterns that developed in the NMS analysis (Figure 4.4a). In contrast, such strong domination by one two bacterial species was not found in the $Biotic_{RCF}$ treatment (Table 4.4). Indeed, the distribution of taxa over a wide range of taxonomic groups explains the high evenness in $Biotic_{RCF}$ treatment.

Growth of previously uncultured bacteria

The bacterial community composition of the treatments was unevenly distributed among different phyla (Table 4.4). A search for similar sequences with RDP revealed that the majority of clones were closely related to environmental DNA instead of bacterial isolates. Hence the taxa we have grown are most closely related to previously uncultivated bacterial taxa. However the majority of taxa associated with $Biotic_{RCF}$ treatment showed low (90-96%) sequence homology to previously cultured bacteria. The Abiotic-HN_{RCF} and Abiotic-LN_{RCF} treatments shared great similarity in community composition. More than 80% of their sequences were shared in 10 OTU's (Table 4.4). *Bacillus megaterium* is the bacteria, for instance, dominated both the culture systems. The high similarity between the communities on these 2 treatments favors the idea that abiotic soil factors such as nutrient availability across the concentrations tested played a small role in the selection of the bacterial communities.

Fatty Acid Methyl Ester analysis

The FAME profiles of the microbial communities growing on four main treatments indicated that the largest differences were related to the growing conditions associated with the RC filters and the CCRA plates. The Abiotic-HN_{RCF} treatment has higher total FAMEs followed by $Biotic_{RCF}$, CCRA and Abiotic-LN_{RCF} treatments (Table

4.5). Relative abundance of fungal fatty acids was significantly greater in the $Biotic_{RCF}$ and Abiotic-HN_{RCF} treatments than the Abiotic-LN_{RCF} and CCRA treatments. Presence of more fungal biomass in the Abiotic-HN_{RCF} treatment likely resulted in greater total FAMEs. The amount of microbial biomass in each treatment was related to the amount of available nutrients in the respective media (Table 4.6). Clustering of $Biotic_{RCF}$ and Abiotic-HN_{RCF} treatments in the NMS plot suggests that soil might promoted the accumulation of similar fatty acids. When NMS ordination was ran on individual fatty acids, 18:2ω 6, 9 and 18:1 ω9c fungal fatty acids were highly positively correlated to the $Biotic_{RCF}$ and Abiotic-HN_{RCF} treatments on axis 1 (Figure 4.4b; Table 5; $r>0.75$). FAME analysis of $Control_{RCF}$ treatment has shown the presence of fatty acids, of which 60% are short chain fatty acids. However, the rest 40% was dominated by bacterial biomarkers like 16:0, 16 ω 7c and 18:1 ω7c gave evidence of bacterial growth on the cellulose filters in $Control_{RCF}$ treatment.

Discussion

The fastidious nature and close ties to the biotic conditions of their native soil habitat may explain why there has previously been success in isolating some of the previously uncultivated bacteria when cultured under in situ conditions (Kaeberlein et al. 2002, Bollmann et al. 2007, Ferrari et al. 2008). Intra- and inter specific interactions among microbial populations influence microbial growth, which in turn can have direct consequence for the culturability of microorganisms. The present study was conducted to test the importance of the soil habitat for cultivating soil derived bacteria, in particular the effect of a co-occurring microbial community for the cultivation of soil microorganisms. Microorganisms associated with decomposing rice (Oryza sativa) straw residues were

inoculated on to the regenerated cellulose filters (Figure 4.1 & 4.2) and traditional cellulose media plates (CCRA). The inoculated regenerated cellulose filters were placed in close association with 1) unsterilized soil (BioticRCF) 2) sterilized soil (Abiotic-HNRCF) and 3) a soil solution amendment but no soil treatment (Abiotic-LNRCF). The Abiotic-HNRCF and Abiotic-LNRCF treatments differ based on the pool of available nutrients that can support microbial growth, and thus represent high and low nutrient treatments, respectively. These two treatments provided different ways to test the effect of the soil-abiotic environment and how strongly it contrasted with the community that developed when grown in association with the living biotic soil community.

Effect of microbe rich habitat on bacterial cultivation

Simulating the native habitat (Kaeberlein et al. 2002) and using soil as substrate (Svenning et al. 2003, Ferrari et al. 2005) has been shown earlier to be important for growing previously uncultivated microorganisms, however, this approach, as far as we know, has not been used to differentiate biotic and abiotic effects of the native soil habitat on bacterial cultivation. Considering the enormous heterogeneity in chemical and physical features that describe soil habitats at microbial scales, and the enormous possibilities for biological interaction, it seemed likely that some microorganisms that are fastidiously dependent upon their environment for survival and growth would benefit from growth in close association with their native habitat. It was not possible to pinpoint the exact mechanisms of influence; however the biotic bacterial community was clearly very different from those found in the abiotic environment.

In $Biotic_{RCF}$ treatment presence of the living microbial community, though separated by ~100 μm strongly impacted the rRNA based composition, richness, and

diversity of the cultivable community on cellulose filters compared to sterile abiotic conditions. These results support the hypothesis that the biotic soil community provides important conditions that aid in the growth /cultivation of a larger array of bacterial types. Our results also confirm few other studies that have reported greater diversity and richness of microbial types when cultured in a simulated natural environment (Kaeberlein et al. 2002, Ferrari et al. 2005, Bollmann et al. 2007). Similarly, the results presented here have also similarly reported the growth of previously uncultured members within *Proteobacteria* and *Bacteroidetes* in simulated natural environments. In contrast, it is interesting to note the different impact of the growth conditions on the fatty acid composition of the microbial communities between the cultivation treatments. The bulk of the differences can be attributed to fungal growth on the plates. Moreover, the growth of fungi was much greater in the Abiotic-HN_{RCF} compared to the Abiotic-LN_{RCF} treatment, and yet apparently had little effect on bacterial community composition. Spatial separation on the plates may have played a role in allowing the fungi to grow without showing any obvious influence on bacterial community composition.

The exact role that biotic activity in $Biotic_{RCF}$ treatment played on the cultivation of the bacteria growing on the RCF cannot be known. However, the effect is supportive of the impact that auto inducer (e.g. quorum sensing) molecules have on bacterial growth (Kaeberlein et al. 2002, Bruns et al. 2003, Williams et al. 2007). In soil, acyl homoserine lactones were detected in 24% of isolates recovered from soil bacterial communities, suggesting that a number of organisms in soil utilize AHL for communication (DeAngelis et al. 2008). The recent discovery of bacterial nanowires that function as long distance pathways (microns) of electron transfer cannot yet be invoked to explain communication between soil micro biota and bacteria growing on cellulose filters ~100m away, however,

the nanowire concept is supportive of the importance that microbial interactions and communication have on microbial growth (Reguera et al. 2005).

The cultivation of bacteria in association with a living soil community favored the growth of bacterial phyla that have only a few cultured representatives. At 97% evolutionary distance, approximately 40% of the bacterial taxa from $Biotic_{RCF}$ treatment and 91% in CCRA treatment were most similar to previously cultured bacterial isolates documented in Gene bank. As such, the majority of taxonomic units in the $Biotic_{RCF}$ were most related to uncultivated environmental sequences. This result is notable for the high degree of novelty in the growth of rarely cultivable bacteria associated with a simulated biotic environment, but also from the standpoint that rather common cultivation methods such as the CCRA may not have been fully probed for their diversity of bacterial types.

The even distribution of different taxa indicates that the $Biotic_{RCF}$ habitat, in contrast to most other cultivation habitats, did not strongly select for specific microbial groups. Members of hard to culture phyla like *Verrucomicrobia* and *OP10* were fairly well represented in $Biotic_{RCF}$. However, other approaches to cultivation of previously uncultured biota from soil, such as diluting the nutrient content of growth media and especially the extension of incubation times have also successfully grown bacteria considered cultivation-resistant, such as *Acidobacteria, Actinobacteria, Verrucomicrobia* (Janssen et al. 2002, Janssen 2003). It has been speculated that the cultivation of *Verrucomicrobia* may be enhanced when the abundance of other microbes in culture are low (Sangwan et al. 2005). In this regard, the lack of a dominant bacterial group associated with the BioticRCF treatment would have lowered the likelihood of a strong antagonist taking over the culture and thus increases the potential for the growth of many other bacteria taxa.

