Molecular Analysis of the Rhizosphere Fungal Diversity on a Regenerating Louisiana Oil Brine Spill: Perspectives in Biorestoration

By

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Abstract

Chapter 1

Introduction/Literature Review

Oil spills

Louisiana ranks as one of the largest crude oil producing states in the country behind Alaska. In fact, Louisiana is considered to be the nation's third largest producer of crude oil, not including offshore federal drilling (OSRADP). This large extraction of crude oil not only damages the local environment but does so in latter stages of production as well.

Extracted crude is pumped to local separators where it is separated from the extracted salt water (brine or produced water), a byproduct of the extraction, while the brine is reinjected back into the reservoir. Reinjection is often necessary to acquire more oil and as reservoirs age the amount of brine water required increases. Accidental spills are often frequent because of old or unattended machinery including pipes separating the wellhead from the separators. In Louisiana alone between 1990 and the middle of 1998, Bass stated that there were 567 reported cases of accidental brine spills (1999). This necessitates the need for an economical bioremediation strategy that will reinitiate ecosystem processes without harming the already compromised area.

Effects of spills on soil

Oil-brine spills cause a myriad of negative ecological effects within the spill area. In general, ecosystem function of the contaminated area is diminished leaving it uninhabitable to most organisms. The soil at a spill site contains large deposits of hydrocarbons, phenols, chemicals, aromatics, salts and heavymetals (Reis 1992) which in turn supports poor vegetation due to its high pH, high salt content and low nutrient availability (Raghuwanshi *et al*. 2003). The salinity of oil-brine can range from 1,000 to 400,000mg L-1 while the salinity of seawater is in the range of 35g L-1 (Carlile *et al.* pg 350). Jones (1945) stated that sea water contains 20,000 part per million of chlorides while a brine spill can possess upwards of six times that amount. To put this into perspective, Jones (1945) also noted that a particular oil-brine spill contained 248 times the allowable amount of total solids by the U.S. Public Health Service in standard drinking water.

Ultimately, this creates a need for the restoration of these spills. The available options are limited and mainly include: bioremediation which is accomplished fairly

easily and economically or the extraction and replacement of the contaminated soil. The replacement of contaminated soil is not very feasible for large areas, being very laborious, costly and mainly unsuccessful.

Effects on Plants

The high salt content of affected soil poses as the most direct threat to vegetation. This creates osmotic stress therefore making available water harder to acquire in addition to leaching water directly from the plant (Vavrek & Colgan). Salt toxicity can result through the accumulation of ions (Cl or Na) in the leaves thereby reducing the photosynthetic leaf area, stunting growth, altering respiratory pathways, and causing leaf injuries (Bernstein 1975). In addition, the excess Sodium and hydrocarbons alter the overall soil structure by dispersing clay particles, therefore limiting water and gas movement through soils. Overall, these stress imposed on vegetation alter the plants metabolic, reproductive, and physiological mechanisms making sustainable vegetation challenging.

Effects on Fungi & Microbes

The indigenous fungi at an oil-brine spill are thought to be adversely affected by the soil disturbance encountered on an oil brine spill. Studies have shown that Arbuscular Mychorrizal Fungi (AMF) are primarily effected through a reduction in propagule number, population density, vigour of hyphal growth and also a delay in the germination time of spores; a change in the overall diversity and community structure (Guar *et al.* 2004; Juniper *et al.)*. Despite this, it has also been shown that the number of propagules and fungal hyphae are severely reduced in polluted vs. non-polluted habitats, seven and a half times higher reduction in polluted soils (Cabello 1997). The disturbance encountered on an oil-brine spill ultimately reduces the overall capacity and ability of forming mycorrhizal associations, yet there have been many documented cases of mycorrhizae performing well in harsh and stressful environments. Overall, high concentrations of soil salinity and heavy metals have been shown to reduce the capacity to form mycorrhizae (Brown *et al.* 1996;).

While thought to reduce the overall microbial community profile, oil-brine stress could possibly act as a natural selection process, therefore eliminating less tolerant species and making tolerant species more numerous or detectable. The overall microbial community structure and dynamics following an oil-brine spill vary by report. Several studies (Kastner et al 1998; Hood et al 1975) have revealed that the microbial community within a spill zone contains more species capable of degrading polycyclic aromatic hydrocarbons (PAH) when compared to adjacent non-disturbed sites. While Allen and Wagner (2000) found that rhizosphere microbial diversity was largely unaffected by an oil-brine spill, and remained relatively similar to adjacent undisturbed areas. The chemical/physical disturbance alters microbial

physiology and reproduction therefore theoretically leaving only stress tolerant species. Several oil spill studies have documented the inefficiency of specific bacterial strains to compete with more adapted indigenous microbes (Pritchard 1992; Barriault et al 1993 as referenced in Heinonsalo et al 2000). Stress tolerant species would be more adapted to local conditions and could potentially serve as an inoculum source for re-vegetation studies through their ability to stimulate plant growth better than non-indigenous species (Guar 2004). Based on this, stress-tolerant fungi can be selectively isolated and propagated then combined with an appropriate stress-tolerant plant host to form an effective oil-brine spill bioremediation tool.