It has been observed that *Verrucomicrobia* have been found in a wide array of environments and that their activity and abundance in nature may be partially linked to available water resources (Buckley and Schmidt 2001, Tarlera et al. 2008). The abundance of *Verrucomicrobia* in soil can range spectacularly, from 0 to 21% of the Division level census, so it is clear that these organisms are sensitive to environmental and habitat conditions. From this study it is difficult to speculate the conditions that allow for growth of previously uncultivated bacteria in culture. However, the proximity of other microorganisms in the associated soil habitat next to the culture in $Biotic_{RCF}$ may have allowed for the diffusion and exchange of important metabolites for their growth (Greene 2002, Bollmann et al. 2007).

Selection of bacterial community in abiotic environment under different nutrient status

In the case of the $Abiotic_{RCF}$ treatments, it was suspected that the nutrient availability would have strongly influenced the composition of the cultivable community. Autoclaving the soil resulted in the release of a flush of soluble organics and nutrients into the soil solution. Even though the Abiotic-LN_{RCF} treatment received only a portion of the soil solution and its associated nutrients compared to what would be found in the Abiotic-HN_{RCF} treatment, the bacterial community members residing in both $Abiotic_{RCF}$ systems were very similar. This would suggest that nutrient availability across the range utilized in the experiment had little impact on the composition of the cultivated bacterial communities.

The most obvious resemblance between the high and low nutrient treatments comes from the observation that ~ 50% of clones showed high sequence similarity(>99%) to *Bacillus megaterium. Bacillus megaterium* is a fairly well described

bacterium with a large genome and wide industrial utilization (Vary et al. 2007). While it is not know whether the specific clones that we have identified have novel ecological roles or metabolic capacities compared to those strains previously isolated, there are numerous methods already available for the cultivation of *Bacillus megaterium*. Nevertheless, it is an intriguing outcome that *Bacillus megaterium* was able to dominate growth in the presence of RCF-cellulose in Abiotic treatments but that this growth was completely muted when its RCF-habitat was associated with a soil containing an active microbial community.

In spite of having greater dominance of *Bacillus megaterium* related clones, the high nutrient media (Abiotic-$HN_{RCF)}$ was represented by ~13% of clones most closely related to taxa in *Planctomycetes*. *Planctomycetes* are a group with sparse representation in culture and that have an array of unusual traits that include the production of rare fatty acids and the lack of peptidoglycan in the cell wall (Wagner and Horn 2006). *Planctomycetes* are typically rare, but are widespread inhabitants in numerous soil and aquatic environments (Bauld and Staley 1976, Stackebrandt et al. 1993). Recently, however, it has been shown that they often dominate the intestinal tracts of various animals, especially termites (Kohler et al. 2008). Termite hindguts are zones that receive periodic influxes of nutrients and cellulose rich organic matter. This habitat may be mimicked by the Abiotic-HN_{RCF} habitats. However, it must be acknowledged that the exact phylogenetic relationship between the clone groups in our work and those of Tholen and Brune (Tholen and Brune 2000) are not known.

Regenerated cellulose filter method vs. traditional plating media

Bacterial communities cultivated on cellulose filters (RCF) were clearly very different from those growing on traditional carboxycellulose in agar (CCRA). The CCRA media resulted predominantly in the growth of fairly well described members of the *Proteobacteria, Bacteroidetes*, and *Firmicutes* which were earlier reported as cellulose degrading bacteria (Lynd and Zhang 2002, Yang and Zhang 2007, Danon et al. 2008, Ulrich et al. 2008, Schellenberger et al. 2010). These results agreed well with other studies that traditional plating methods are good at selecting certain bacterial groups redundantly (Bockelmanna et al. 2000, Kopke et al. 2005, Bollmann et al. 2007) instead of recovering the novel members of the bacterial taxa.

From the standpoint of ubiquity, cellulose is a good source of C that dominates soil ecosystems and can thus be rationalized as a strong selective force in shaping the evolution of microbial catabolism across numerous taxonomic groups. It would thus be interesting to know how many of the bacteria in our experiments are capable of decomposing cellulose. In contrast to the obvious clearing zones that are indicative of cellulase activity when using CCRA, the observation of cellulose degradation using RCF is less easily attributed to a single colony. It is also difficult to ascertain whether the cellulose decomposition is related to bacterial rather than fungal activity. However, when we tried to isolate and regrow the colonies from regenerated cellulose filters on low nutrient soil extract media, bacterial growth was noticed on approximately 70% of the plates. When some of these bacteria were transferred to CCRA media ~50% of them formed clearing zones indicating that the capability to degrade cellulose was common among RCF community members (Hendricks et al. 1995, Ulrich et al. 2008). The capacity to regrow many of the RCF-cultivated bacteria on CCRA also indicates that

once these organisms are coaxed into culture they may then be grown in isolation using standard methods and thus further characterized for their ecological relevance and metabolic functioning.

Impacts of contamination on bacterial cultivation

Despite the utilization of highly sterile and clean techniques, evidence of bacterial invasion onto cultures was observed on non-inoculated RCF. It is thought that bacterial movement from the soil and onto the surface of the un-inoculated RCF may have occurred via aerial transport or through the movement of mites around the 0.003 micron polycarbonate filter.. Nevertheless, while there was evidence that his occurred, the result is primarily interesting and does not detrimentally impact the conclusions in this study. In fact, each cultivation system, with the exception of the nutrient level experiments, selected for very different bacterial communities. Hence, even with the potential for unintended bacterial inoculation, the cultivation systems selected for unique communities unlike the bacterial contaminants that were most closely related to genera *Cellvibrio* and *Rhizobium*.

Conclusion

In conclusion, it has been shown that the biotic soil environment provides congenial conditions that support the growth of a unique and diverse bacterial community containing numerous previously uncultivated bacterial groups. While the novel methods that we attempted grew a number of previously uncultivated bacterial taxa, it was also noted that the traditional CCRA technique has the potential to provide some previously uncultivated taxa. The huge variations we find in the microbial communities grown on different treatments suggests the sensitiveness of microbes to the biotic and abiotic

factors in the growth media. Indeed, the likely impact of cultivating bacteria associated with a living soil environment speaks to the power of microbial interactions for shaping microbial communities and provides new insights into cultivating previously difficult to cultivate bacteria.

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Table 4.1 Description of treatments

Treatment	Media	Growth support	Microbial activity in associated soil	Available nutrients
$Biotic_{RCF}$[†]	Unsterilized soil	RCF‡	Present	Low
Abiotic-HN_{RCF}[§]	Sterilized soil	RCF	Undetectable	High
Abiotic-LN_{RCF}[¶]	Sterile soil organics	RCF	Undetectable	Low
No-$Innoc_{RCF}$[#]	Unsterilized soil	RCF	Present	Low
CCRA[††]	CCRA	CCRA	N/A[‡‡]	N/A

[†] $Biotic_{RCF}$ - Inoculated Regenerated Cellulose Filter on unsterile soil.
[‡] RCF- Regenerated Cellulose Filter.
[§] Abiotic-HN_{RCF} - Inoculated Regenerated Cellulose Filter on sterile soil.
[¶] Abiotic-LN_{RCF} - Inoculated Regenerated Cellulose Filter amended with sterile soil extract.
[#] No-$Innoc_{RCF}$ - Uninoculated Regenerated Cellulose Filter on unsterile soils.
[††] CCRA - Cellulose Congo Red Agar.
[‡‡] N/A - Not Applicable.

Table 4.2 Phylogenetic distribution of sequences among different phyla.[†]

Phylum	Class	Order	$Biotic_{RCF}$	Abiotic-HN_{RCF}	Abiotic-LN_{RCF}	CCRA
Acidobacteria			**1**	**1**	**0**	**0**
	Acidobacteria		*1*	*1*	*0*	*0*
		Acidobacteriales	1	1	0	0
Verrucomicrobia			**3**	**1**	**1**	**0**
	Verrucomicrobiae		*3*	*1*	*1*	*0*
		Verrucomicrobiales	3	1	1	0
Proteobacteria			**43**	**18**	**26**	**82**
	Deltaproteobacteria		*4*	*1*	*1*	*0*
		Myxococcales	4	1	1	0
	Gammaproteobacteria		*18*	*2*	*0*	*23*
		Altermonadales	8	0	0	0
		Legionellales	2	1	0	0
		Xanthomonadales	4	1	0	4
		Pseudomonadales	0	0	0	19
		Unclassified Gammaproteobacteria	4	0	0	0
	Betaproteobacteria		*3*	*9*	*10*	*57*
		Burkholderiales	3	8	10	57
		Unclassified Betaproteobacteria	0	1	0	0
	Alphaproteobacteria		*18*	*6*	*13*	*2*
		Sphingimonadales	6	1	2	1
		Caulobacterales	2	1	1	0
		Rhizobiales	7	3	6	1
		Rickettsiales	0	0	2	0
		Unclassified Alphaproteobacteria	3	1	2	0
	Unclassified Proteobacteria		*0*	*0*	*2*	*0*
Firmicutes			**1**	**54**	**39**	**2**
	Bacilli		*1*	*54*	*39*	*2*
		Bacillales	1	54	39	2
Planctomycetes			**2**	**12**	**7**	**0**
	Planctomycetacia		*2*	*12*	*7*	*0*
		Planctomycetales	2	12	7	0
Bacteroidetes			**23**	**3**	**10**	**7**
	Flavobacteria		*0*	*0*	*0*	*1*
		Flavobacteriales	0	0	0	1
	Sphingobacteria		*22*	*3*	*10*	*6*
		Sphingobacteriales	22	3	10	6
	Unclassified Bacteroidetes		*1*	*0*	*0*	*0*
OP10			**2**	**0**	**0**	**0**
unclassified_Bacteria			**2**	**0**	**1**	**0**

[†] The distribution of sequences (based on 16s rRNA gene analysis) in different treatments when matched to RDP (Ribisomal Database Project) database. Each number indicates the number of clones in respective treatment matched to respective Phylum, class or order.

Table 4.3 Diversity indices at 0.03% evolutionary distance.[†]

Diversity Indices	$Biotic_{RCF}$	$Abiotic\text{-}HN_{RCF}$	$Abiotic\text{-}LN_{RCF}$	CCRA
No. of Clones	77	89	84	91
No. of OTU's	44	41	39	14
Simpson (1/D)[‡]	33.63	9.25	10.53	5.03
Shannon (H)	3.49	3.01	3.02	1.94
Evenness(H/Hmax)	0.92	0.81	0.82	0.73
Chao 1	102.12	113.5	82.63	16.5
95% COI	66.65-193.12	67.93-236.21	55.41-156.26	14.36-30.98

[†] Calculations were based on OTU's formed using DOTUR at an evolutionary distance of <0.03.
[‡] Simpson (1/D) – Simpson Reciprocal Index.

Table 4.4 Distribution and phylogenetic affiliation of the most abundant OTU's in Genbank.

No.of OTU's	$Biotic_{RCF}$	$Abiotic\text{-}HN_{RCF}$	$Abiotic\text{-}LN_{RCF}$	CCRA	Phyla	Closest Match[†]	Closest Cultured organism[‡]
52		28	24		*Firmicutes*	*Bacillus megaterium*; AceR-2; FJ605385 (99.2)[§]	*Bacillus megaterium*; AceR-2; FJ605385 (99.2)
42		4	5	33	*Proteobacteria*	*Achromobacter insolitus* (T); LMG 6003; AY170847(99)	*Achromobacter insolitus* (T); LMG 6003; AY170847(99)
20	1	1	2	16	*Proteobacteria*	*Ralstonia sp.* MCT1; DQ232889 (98.7)	*Ralstonia sp.* MCT1; DQ232889 (98.7)
17				17	*Proteobacteria*	*Pseudomonas sp*. MG1; AF326378 (99.3)	*Pseudomonas sp*. MG1; AF326378 (99.3)
14		5	9		*Firmicutes*	*Bacillus megaterium*; PC IW 13; AM992177 (99.9)	*Bacillus megaterium*; PC IW 13; AM992177 (99.9)
10	2	7	1		*Planctomycetes*	*uncultured bacterium;* OTUc55; EU834799 (98.5)	*Gemmata-like str*. CJuql4; AF239693 (90.3)
8	8				*Proteobacteria*	*uncultured bacterium;* nbw778f06c1; GQ009721(97.3)	*Saccharophagus degradans* 2-40; CP000282 (90.9)
7	5	2			*Bacteroidetes*	*Chitinophaga soli;* Gsoil 219; AB267723 (95.7)	*Chitinophaga soli;* Gsoil 219; AB267723 (95.7)
7	7				*Bacteroidetes*	*uncultured bacterium*; 28RHF48; AJ863367(96.8)	*Terrimonas lutea (T); DY*; AB192292 (95.2)
6		5	1		*Firmicutes*	*Paenibacillus pocheonensis;* Gsoil 1138; AB245386(99.5)	*Paenibacillus pocheonensis;* Gsoil 1138; AB245386(99.5)
6		2	1	3	*Proteobacteria*	*Cupriavidus sp*. A2; EU363682(99.2)	*Cupriavidus sp*. A2; EU363682(99.2)
6	6				*Bacteroidetes*	*uncultured bacterium*; WC2_183; GQ263931(99.3)	*Niastella sp. Gsoil* 221; GQ339899 (96.5)
6				6	*Bacteroidetes*	*uncultured bacterium;* FW1_a34; GQ263287(98.2)	Bacteroidetes bacterium CK32 5.3; FJ688408(98.2)
5	1			4	*Proteobacteria*	*uncultured bacterium;* S1-3-CL17; AY725259(99)	*Pseudoxanthomonas sp.* RN402; FJ032195(98.9)

Table 4.4 Continued

No.of OTU's	$Biotic_{RCF}$	Abiotic-HN_{RCF}	Abiotic-LN_{RCF}	CCRA	Phyla	Closest Match†	Closest Cultured organism‡
4		1	2	1	*Proteobacteria*	Rhizobium sp. Cg-A3; AB456621(99.3)	*Rhizobium sp*. Cg-A3; AB456621(99.3)
4		2	2		*Firmicutes*	*Bacillus megaterium*; ZFJ-14; EU931553 (99.4)	*Bacillus megaterium*; ZFJ-14; EU931553 (99.4)
4			4		*Bacteroidetes*	*Chitinophaga sp.* 37C1; GQ281771(96.5)	*Chitinophaga sp*. 37C1; GQ281771(96.5)
3		3			*Firmicutes*	*Bacillus muralis*; REG126; GQ844961(99.4)	*Bacillus muralis*; REG126; GQ844961(99.4)
3		2	1		*Planctomycetes*	uncultured bacterium; p26m12ok; FJ478560 (98.5)	*Planctomyces sp*.; Schlesner 658; X81954(94.8)
3		1		2	*Firmicutes*	*Bacillus sp.* P05; AY822613(98.9)	*Bacillus sp*. P05; AY822613(98.9)
3	2		1		*Proteobacteria*	*Caulobacter sp.;* FWC33; AJ227772(97.6)	*Caulobacter sp.;* FWC33; AJ227772(97.6)
3	3				*Proteobacteria*	uncultured bacterium; UWL_CL-080514_OTU-34; EU809244(98.6)	Rhodospirillaceae bacterium KNA-P; AB539973(98.9)
3			2	1	*Proteobacteria*	*Cupriavidus campinensis*; LMG 20576; AY040355(99.7)	*Cupriavidus campinensis*; LMG 20576; AY040355(99.7)
2		2			*Planctomycetes*	*uncultured bacterium*; 1-2D; EU289425(92.6)	Nostocoida limicola III; Ben223; AF244750(92.4)
2	1	1			*Proteobacteria*	*Nordella oligomobilis*; N21; AF370880(98.6)	*Nordella oligomobilis*; N21; AF370880(98.6)
2	1	1			*Firmicutes*	*Bacillus thuringiensis;* DQ286358(99.5)	*Bacillus thuringiensis*; DQ286358 (99.5)
2	2				*Verrucomicrobia*	*uncultured Opitutales bacterium*; B15-Capima; AB479055(97.3)	Opitutaceae bacterium TAV1; AY587231(94.1)
2	2				*Proteobacteria*	*Legionella quinlivanii*; sreogroup 2, nctc 12433; Z49733(98.3)	*Legionella quinlivanii*; sreogroup 2, nctc 12433; Z49733(98.3)