Fungal Diversity and Roles

Fungi are ubiquitous organisms occupying a wide array of niches and performing many ecological functions, particularly those associated with nutrient cycling. Estimates of fungal biomass in soil include Christensen's (1989) report finding 66,900m of fungal mycelium in one gram of dry soil (referenced in bridge et al 2001). Fungi thrive in habitats from soil to plant roots to the rumen of cows. Their relationship can be beneficial, (mutualistic) negative (parasitic) or they can exist as free-living organisms decaying plant matter on the forest floor. Fungal diseases have been responsible for such devastating events as the Irish potato famine and can even cause a variety of ailments in humans such as athletes foot and yeast infections. Many advancements have come from fungi, such as common prescribed medications like Penicillin, a by product of fungal metabolism released to inhibit competing bacteria, fermentation of various alcoholic beverages, other pharmaceutical drugs, enzymes, and biological control agents (Carlile *et al.* 2001). Agricultural practices have now begun to realize the importance of incorporating mycorrhizal fungi because of their ability to increase nutrient uptake in nutrient poor soils, making plants resistant to pathogens, thus increasing crop yield and decreasing the need for fertilizers (Carlile *et al.* 2001)

The Kingdom Fungi encompasses an eclectic group of organisms contained in many representative phyla, ranging from the common mushrooms to bread moulds to lichens. This Kingdom contains the masters of chemical production, secreting a cornucopia of enzymes to digest and obtain the necessary substrates required for growth. Their anatomy and growth has adapted to maximize the surface area covered therefore increasing nutrient uptake and ultimately sporulation.

Benefits of Mycorrhizae

Mycorrhizal associations are ubiquitous in nature and constitute a two-way beneficial relationship between a plant host and a fungus and are responsible for various ecosystem functions. In fact it has been shown that more than 60% of all terrestrial plant species form mycorrhizal associations (Trappe 1962). They are known to be key players in the establishment of plant communities by leading to greater plant densities in early

succession. Mycorrhizae act as an intermediary in the bridging of the above and below ground ecosystems, basically by acting as an extension of the plants root system exploring areas not accessible by plant roots. The mycelium benefits by receiving carbon from the plant host in turn delivering water and various nutrients to the plant. Allen et al (2003) states that mycorrhizal roots acquire phosphorus, nitrogen, zinc, copper, nickel, sulfur, manganese, boron, iron, calcium and potassium better than nonmycorrhizal roots, with more evidence in low fertility soils. Plants benefit from the fungus in numerous ways including an increase in iron availability via fungal excretion of chelating compounds (Marschner *et al.*), increasing resistance to pathogens, and by increasing overall plant and seedling growth and reproduction. In one study, Stanley *et al.* (1993) showed that mycorrhizal *Abutilon theophrasti* (velvetleaf) had a significant increase in seed production, weight and phosphorus content in addition to a large increase in seedling recruitment the following year. In another study with *Abutilon theophrasti,* mycorrhizal colonization increased the plants weight, height, total leaf area, root formation and the percentage of offspring that flowered (Koide and Lu 1995). Koide et al (2002) found that mycorrhizal tomato plants (*Lycoperdon esculentum*) showed a significant increase in pollen tube growth rate, which could possibly allow mycorrhizal plants to outcompete nonmycorrhizal plants by producing seeds faster; thus becoming primary successors on diminished soils.

Numerous studies have reported the ability of mycorrhizae to alleviate the stresses associated with soils contaminated with heavymetals. Arbuscular mycorrhizal fungi are known to regulate heavy metal translocation into plants, and can therefore be a useful tool in phytoextraction of contaminants from soils. Yet, the effectiveness of these fungi is largely determined by the concentration and composition of the metals present, the host plant, soil properties and the AMF assemblage (Hovsepyan et al 2004). In summary, certain AMF species prevent heavy metal uptake while others facilitate the process. For instance, Hetrick et al (1994) showed that mycorrhizal plant leaves contained reduced concentrations of Cd, Zn, and Mn (referenced in Christie et al 2004). On the other hand, Hovsepyan found increased concentrations of zinc and copper in corn leaves when mycorrhizae were present (2004). The mechanism behind decreased metal translocation is not entirely known, but is thought to be the result of binding of the metals to the fungal hyphae in or around the rhizosphere, thereby immobilizing the metals and preventing translocation into the plant.