Table 4.4 continued

No.of OTU's	Biotic$_{RCF}$	Abiotic-HN$_{RCF}$	Abiotic-LN$_{RCF}$	CCRA	Phyla	Closest Match†	Closest Cultured organism‡
2	2				*unclassified_Bacteria*	uncultured bacterium; FFCH4309; EU134282 (98.3)	Vampirovibrio chlorellavorus; ICPB 3707; HM038000(93.4)
2	2				*Proteobacteria*	*Pseudoxanthomonas sp*. D7-5; AM403203(99.5)	Pseudoxanthomonas sp. D7-5; AM403203(99.5)
2	2				*Proteobacteria*	*Sphingomonas mali* (T); IFO 10550-T; Y09638(98)	*Sphingomonas mali* (T); IFO 10550-T; Y09638(98)
2			2		*Bacteroidetes*	*Sphingoterrabacterium composti*; TR6-03; AB267719(97.6)	*Sphingoterrabacterium composti*; TR6-03; AB267719(97.6)
2			2		*Proteobacteria*	*Rickettsia conorii str. Malish 7*; AE008647(81.5)	*Rickettsia conorii str. Malish 7*; AE008647(81.5)
2			2		*Proteobacteria*	uncultured bacterium; c5LKS72; AM086142(94)	*bacterial symbiont of Diophrys sp.;* AJ630204(86.3)
2			1	1	*Proteobacteria*	*Sphingomonas sp*. MN57.2a; AM159534(98.8)	*Sphingomonas sp*. MN57.2a; AM159534(98.8)
2				2	*Proteobacteria*	*Pseudomonas sp*. LAB-18; AB051696 (100)	*Pseudomonas sp*. LAB-18; AB051696 (100)
2				2	*Proteobacteria*	*Ralstonia sp*. MCT1; DQ232889 (98.9)	*Ralstonia sp*. MCT1; DQ232889 (98.9)

† The closest match identified among environmental and cultured organisms in Genbank with their corresponding accession number.

‡ The closest match identified among the cultured bacteria in GenBank with their corresponding accession number.

§ Numbers in Parenthesis indicates the similarity percentage of cultured clones in our experiment to their closest match in GenBank.

Table 4.5 Abundance of FAME from each cultivation treatment. [†]

Property	$Biotic_{RCF}$	$Abiotic\text{-}HN_{RCF}$	$Abiotic\text{-}LN_{RCF}$	$Non\text{-}Innoc_{RCF}$	CCRA
Total FAMEs (nm) [‡]	65.9 (5.3)	131 (3.2)	48.3(1.4)	16.5(0.17)	70.90 (14.1)
Bacterial FAMEs (mol%)[‡]	30.7(1.9)	32.2 (1.8)	23.5 (3.9)	38.2 (2.4)	30.41 (4.5)
Fungal FAME (mol%)[§]	12.8(3.4)	22.9 (7.5)	1.50 (0.59)	0	0.098 (0.01)
[b]Fungal/bacterial Ratio[¶]	0.41(0.09)	0.72 (0.27)	0.06 (0.01)	0	0.002 (0.0001)

[†] Values for each row reflect the mean (left of parentheses) and standard error (in parentheses).
[‡] Total FAMEs were measured in nanomoles and were calculated using 16:0 as the abundance standard.
[§] Bacterial and Fungal FAMEs were expressed in mol% to the total FAMEs.
[¶] Fungal/Bacterial ratio is the ratio of mole % of 18:2ω6 and total bacterial fatty acids.
[¶]The figures in the parenthesis indicates the standard error with in the treatment.

Table 4.6 Microbial demand and supply for N among different treaments. [†]

Treatment	Microbial Carbon (µg) [‡]	Microbial N demand (µg) [§]	Estimated Nitrogen supply in cultures (µg) [¶]	N Index [#]
$Biotic_{RCF}$	370.3(33.77)	61.72(5.62)	12.79 (0.26)	0.21
Abiotic-HN_{RCF}	744.06(24.54)	124.01(4.09)	141.67 (2.92)	1.14
Abiotic-LN_{RCF}	268.81(15.78)	44.80 (2.63)	4.51 (0.16)	0.10
CCRA	360.41(33.35)	60.07 (5.55)	30.06 (0.01)	0.50

[†] Values for each row reflect the mean (left of parentheses) and standard error (in parentheses).
[‡] Microbial Carbon was calculated based on total fatty acids and expressed in µg.
[§] Microbial N demand for each treatment was based on a microbial C: N ratio of 6:1. Nitrogen demand was used as an indicator of total nutrient demand.
[¶] Nitrogen available in each cultivation treatment. N supply for 1 and 2 was calculated based on water soluble soil N and nitrogen available in 3 and 4 based on added N amendments. Ninhydrin analysis was used to estimate N pools.
[#] N index was the ratio of the nitrogen supply/microbial N demand.

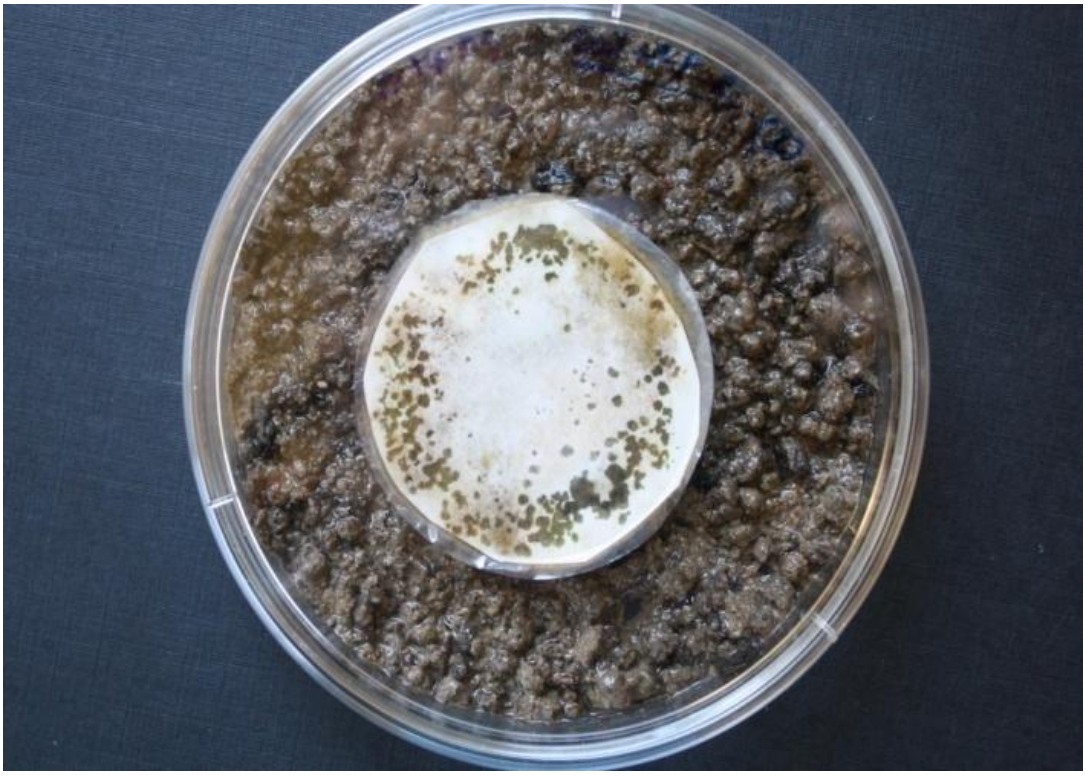

Figure 4.1 Photograph of representative $Biotic_{RCF}$ treatment (inoculated regenerated cellulose filter on unsterile soil) after 20 days of incubation.