Benefits of Plants and Microbes in Bioremediation

Vegetation is of primary significance in biorestoration primarily because plants constitute the foundation of the ecosystem as primary producers. Vegetation aids in restoration by improving the overall soil structure and chemistry, adding organic matter, and by stimulating microbial populations that can biodegrade pollutants and initiate nutrient cycling. Many common soil microbial communities possess the ability to degrade aliphatic and aromatic hydrocarbons including bacterial and fungal species, and both are known to be widely distributed in nature (Li et al 2002 and Atlas et al 1981). The introduction of vegetation and microbes should allow the reinitiation of the diminished ecosystem processes necessary for biorestoration of these sites to begin.

Symbiosis Formation

The process of forming the intimate relationship between fungus and host is an intricate and highly orchestrated process that is still not fully understood. It is now known that chemical signals between the two partners is the impetus of the symbiosis, driving and directing the fungal hyphae towards roots. Researchers have reported increased directional hyphal growth and morphological changes in the presence of plant roots and exudates (Becard et al 2004).

First in an AM fungus symbiosis, a complex array of chemical signals are exchanged between the symbionts, allowing the plant to recognize the fungus as a nonpathogen, followed by subsequent dramatic branching of the fungal hyphae. After recognition, physical contact occurs resulting in the fungus forming a swollen structure known as an appressorium, which aids in root tissue entry. Next, the hyphae enters the root via enzymatic and mechanical processes penetrating the intra and inter cellular spaces of the plant's cells, dichotomous branching occurs and ultimately arbuscules are formed which are indicative to this mycorrhizal fungus. Abruscules are thought to be the main site of nutrient exchange between the two organisms (Guinel et al 2002).

Mycorrhizal Ecology

There are several types of mycorrhizae mainly characterized based on morphology, host preference and mode of infection. The most widespread and researched groups are the following: endomycorrhizae, ectomycorrhizae, Vesicular Arbuscular mycorrhizae (VAM), Arbuscular mycorrhizae (AMF), orchidaceous mycorrhizae, and ericaceous mycorrhizae. Ectomycorrhizas are mainly formed on the roots of woody plants including oak, beech, birch and coniferous trees, and consist of an Ascomycete or Basidiomycete fungus. Fungal hyphae penetrate the intercellular spaces of the epidermis and cortical region yet do not invade or penetrate the cells.

Endomycorrhizae penetrate the walls of cells, and fill the intracellular space with hyphal clusters (Atlas et al 1998).

Vesicular arbuscular mycorrhizas

Community Determination/Diversity

Fungal diversity is estimated at 1.5 million species, yet only about 5-10% of these have been discovered (Viaud *et al.* 2000). Traditional methods of observing and characterizing fungal communities include direct isolation on culture media and the observation of fruiting bodies, both of which are limited by the inability of many fungal species to grow on culture media (Valinsky *et. al* 2002*.,* Smit *et. al.*1999). In addition, fungal growth on culture media does not provide a natural profile of the community

diversity and structure as this technique is extremely biased towards certain species. Moreover, this traditional approach has greatly hampered the understanding of fungal ecology.

Identification of mycorrhizae (AMF) is an even more daunting challenge, as these fungi have been traditionally classified based on the morphology of their spores or intraradical structures. In the absence of spores the morphological structures provide identification to the family level at best (Reddecker et al 2003). Morphological identification is extremely biased as certain mycorrhizal species produce vast quantities of spores, therefore appearing to be the dominant species present.

The use of molecular profiling techniques is an evolving and reliable way of characterizing natural microbial communities *in-situ*. In particular, the advent of the polymerase chain reaction (PCR) has greatly advanced this area of research, through its ability to target certain organism specific genes which can be used for taxonomic assignments, while only requiring a small amount of DNA. The first task is to develop PCR primers that provide selective amplification of fungal DNA with high specificity, because fungal DNA only constitutes a small portion of the total DNA extracted from most samples (Valinsky et al 2002).