Notes: The dark spots on the filter paper represent the degradation of filter paper.

Figure 4.2 Photograph of representative Abiotic-HN_{RCF} (top left), $Biotic_{RCF}$ (top right) and $Control_{RCF}$ (Bottom) treatments after 20 days of incubation.

Notes: biotic-HN_{RCF} treatment (inoculated regenerated cellulose filter on autoclave sterile soil), $Biotic_{RCF}$ treatment (inoculated regenerated cellulose filter on unsterile soil) and non-$Innoc_{RCF}$ treatment (un-inoculated regenerated cellulose filter on unsterile soil).

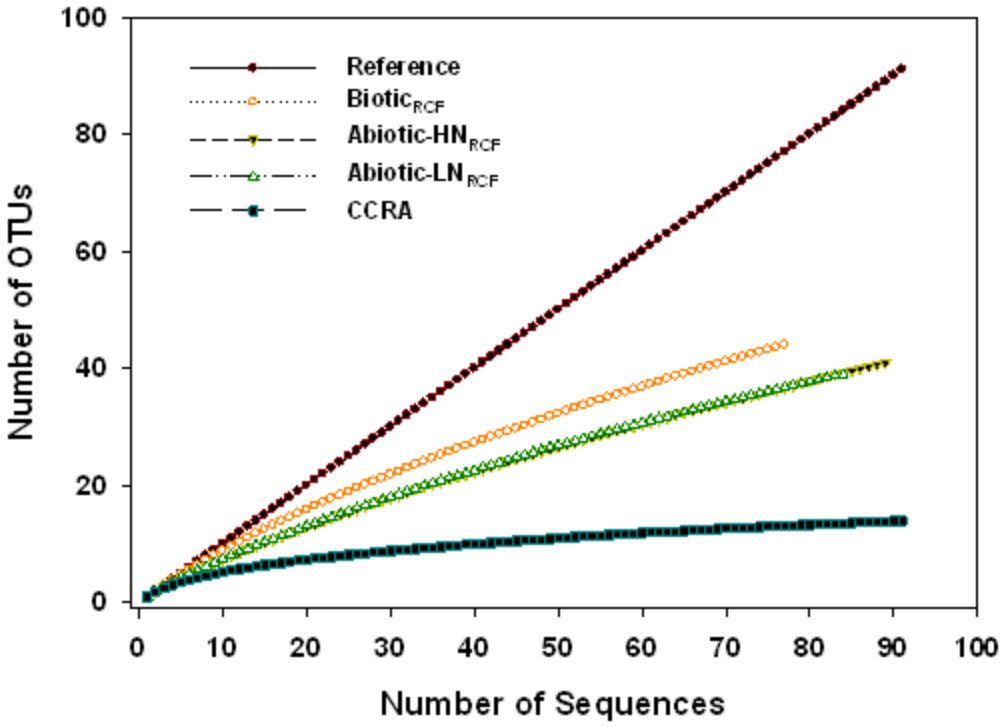

Figure 4.3 Bacterial diversity in four treatments (3 RCF and 1 CCRA) after 20 days of incubation. Rarefaction curves were calculated with DOTUR at 0.03% evolutionary distance.

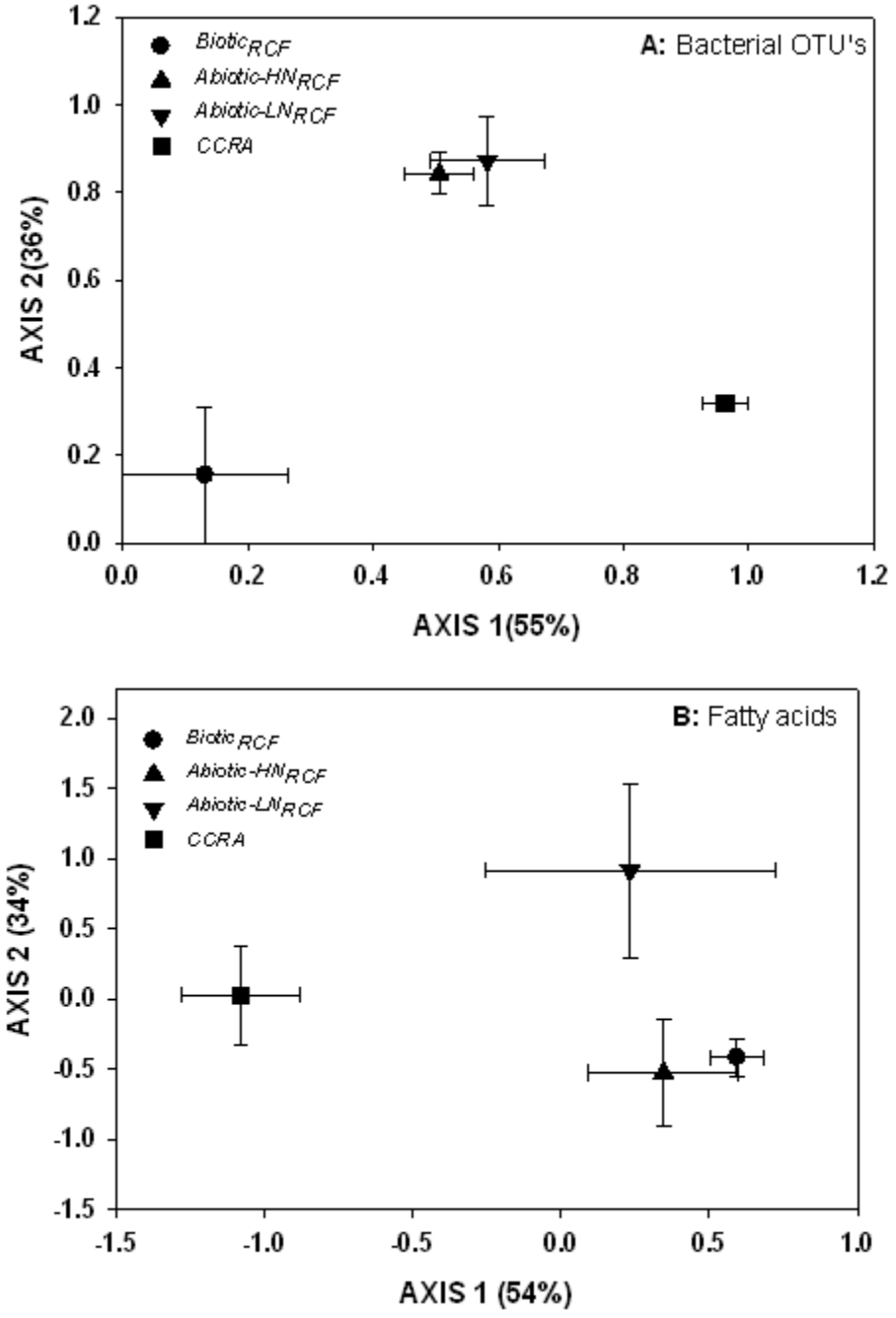

Figure 4.4 Differences in the structure of the microbial community associated with the different treatments using non-metric multidimensional scaling (NMS) based on A) bacterial OTU (D=0.03) and B) mole percentage of FAMEs. The designations noted in the legend represent the four treatments (3 RCF and 1 CCRA treatment).

Notes: Percentages denote the amount of variability associated with each axis. The standard errors of the treatments are noted for each symbol.

CHAPTER V

GENERAL DISCUSSION

Soil microorganisms comprise a large portion of the genetic diversity on earth (Whitman et al. 1998) and have influential role in crucial biogeochemical process. There is growing recognition that environmental stressors and perturbations have marked effects on microbial physiology and community composition, indirectly affecting the energy and nutrient flows in terrestrial ecosystems (Schimel et al. 2007). We have focused our research on investigating the effects of two important factors (one abiotic and one biotic) on soil microbial communities. Specifically, we assessed how 1) soil water status and 2) biotic interactions along with nutrient status influence microbial communities.