The region of the fungal genome that is most widely exploited is ribosomal DNA (rDNA), which is available in large copies and possesses highly-conserved as well as variable regions, thus allowing taxonomy at various levels (Reddecker et. al 2003). Located between the 18S and 28S rRNA genes and including the 5.8S rRNA gene is the internal transcribed spacer (ITS). The ITS region is noncoding, resulting in a faster rate of evolution and greater sequence differences between species, therefore providing greater taxonomic ability than coding regions (Anderson et al 2004). In addition, ITS sequence variability has been documented to be sufficient enough to distinguish between many different species within a genus (Viaud et al 2000).

Several approaches are used widely to separate mixed amplicons from PCR reactions, including temperature gradient gel eclectrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), and cloning. Both TGGE and DGGE separate DNA fragments based on melting behavior, with DNA of different lengths and nucleotide composition denaturing and migrating at different rates. These two techniques have been primarily used to observe changes or shifts in microbial community composition. A major advantage of using gel based community profiling techniques is the ability to excise bands of particular interest, thereby allowing unusual or specific community members to be analyzed (Anderson et al 2004). Cloning, while providing a good microbial community profile, also allows the screening of clones prior to DNA sequencing thus eliminating costly and time consuming processes. Clones can be screened by subjecting them to RFLP analysis, once a RFLP pattern has been documented, new patterns can be compared and matched without the need for sequencing (Redecker et al 2004).

Environmentally obtained sequences can be rapidly compared to public data bases for identification using the basic linear alignment search tool (BLAST), which is available on the internet through NCBI. This process is very efficient and can be accomplished easily without any cost to the user.

Hydroseeding Advantages

The application of biorestoration agents, vegetation and microbes, may be accomplished fairly economically through the use of a hydroseeder. A hydroseeder is a mobile unit that allows the simultaneous application of plant seeds, microorganisms, mulch, fertilizer and water. This technique is noninvasive, thereby minimizing disturbance to the compromised spill ecosystem and utilizes minimal man power.

The objectives of this research project are to analyze the rhizosphere fungal community diversity on a regenerating Louisiana oil brine spill and to determine the rhizosphere fungi which possess the ability to tolerate the physiological stresses associated with oil brine spills, thereby enhancing plant sustainability. This study will also analyze the. Ultimately, this will hopefully provide the foundation for biorestoration of oil brine spills by allowing the propagation of a particular species of fungus (or multiple species) that can be used to field inoculate seedlings or that possesses the innate ability to biodegrade hydrocarbons.

CHAPTER 2

MATERIALS AND METHODS

The oil brine spill site is located near the town of Jena, La Salle parish Louisiana. The site was indicative of a typical brine spill in that it contained little or no vegetation in the vicinity of the spill. Approximately 750 barrels of oil brine were spilled in February of 2003 damaging 0.3 hectares (Clark 2005). All hydroseeding, vegetation growth and effect of fungal inocula were performed and documented by Barret Clark and need not be mentioned here (See Clark 2005 for details). As mentioned earlier, the main purpose of this study is to analyze the fungal rhizosphere diversity on a regenerating oil brine spill site via molecular profiling techniques.

Rhizosphere fungal diversity will be compared between trap cultures (inoculum source) of *Sorghum sudanese* inoculated with fungi from previous brine spills that displayed the greatest vegetative performance, and from plots that received these fungi via hydroseeding. This will track the fate of the introduced fungi and will help assess their tolerance to this environment, thereby selecting and identifying stress tolerant species. A total of nine samples were collected, four from the nursery trap cultures and five from the Jena, LA spill plots, with one sample serving as a control containing no fungal inoculum.

Table 1
Location of sample sources

Sample Name	City, State	Vegetation Type	Coordinates
Ranger 1	Ranger, TX	Bermuda	32.22.21 N, 98.41.13 W
Ranger Old	Ranger, TX	Bermuda	32.22.21 N, 98.41.13 W
Smack 6	Smackover, AR	Bermuda/halophytes	33.20.53 N, 92.37.40 W
PPB	Abita Springs, LA	Various grasses	30.30.42 N, 89.59.55 W
Five others	Jena, LA	*Sorghum sudanese*	

Table 1. Location of spill sites and fungal inoculum source

DNA Extraction

Approximately 0.2g of feeder roots and associated soil were collected from *Sorghum Sudanese* for DNA extraction. DNA was extracted using the Ultraclean™ Soil DNA Kit (MoBio, Solano Beach, CA) by following the instructions from the manufacturer. To ensure efficient DNA extraction, each sample was visualized on a 1% agarose gel prior to PCR amplification.

PCR Parameters

DNA from each sample was amplified in 0.2ml plastic tubes using a PCR Express thermocycler (Thermo Labsystems, Vataa, Finland). Each PCR reaction contained a total of 25µl which consisted of the following: 12.5µl of MasterAmp 2X premix B (Epicentre, Madison WI) which contained dNTP's and buffers, 10.5µl of ddH20, 0.5µl (10 pMol) of each primer, 0.15µl (2.5 U) of DNA *Taq* polymerase and 1µl (30ng) of sample DNA. The internal transcribed spacer primers ITS1F (5'CTTGGTCATTTAGAGGAAGTAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') were used as these are known to specifically amplify fungal DNA from a pool of mixed amplicons (White et al, 1990). Thermocycling parameters consisted of an initial denaturation step of 94ºC for 2min, followed by 30 cycles of 94ºC for 20s, annealing at 52ºC for 20s, and elongation at 72ºC for 45s. A final elongation cycle at 72º for 2min was allowed. The thermocycler heating lid was used on all samples and cycles. Samples were then held at 4ºC until they were removed for subsequent processing. Each reaction was visualized using a 1% agarose gel stained with ethidium bromide in TBE buffer (75V for 30min) to ensure proper amplification occurred. Gels were visualized using the UV light box AlphaImager™ 1220 Documentaion & Analysis System (Alpha Innotech Corp, San Leandro, CA).

ITS rDNA clone library construction

Each PCR reaction was cleaned using the Ultraclean PCR Cleanup Kit (MoBio, Solano Beach, CA) following the instructions of the manufacturer. Clone library construction was accomplished by ligating the fungal amplicons into the pGEM-T Easy Vector™ (Promege, Madison, WI) subsequently transforming the plasmids into competent JM109 (Promega), by following the instructions provided by the manufacturer. 100µl of each transformation solution was plated on to a TSA/X-Gal (100µl) with IPTG (100µl) and ampicillin (50mg/ml) Petri plate. This allowed for the visual screening of successful transformation via α-complementation—blue white screening. Plates were then incubated inverted for approximately 16hrs at 37ºC. Twenty-five colonies from each sample (plate) that showed signs of complementation (white colonies) were randomly selected for clone library construction by transferring colonies with a sterile toothpick to TSA/Ampicillin (50mg/ml) plates and incubated inverted at 37º for 16 hours.

rDNA clone library analysis

Each clone was subjected to whole cell PCR by transferring cells directly to the PCR reaction via a sterile toothpick. PCR parameters are identical to those described

above, except cloned cells served as the DNA template. To ensure proper amplification occurred, reactions were visualized on 1% agarose gels stained with ethidium bromide. Operational taxonomic units were then assigned by treating each whole cell PCR reaction with the restriction enzymes AluI and HinfI (Invitrogen). Each restriction digest consisted of the following: 10µl from the PCR reaction, 2µl buffer II (Invitrogen), 0.5µl (2.5 units) of each enzyme and 7µl ddh20, for a final concentration of 20µl. The digests were incubated at 37ºC for 12 hours. Fragment patterns were visualized on 3% super fine resolution (SFR) agarose gels at 175V for 2 hours stained with ethidium bromide. Each unique fragment pattern per sample was transferred into 5ml of LB/ampicillin (50mg/ml) media and grown for 16 hours at 37ºC with constant shaking at 175rpm. Plasmids from each liquid culture were extracted using the Ultraclean™ Mini Plasmid Prep Kit (MoBio, Solano Beach, CA) as described by the manufacturer. The plasmid solution was subsequently vacuum dried in a speed vacuum and then rediluted in 12µl ddh20 to facilitate sequencing, for a total DNA concentration between 40 and 80ng/µl. Samples were sent to Gene Gateway (Hayward, CA) for sequencing. Gene Gateway provided the universal clone primer T7 in addition to performing the reaction and read. Each sample (clone) returned approximately 700bp of nucleotide sequence, each consisting of vector and fungal DNA. Sequences were viewed and edited in Sequencher® version 4.2. Vector sequence contamination was removed using the automatic vector trim feature in sequencher and ambiguous base calls were manually edited for later phylogenetic analysis. All sequences were compared to sequences from the GenBank database using Blastn to determine the closest taxonomic match to known sequences.

Phylogenetics

The closest matching two sequences were downloaded from Genbank and added to the sample sequences for alignment of the 5.8S region. Alignment was performed using ClustalW (http://clustalw.genome.jp/). The alignment was manually edited using Bioedit (Tom Hall, NCSU, Raleigh, NC) then exported in nexus file format for phylogenetic tree creation using PAUP 4.0b10 (Swafford, 2000). Relationships were inferred from the 5.8S gene, by using the Neighbor Joining algorithm.