One of the major goals of my research is to determine how the soil microbial communities under in situ soil conditions adapt to and/or survive under low water potentials caused by drying of soils. While drying and rewetting (DRW) of surface soils is common in majority of the ecosystems, it is more relevant in seasonally dry climates where there is often a great variability in precipitation. DRW alters the soil water potential creating matric and osmotic stress (Halverson et al. 2000), leading to microbial death and cell lysis (Bottner 1985, Turner et al. 2003) unless they are able to resist the stress by adjusting the cytoplasmic concentration to external conditions (Griffiths et al. 2003) or become dormant until conditions become more favorable (Schimel et al. 2007). Drought also subjects soil microbes to physiological stresses by decreasing substrate

diffusion leading to changes in metabolism (Stark and Firestone 1995). Ultimately, all these processes occur simultaneously resulting in physiological and structural changes in the soil microbial communities.

But it was obvious that the microorganisms adapt and survive to the fluctuations in water potentials. Tremendous work has been done to understand the adaptation strategies of microorganisms under low water potentials and reported that the microorganisms adjust their cytoplasmic concentration to adapt to external water potentials by accumulating/releasing certain kind of low molecular organic compounds (sugars and amino acids) called compatible solutes (Killham and Firestone 1984, Schimel et al. 1989, Poolman and Glaasker 1998, Shen et al. 1999, Halverson et al. 2000).However, majority of the research on osmolyte accumulation was done on culture based studies by introducing salts to lower the water potentials and these results were often relate to the microbial response to matric stress (drying) in soils. But, unlike culture plates the soils are oligotrophic and are depreviated of nutrients and further more matric stress is a completely different phenomenon compared to osmotic stress. With this regard, we executed a study to determine the microbial communities' response to low water potentials (matric and osmotic) under in situ soil conditions. We hypothesized that the microorganisms accumulate organic osmolytes to overcome the matric stress under in situ soil conditions.

We chose two soils (Marietta and Sumter) to study the response of microbial communities to varying intensities of matric and osmotic stress under in situ soil conditions. The two soils, Marietta and Sumter were selected for our study because of their different water status and apparently drying histories. Sumter is an upland soil and relatively quick to drain. Marietta is a lowland soil along stream banks with a shallow

water table, making it a relatively moist environment compared to the Sumter series. So, we assume to have different microbial communities in the two soils that differ in their sensitivity to moisture availability, as the communities might adapt to the water potential fluctuations after few DRW events (Van Gestel et al. 1993, Lundquist et al. 1999). The PLFA analysis of the bulk soils showed that the microbial communities inhabiting the two soil types were distinct from one another. While there are number of differences in the abiotic characteristics of the soils (e.g. organic matter content, texture) water availability is a key factor contributing to differences in the two soils and soil ecosystems processes. Water status has been shown to have large a large impact on microbial community structure before (West et al. 1992, Schimel et al. 1999, Fierer and Schimel 2002, Wilkinson et al. 2002, Williams 2007).

Overall, our results were in partial agreement with the osmolyte accumulation hypothesis under in situ soil conditions. The colorimetric analysis of microbial metabolites showed a significant increase in carbohydrate concentrations in the Marietta (~25%) and Sumter (~14%) soils along drying gradient up to -10 MPa compared to moist controls. An increases in cytoplasmic C content ranging from 10% to 40% and N content from 11% to 60 % at low water potentials was reported in few culture based studies (Koujima et al. 1978, Killham and Firestone 1984b, Schimel et al. 1989) supports our hypothesis of osmolyte accumulation by in situ soil microbes to survive under low water potentials. However, a slight to significant decrease in sugar concentrations was noticed in the two driest treatments -20 MPa and -40 MPa in both the soils is a deviation from our hypothesis of osmolyte accumulation. The restraints in substrate diffusivity and nutrient availability in soils under extreme dry conditions (Stark and Firestone 1995) unlike in cultures might be a reason for the decrease in carbohydrate concentration.

One important difference we noticed between culture-based studies and our study is the accumulation of amino acids. While amino acids are thought to be important osmolytes accumulated in the bacteria during stress (Csonka 1989), we observed a decrease in the Ninhydrin reactive N concentrations with stress. As soils are highly oligotrophic, nutrient limitation might be a major determining factor of microbial response to water stress in terms of using different kinds of osmolytes (Gleason et al. 2010). Since accumulation of osmolytes is energy expensive process, the soil microbes might have adapted other acclimatization strategies that are less expensive like lowering the metabolism or undergoing dormancy under in situ soil conditions.

A significant trend that was noticed on characterizing the microbial metabolites on GC-Ms was an increase in the relative proportions of polyols concentration with the stress and it was consistent in the two soils. Our results indicate that the variations in the sugar concentrations in the Sumter soil are correlated to the fungal fatty acid signatures. The increase of intracellular polyols concentration with increasing osmotic stress in numerous fungal isolates strongly supports the function of this polyols as organic osmolytes under low water potentials (Wethered et al. 1985). While the diversity in metabolites show limited differences between the two soils, they had sufficient resolution to separate the microbial communities of the two soils on the basis relative abundances of metabolites. The non-metric multidimensional analysis of metabolites shows the clustering of matric and osmotic stress treatments separately in the two soils. It has been shown that the two forms of stress affect physiological performance of heterotrophic bacteria (Chang et al. 2007) and fungi (Ramirez et al. 2004) differently.

While the physiological response was not very clear nor consistent in the two soils, slight disparity was observed in the structural response too. Total amount of

PLFAs, a measure of microbial biomass, decreased in Marietta soil along the water stress gradient whereas a significant increase was recorded in Sumter soil. Among the two soils, Sumter which is more mesic soil harbored more fungal biomass than the Marietta soil. we observed a significant increase in the fungal biomass (fungal/bacterial ratio) with stress suggesting that the fungi are more adapted to the water stress (Harris 1981) so as the community present in the Sumter soil. While Sumter soil did not show much of a response in terms of sugar and amino acid accumulations to water stress, reallocation of C in the cell walls or new cell growth during stress periods might be an adaptation strategy to survive under the matric stress conditions (Paustian and Schnurer 1987). This suggests that the long term exposure to fluctuations in water availability might result in the selection of resistant microbial species with different adaptation strategies that are less expensive to surviving microbial biomass.

The second major goal of this research was to understand the importance of biotic or community interactions in the selection of the microbial community in the soil ecosystems and we adapted a culture based approach for determining the objectives. While the cultivation and isolation of microorganisms is essential to completely explore their physiology and ecology, 99% of microbes resist growing in culture plates (Amann et al. 1995, Rappé and Giovannoni 2003). Establishing the metabolic properties and potential of these diverse organisms in the absence of pure culture presents an immense challenge for microbial ecologists (Kaeberlein et al. 2002, Zengler et al. 2002). Over decades the conventional cultivation of microorganisms has relied upon growth under very unnatural conditions, for example, growth in high nutrient media at standard temperature and humidity which are selective and biased for the growth of specific microorganisms (Eilers et al. 2000). Recently studies in marine habitats have indicated

that mimicking the native environment of an organism can enhance the cultivability of previously uncultured bacteria and we adapted thy approach for studying the importance biotic interactions in the growth of bacterial community.