CHAPTER 3

RESULTS

A total of 92 unique fingerprints were obtained, with 52 of these from the Jena samples and 40 from the trap (inoculum) cultures. Two sequences were excluded (LR 25 and LR 32) due to a high proportion of ambiguous base calls, for a total of 90 sequences.

The field samples produced the highest proportion of RFLP patterns consisting of the following: Smack 6 produced 13 unique fingerprints, Ranger 1 produced 14, the control returned 9, PPB returned 11, and Ranger old returned 10. The trap cultures returned: Ranger one 9, PPB 10, Smack six 8, and Ranger Old with 5 (See Table 2).

Table 2. Comparison of OTUs by sample source

Sample Source	OTUs	Identical Taxa between sample sources
Smack 6 inoculum	8	None
Jena Smack 6	13	
Ranger old inoculum	6	Ascomycete sp. *Chrysosporium pseudomerdarium*
Jena Ranger old	10	
PPB inoculum	10	None
Jena PPB	11	
Ranger 1 inoculum	9	*Alternaria* sp. *Aspergillus oryzae*
Jena Ranger 1	14	
Control	9	N/A

Table 2. Cross analysis between sample sources and OTU's by sample

As evident in table 2, very low taxonomic similarities were found between the inoculum sources and the root samples from Jena. Only two definitive species were successfully detected in both sample origins, one in the Ranger old samples and one in Ranger 1.

Genbank Database Similarity

Comparison of the environmental sequences with the Genbank database revealed a large range in percentile similarity, between 82 and 100 percent and revealed that all sequences were of fungal origin. The highest number of base matches was 649 of 664 and returned an uncultured fungus clone as the closest taxon. The lowest number of matching bases 67 of 67, returned *Cercospora kikuchii* as the closest taxon (See Tables 3-5).

The genus *Alternaria* was found to be related to eight of the sequences, with five of these belonging to *Alternaria alternata*. No definitive species was given for the other three sequences. Uncultured Ascomycetes returned 8 closest matches, uncultured fungus/fungus clone produced 7 matches, six sequences returned *Chyrsosporium pseudomerdarium* as the closest related species, *Terfezia leptoderma* was related to five, *Debaromyces polymorphus* 4, 4 were in the genera geomyces with 2 of these belonging to *Geomyces pannorum*, uncultured hypocreales clone 3, *Fusarium proliferatum* 2, *Ramaria spinulosa* 2, *Paecilomyces inflatus* 2, *Pseudotomentella nigra* 2, *Aspergillus oryzae* 2, (See tables 3-5)

Phylogenetic Analysis

Table 3: OTU data as determined from Genbank

OUT ID	Closest Taxa	Ascension No.	No. of matching bases	Similarity %
LR1	*Paecilomyces inflatus*	AB099943	577/597	96

LR2	*Chyrsosporium pseudomerdarium*	AJ390386	467/482	96
LR3	*Ramaria spinulosa*	AJ292293	176/185	95
LR4	*Chyrsosporium pseudomerdarium*	AJ390386	467/481	97
LR5	*Debaromyces polymorphus*	AB054100	229/236	97
LR6	*Phoma tracheiphila*	AY531679	73/85	85
LR7	*Ascomycete* sp. A1	AJ279460	440/442	98
LR8	*Debaromyces polymorphus*	AB054100	229/235	97
LR9	*Geomyces pannorum*	AJ509872	318/386	82
LR10	Uncultured *Leptosphaeriaceae*	AJ87964	566/596	94
LR11	*Paecilomyces inflatus*	AB099943	417/426	97
LR12	*Stagonospora* sp. Po41	AY208791	408/437	93
LR13	*Epacris microphylla* root fungus	AY268219	491/503	97
LR14	*Debaromyces polymorphus*	AB054100	228/235	97
LR15	*Cladosporium sp. 4/97-17*	AJ279487	586/588	99
LR16	*Chyrsosporium pseudomerdarium*	AJ390386	468/481	97
LR17	*Stagonospora sp. Po41*	AY208791	408/437	93
LR18	*Ramaria spinulosa*	AJ292293	176/185	95
LR19	*Ascomycete sp. A1*	AJ279460	591/598	98
LR20	*Geomyces sp. GFI 22*	AJ608988	350/367	95
LR21	Uncultured fungus clone 5	AY702074	649/664	97
LR22	Ascomycete sp. ITS 423	AF502902	414/435	95
LR23	*Fusarium proliferatum*	AF291061	595/596	99
LR24	*Ramaria stricta*	AF442097	168/174	96
LR26	Uncultured fungus	AJ920018	146/161	90
LR27	*Ramaria bataillei*	AF441082	167/170	98
LR28	*Fusarium proliferatum*	AF291061	596/599	99
LR29	Uncultured fungus	AJ920018	147/160	91
LR30	Ascomycete sp. Ae	AY303610	456/472	96
LR31	*Alternaria alternate*	DQ023279	600/604	99
LR33	Ascomycete sp. A1	AJ279460	594/600	99
LR34	*Chyrsosporium pseudomerdarium*	AJ390386	469/482	97
LR35	*Penicillium verruculosum*	AF510496	598/620	96
LR36	*Cladosporium tenuissimum*	AJ300331	459/465	98
LR37	*Apiosordaria nigeriensis*	AJ458184	242/262	92
LR38	*Antrodia camphorate*	AJ496405	69/74	93
LR39	Uncultured fungus clone LP55	AY615959	183/199	91
LR40	*Chyrsosporium pseudomerdarium*	AJ390386	469/481	97
LR41	*Cladosporium* sp. 4/97-17	AJ279487	310/316	98
LR42	*Ampelomyces humuli* isolate wb29	AF455485	470/480	97
LR43	*Talaromyces flavus*	AF455513	163/171	95
LR44	*Cochliobolus spicifer*	AJ303084	343/356	96

Table 3. Sequence ID, Closest Genbank match, Genbank Ascension Number, Matching Bases and Percent Similarity

Table 4: OTU data as determined from Genbank (Continued from Table 3)

OUT ID	Closest Taxa	Ascension No.	No. of matching bases	Similarity %
LR45	Alternaria sp. PBN4	AY923863	562/567	99

LR46	Ascomycete sp. A1	AJ279460	168/170	98
LR47	*Talaromyces flavus*	AF455513	387/395	97
LR48	*Bipolaris spicifera*	AY253918	293/306	95
LR49	*Chyrsosporium pseudomerdarium*	AJ390386	370/383	96
LR50	Ascomycete sp. A1	AJ279460	575/600	95
LR51	*Aspergillus oryzae*	AY373857	382/387	98
LR52	*Alternaria* sp. IA215	AY154686	444/450	98
LR53	Fungal sp. GFI 143	AJ608964	525/537	97
LR54	Alternaria sp. PBN4	AY923863	570/570	100
LR55	*Fusarium Chlamydosporium var. fuscam*	AY213655	498/508	98
LR56	*Pseudotomentella nigra*	AF274770	151/157	96
LR57	*Pseudotomentella nigra*	AF274770	151/157	96
LR58	*Terfezia leptoderma* strain Val06	AF396864	119/129	92
LR59	*Alternaria alternate*	AF455540	242/257	94
LR60	Fungal sp. GFI 143	AJ608964	453/468	96
LR61	*Polyporus tuberaster*	AF518763	68/71	95
LR62	*Geomyces* sp. GFI 22	AJ608988	455/490	92
LR63	*Terfezia leptoderma*	AF396864	164/164	100
LR64	*Verticullum coccosporum*	AF110531	507/516	98
LR65	Uncultured fungus clone 5	AY702074	369/374	98
LR66	Uncultured fungus clone 5	AY702074	351/365	96
LR67	*Apiosordaria nigeriensis*	AJ458184	288/311	92
LR68	*Cladosporium tenuissimum*	AJ300331	252/266	94
LR69	*Stagonospora* sp. SAP 44	AF422976	190/201	94
LR70	*Uncultured Leptosphaeriaceae*	AJ879641	373/386	96
LR71	*Fusarium incarnatum*	AY633745	381/381	100
LR72	*Debaromyces polymorphus*	AB054100	135/143	94
LR73	*Fungal sp. GFI 22*	AJ608973	307/320	95
LR74	*Fungal sp. GFI 22*	AJ608973	307/320	95
LR75	*Alternaria alternate*	DQ023279	607/608	99
LR76	*Uncultured hypocreales clone 4.3*	AY615879	117/121	96
LR77	*Alternaria alternate*	DQ023279	607/608	99
LR78	*Alternaria sp. IA215*	AY154686	407/415	98
LR79	*Ascomycete sp. A1*	AJ279460	440/442	99

Table 4. Sequence ID, Closest Genbank match, Genbank Ascension Number, Matching Bases and Percent Similarity (Continued from Table 3)

Table 5: OTU data as determined by Genbank (Continued from Table 4)

OTU ID	Closest Taxa	Ascension No.	No. of matching bases	Similarity %
LR80	*Alternaria alternate*	AY445812	396/404	98