A culture-based study was conducted whereby soil microorganisms were grown in association with their native habitat. The objective of the study was to assess if mimicking native conditions could help to promote the growth of previously uncultivated microorganisms. Moreover, the importance of biotic communities (microbe-microbe) and abiotic soil effects were assessed on bacterial growth. The results strongly indicated that the presence of a living and diverse soil microbial community in the vicinity of the target culture resulted in the cultivation of novel and rare bacterial taxa from phyla *Verrucomicrobia, Bacteroidetes, Proteobacteria,* and *Planctomycetes*. The richness, evenness and diversity of the cultivable community in the presence of living community were significantly greater compared to microbial community grown under sterile abiotic conditions. Our results also confirm few other studies that have reported greater diversity and richness of microbial types when cultured in a simulated natural environment (Kaeberlein et al. 2002, Ferrari et al. 2005, Bollmann et al. 2007). Even though, the exact role that biotic activity played on the cultivation of the bacteria growing on the RCF media cannot be known. However, the effect is supportive of the impact that auto inducer (e.g. quorum sensing) molecules have on bacterial growth (Kaeberlein et al. 2002, Bruns et al. 2003, Williams et al. 2007)

It was suspected that the nutrient availability would have strongly influenced the composition of the cultivable community, in the abiotic RCF treatments. Even though the Abiotic-LN_{RCF} treatment received only a portion of the soil solution and its associated nutrients compared to what would be found in the Abiotic-HN_{RCF} culture, the bacterial

community members residing in both RCF systems were very similar. The most obvious resemblance between the high and low nutrient treatments comes from the observation that ~ 50% of clones showed high sequence similarity(>99%) to *Bacillus megaterium.* This would suggest that nutrient availability across the range utilized in the experiment had little impact on the composition of the cultivated bacterial communities.

Conclusions

The biotic and abiotic environmental conditions in majority of the ecosystems change rapidly. It is obvious that the microbial communities indeed adapt to stressful environment conditions. While drought is a common phenomenon in majority of the ecosystems very little is known about its impact on the surviving soil microbial communities. The water potentials in the soil ecosystems change so rapidly that the microorganisms living intact with it should adapt to stressful environment conditions. While significant flushes of respiration and available nutrients were observed in a wide range of soil types after rewetting of the dry soil which were attributed to soil microbial communities either directly (cell lysis and osmolyte accumulation) or indirectly (mineralization of substrate available after slaking). Either way microorganisms are having huge impact on the soil carbon and nutrient turnover. We have found that drying of soil resulted in the accumulation of a measurable amount of organic C in the soil biomass. Inherent soil properties seem to have significant effect on selection of the microbial community and it's the physiological response to environmental stresses.

Although culturing majority of the soil microorganisms is considered a challenge based on their specific growth needs, in situ cultivation method hold a distinct promise to access the previously uncultured bacteria. Considering the enormous heterogeneity in

chemical and physical features that describe soil habitats at microbial scales, and the enormous possibilities for biological interaction, it seemed likely that some microorganisms that are fastidiously dependent upon their environment for survival and growth would benefit from growth in close association with their native habitat. It was not possible to pinpoint the exact mechanisms of influence; however the biotic bacterial community was clearly very different from those found in the abiotic environment.

Future Research

Although the results of my research to understand the effects of biotic and abiotic factors gave certain preliminary hints on key mechanisms adapted by soil microbial communities to adapt to different environmental factors, the variations in certain results have reiterated the complexity in studying the soil ecosystems. Specifically, while my study on adaptation strategies of soil microbial communities to water stress have given a hint of osmolyte adaptation mechanisms under in situ soil conditions; the factors that dictate to choose certain kinds of osmolytes under soil conditions yet remain unanswered. We still could not detect the important osmolyte group 'amino acids' and some other solutes that are present in low concentrations. Since a detailed knowledge on the chemistry of soil osmolytes is essential to determine the impact of soil microbial osmolytes in the soil C turnover there is need to work on it. While we have seen the changes in the microbial community composition with drought using the PLFA techniques, the use of molecular based techniques (CDNA/RNA) will give a more detailed account on the microbial community, change, the resistant species to drought and their link to the soil functions. Simultaneously, in our culture experiment, the isolation of

some of these rare bacteria which were grown on the filter papers still remain a challenge and needs to be done to characterize and understand the hidden microbial potential.

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APPENDIX A

WATER RETENTION CURVES OF MARIETTA AND SUMTER SOILS

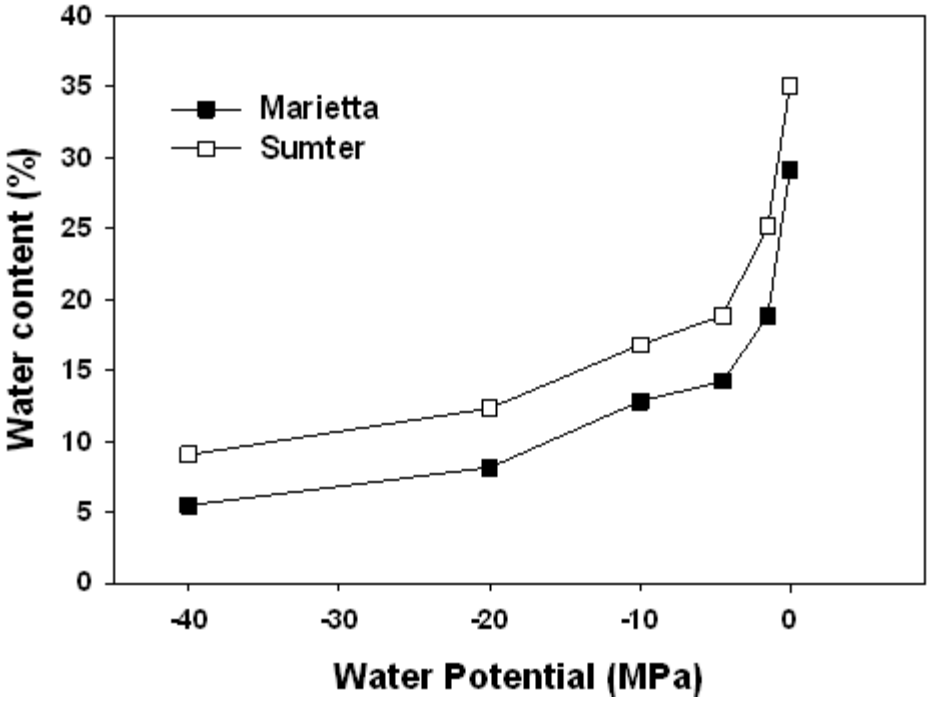

Figure A.1 Water retention curves of the Marietta and Sumter soils (Percent moisture content) as a function of water potential (MPa).

APPENDIX B

PRELIMINARY DATA RELATED TO IN SITU CULTIVATION METHOD

Table B.1 Diversity indices at 0.03% evolutionary distance.[†]

Diversity Indices	RCF[‡]	CCRA[§]	CCRA (No cyclohexamide)[¶]
No. of Clones	46	47	45
No. of OTU's	25	11	19
Simpson (1/D)[#]	22.5	7.45	10.1
Shannon (H)	2.98	2.08	2.51
Evenness(H/Hmax)	0.93	0.87	0.85
Chao 1	55.00	12.00	35.50
95% COI	33.7-128.2	11.1- 21.7	23.2-82.9

[†] Calculations were based on OTU's formed using DOTUR at an evolutionary distance of <0.03.
[‡] RCF- Regenerated Cellulose Filter.
[§] CCRA - Cellulose Congo Red Agar medium.
[¶] CCRA (No Cyclo) - Cellulose Congo Red Agar medium without cyclohexamide.
[#] Simpson (1/D) – Simpson Reciprocal Index.