LR81	*Rhodotorula mucilaginosa* strain CBS	AF444541	615/616	99
LR82	*Cladosporium tenuissimum*	AJ300331	588/590	99
LR83	*Terfezia leptoderma*	AF396864	164/164	100
LR84	*Cercospora kikuchii*	AY633841	67/67	100
LR85	*Terfezia leptoderma*	AF396864	163/164	99
LR86	Uncultured fungus clone 5	AY702074	573/589	97
LR87	Uncultured dothideomycete	AJ879690	384/389	98
LR88	Uncultured hypocreales clone 4.3	AY615879	533/537	99
LR89	*Aspergillus oryzae*	AY373857	382/387	98
LR90	Uncultured hypocreales clone 4.3	AY615879	529/537	98
LR91	*Geomyces pannorum*	AY873968	563/568	99
LR92	*Terfezia leptoderma* strain Val06	AF396864	164/164	100

Table 5. Sequence ID, Closest Genbank match, Genbank Ascension Number, Matching Bases and Percent Similarity (Continued from Table 4)

CHAPTER 4

DISCUSSION

Molecular profiling is a well known procedure for analyzing microbial communities *in-situ*, and is heavily documented in the literature. As with most procedures, each step in the molecular profiling of microorganisms contains a source of potential bias, making the goal of obtaining the true microbial diversity in an environment challenging.

Overall, the majority of sequences were related to Ascomyete fungi. This was expected as this phylum contains the largest and most diverse group in the kingdom fungi. Amplification and primer bias could potentially dictate the overall abundance of Ascomycete species detected. In samples containing mixed community DNA, the most abundant DNA (organisms) sequences are most likely to be amplified, thus skewing the detection of certain species; although this has not been determined. Anderson et al (2004) states that, it is unclear how the choice of PCR primers and the quantity of certain fungal templates in a community DNA sample influences amplification, and whether the most abundant fungal templates are preferentially amplified. Extraction of bulk DNA and subsequent amplification does not allow the discrimination between active growing microbes or dormant structures such as spores. Therefore, if a soil sample contains a majority of spores or hyphal fragments from one particular fungal species, then theoretically the probability of amplification and detection of this species is markedly increased.

RFLP fingerprinting of clones serves as another source of bias, as each fingerprint (banding pattern) may not actually be indicative to a species. As with the genus *Cortinarius*, species in this genus can have the same RFLP pattern with two restriction enzymes (Anderson et al 2004). Ultimately, this would decrease the ability to select different species based on banding patterns. In addition, screening of restriction patterns to assign operational taxonomic units (OTU) can become very challenging, as it is tedious to discern different banding patterns on a gel. Due to some low resolution banding patterns, identical patterns could be chosen leading to sequencing of the same insert and detection of identical species. In addition the amount of clones that should be sequenced to gain an adequate picture of the fungal diversity in soil is ambiguous. Landis et al (2004) indicates that 100 or more clones might be necessary to obtain the true AMF diversity in soil.

A larger number of distinct RFLP patterns was expected for the field samples. This is most likely due to the natural diversity and abundance of fungal propagules in the field, although mold spore densities can be substantially higher indoors where ventilation is poor. Another possible cause for this outcome could be from the addition of hay as an organic component on the study plots. While adding organic matter, this also allows for the introduction of large numbers of viable fungal propagules since decaying plant matter is a primary substrate for fungi. One way to circumvent this could be to include sampling adjacent to (outside) the spill area to gain an understanding if the fungal diversity on the spill site is in fact contrasted to local community diversity.

Several mycorrhizal species were detected. One species, *Terfezia leptoderma* is of primary concern because of its natural habitat. This mycorrhizal species, also known

as the desert truffle, thrives in harsh environments where water availability is low; an environment similar to oil brine spill zones. A large number of uncultured fungal species were detected. This is most likely due to the lack of information in gene databases, and the dependence of these databases on sequences generated from culturable fungi. Other significant genera discovered were *Aspergillus, Penicillium and Cladosporium* because some members of these genera have been reported to degrade certain types of petroleum products (Bokhary et al 1993). In addition, Llanos and Kjoller (1976) found that the application of oil favored growth of *Paecilomyces* strains, while important hydrocarbon-utilizing fungi detected included *Paecilomyces*, *Fusarium*, and *Penicillium* strains. All three genera were detected in this project.

The field samples showed a larger number of known petroleum-utilizing fungal genera. This is as expected since hydrocarbons are an energy source for these fungi, therefore population densities are stimulated by the oil. In addition, many laboratory and field studies have documented an increase in hydrocarbon-utilizing microorganisms in response to oil addition (Atlas et al 1981).

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