Table B.2 Bacterial composition in different treatments

Number of OTU's	Closest Match in NCBI GeneBank	% sequence Similarity	RCF	CCRA	CCRA (No Cyclo)
20	Ralstonia sp. AU5957; AY860249	0.995		12	8
19	Paenibacillus sp. DSM 6358; AJ345018	0.997		8	11
12	gamma proteobacterium PI_GH1.1.A2; AY162032	0.995		6	1
7	uncultured beta proteobacterium; AKYG1724; AY921977	0.973	7		
7	Lysobacter sp. TSNRS13; EU263111	0.988		6	1
6	Rhizobium etli; PRF51; AY117629	0.998	1	1	4
5	beta proteobacterium MB7; AB013409	0.996	5		
5	Cupriavidus sp. A81; EF090740	0.999		3	2
3	uncultured bacterium; AB369167	0.957	3		
3	Paenibacillus sp. M-2b; AB089250	0.953	3		
3	Ideonella sp. B508-1; AB049105	0.993	3		
3	uncultured gamma proteobacterium; SWL18; AY528817	0.972	3		
3	Paenibacillus sp. DSM 6358; AJ345018	0.998		1	2
2	Caulobacter sp. PhyCEm-1426; AM921648	0.998	2		
2	Telluria mixta; ACM 5158; DQ005909	0.989	2		
2	Massilia timonae; CP13A2; AJ871459	0.99	2		
2	Rhizobium sp. tpud.40a; AY691399	0.982			2
2	Lysobacter yangpyeongensis; GH 19-3; DQ191179	0.982	2		
2	Burkholderia sp. SJ98; DQ986324	0.995		2	
1	uncultured gamma proteobacterium; BIfciii1; AJ318123	0.901	1		
1	Chitinophaga terrae; Gsoil 238; AB267724	0.959	1		

Table B.2 continued

Number of OTU's	Closest Match in NCBI GeneBank	% sequence Similarity	RCF	CCRA	CCRA (No Cyclo)
1	uncultured Sphingobacteriales ; EF019302	0.983	1		
1	uncultured gamma proteobacterium; EF520573	0.769	1		
1	uncultured bacterium; FCPS618; EF516603	0.974	1		
1	uncultured bacterium; FCPO589; EF516121	0.973	1		
1	glacier bacterium FXI47; AY315164	0.997	1		
1	Bradyrhizobium liaoningense; LMG 18230; AJ250813	0.998	1		
1	Bosea eneae; DS29; EF519707	0.989	1		
1	uncultured bacterium; OS-82; AB206013	0.988	1		
1	Paenibacillus chondroitinus; DJCM 9072; AB073206	0.975	1		
1	uncultured eubacterium; 1_32_415; AJ437465	0.97	1		
1	uncultured Gemmatimonadetes bacterium; EF664151	0.972	1		
1	uncultured bacterium; DGGE band 9; AY487126	0.991	1		
1	unidentified eubacterium 173R6; U35508	0.911	1		
1	Rhizobium mongolense; CCBAU 85033; EU256429	0.952			1
1	Achromobacter xylosoxidans ; AJ491845	0.999			1
1	Gram-negative bacterium CCBAU 25211; AY864080	0.945			1
1	Stenotrophomonas sp. MFC-C; AB183423	0.999			1
1	Rhizobium sp. tpud.40a; AY691399	0.988			1
1	uncultured Burkholderia sp.; KM(type1); AB191228	0.989			1
1	uncultured Bacteroidetes bacterium; AKYG467; AY922021	0.961			1
1	Bacillus megaterium; HDYM-24; EF428248	0.999			1
1	Devosia riboflavina; AY512822	0.982			1

Table B.2 continued

Number of OTU's	Closest Match in NCBI GeneBank	% sequence Similarity	RCF	CCRA	CCRA (No Cyclo)
1	uncultured bacterium; WBI100; EU024391	0.995			1
1	Paenibacillus sp. DSM 6358; AJ345018	0.976			1
1	Rhizobium sp. AC100; AB069724	0.971		1	
1	Ralstonia sp. AU5957; AY860249	0.994		1	
1	Cupriavidus sp. KU-26; AB266608	0.994		1	
1	Rhizobium sp. AC100; AB069724	0.976		1	

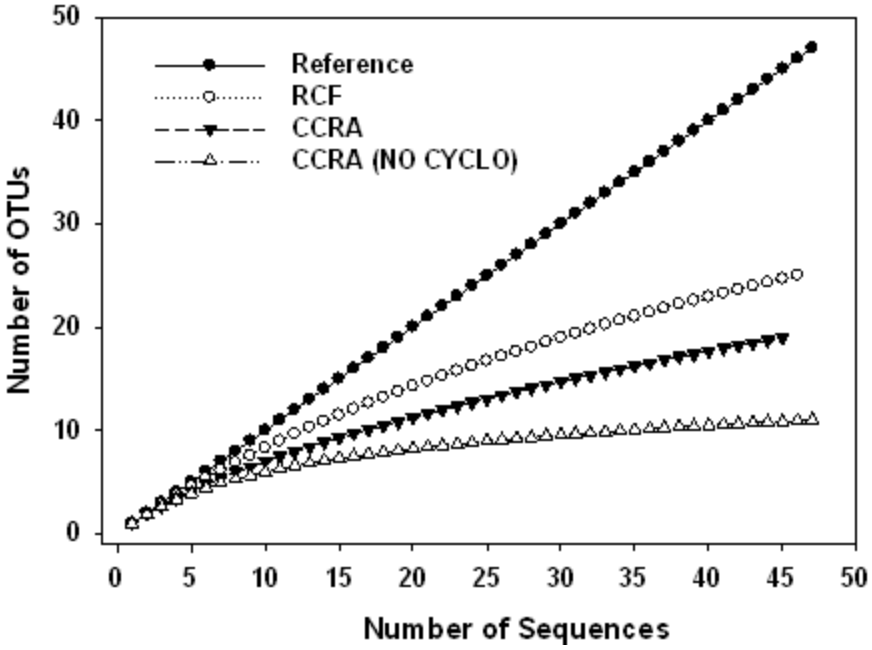

Figure B.1 Bacterial diversity in three treatments after 20 days of incubation. Rarefaction curves were calculated with DOTUR at 0.03% evolutionary distance.

Notes: RCF indicates Regenerated cellulose filters on unsterile soil, CCRA -cellulose Congo Red Agar, CCRA (NO Cyclo) - Cellulose Congo Red Agar medium without cyclohexamide.

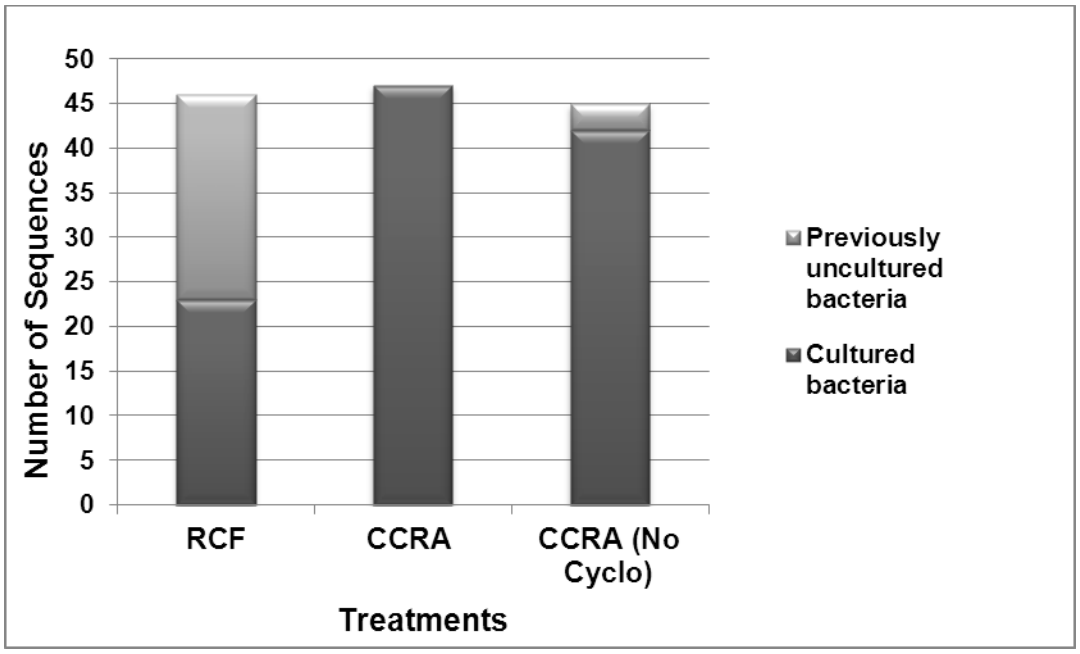

Figure B.2 Number of clones in three treatments (RCF, CCRA and CCRA-No Cyclo) matching to previously uncultured (environmental DNA) and cultured bacteria in NCBI gene bank.