Microbial Community Structure in Hydrocarbon Impacted Sediment Associated with Anomalous Geophysical Signatures

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MICROBIAL COMMUNITY STRUCTURE IN HYDROCARBON IMPACTED SEDIMENT ASSOCIATED WITH ANOMALOUS GEOPHYSICAL SIGNATURES

by
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Joseph W. Duris
The Crystal Refinery is a former refinery site in Carson City Michigan that has had historic releases of refined and unrefined crude oil dating back to the late 1940's. The contamination of soil with light non-aqueous phase liquids (LNAPL) has had a dynamic impact on the subsurface environment in adjacent Carson City Park. Very little is known about the complex interactions between microbial communities, geochemistry and geophysics. In order to investigate possible connections between these parameters a multidisciplinary study was undertaken to investigate the hypothesis that the degradation of LNAPL by resident microbial communities causes a local increase in organic acid concentrations, which in turn cause an increase in native mineral weathering and a concurrent decrease in the bulk electrical resistivity of soil. Microbial community structure was analyzed using a 96-well most probable number (MPN) method and rDNA intergenic spacer region analysis (RISA). In addition to the observational study of this field site, a controlled lab experiment was preformed in an attempt to simulate field conditions and responses.

Microbial community structure was found to change in the presence of LNAPL and was consistently observed in regions of anomalously low resistivity. Thus, geophysical methods for monitoring the subsurface are a promising new technology for monitoring changes in microbial community structure and simultaneous changes in geochemistry that are associated with LNAPL degradation.
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I. INTRODUCTION

The pollution of soil and water with unrefined crude oil and other petroleum related products is a problem of increasing magnitude. Because microorganisms are generally the first organisms to encounter hydrocarbon pollution, it is very important to determine whether microbial populations are interacting with the pollution in any way. In the past two decades bioremediation, the use of living organisms for the removal of environmental pollutants such as hydrocarbons or light non-aqueous phase liquids (LNAPLs), has gained more interest as a result of the Exxon Valdez oil spill and the post-war fallout of the Gulf War. Bioremediation processes can have a broad range of desirable effects ranging from total removal of a contaminant to simply detoxifying the compound (Shannon & Unterman, 1993). Regardless of the effects of bioremediation, one important factor when dealing with the in situ degradation of a xenobiotic is the monitoring of the process. In order to successfully monitor natural attenuation (the naturally occurring bioremediation of a xenobiotic substance by resident microbial communities), the complex interactions of biological, chemical and physical parameters of the soil must be understood.

Most currently available methods of characterizing and monitoring bioremediation involve direct core sampling and drilling. The major problem with coring methods is that they sample from a relatively small fraction of the actual volume of a contaminant plume, which may contain vast heterogeneity especially on a vertical axis. This heterogeneity applies not only to microbiological conditions, but also to chemical conditions such as dissolved gas and ionic concentrations. Thus, while data acquired from these individual points are valid for those points, they may not be indicative of the entire contaminated zone (Smith et al., 1991). Additionally,
discrete sampling methods are often very expensive and involve invasive procedures that can disturb a fragile microbial balance that is imperative for naturally occurring biodegradation, hence the need for non-invasive techniques to adequately address this problem.

In this regard, geophysical techniques hold great promise as alternative technologies for the monitoring of intrinsic bioremediation. Recently, geophysical methods have been described that can characterize sediments and potentially monitor bioremediation (Atekwana et al., 1998; Sauck, 1998; Sauck, 2000; Sauck et al., 1998). One of the most promising geophysical methods described is *in-situ* Vertical Resistivity Probe (VRP) measurement. This measurement provides information about the resistivity of the pore fluid in the soil particles within the area of the 4 soil contacting electrodes (Sauck, 1998). Typically, in fresh LNAPL spills hydrocarbon will displace water from the pore space of soil particles. Because LNAPLs have a high resistivity compared to water, a concurrent increase in the apparent resistivity of the soil is expected upon this replacement (DeRyck et al., 1993). As a result of this observation, field measurements should measure a resistive layer coincident with the zone of LNAPL contamination. However, instead of the predicted high resistivity responses, field investigations of LNAPL spills have instead observed a region of anomalously low resistivity (Atekwana, et al., 1998; Sauck, 1998; Sauck, 2000; Sauck, et al., 1998). The insulative model accounts for changes in pore water chemistry, but neglects the potential of resident microbial communities to modify the LNAPL to a different physical or chemical state. It is well documented that LNAPL contamination causes shifts in microbial community structure to communities that are capable of hydrocarbon degradation. LNAPL degradation also results in the formation of low molecular weight organic acids, which lower the pH of the soil pore water (Cozzarelli et al., 1994; Cozzarelli et al., 1990). Increases in acidity cause a
concurrent increase in mineral dissolution. Thus is is expected that as biodegradation proceeds increases in mineral dissolution from secondary weathering reactions should cause decreases in resistivity of the impacted medium (Cassidy et al., 2001; Hiebert & Bennett, 1992; Hiebert et al., 1995; McMahon & Chapelle, 1991; McMahon et al., 1995). However, the potential impact of microbial mediated processes on geoelectrical properties has remained relatively unrecognized by the geophysical community. Nonetheless, in order to establish the link between biodegradation and lowered resistivity response a careful analysis of the spatial and compositional distribution of the resident microbial communities was carried out. Studies into these hypotheses have focused on microbial communities isolated from soil in the City Park in Carson City, MI that borders a well-characterized contamination plume stemming from the former Crystal Refinery that abuts the park. The long-term goals of this study were to develop a model that relates changes in microbial community structure to subsurface geophysical changes while at the same time building a foundation of information that may be used for future studies. The short-term goals of this study were to determine how community composition is affected by LNAPL in the subsurface while at the same time determining how microbial communities are distributed within the soil at Carson City Park.

II. LITERATURE REVIEW

Degradation of petroleum hydrocarbons for remediation purposes is an area of intensive research. The process of biodegradation can be affected by many different factors. The nature of the LNAPL, the temperature, the availability of oxygen and nitrogen, the composition of the resident microbial communities, and the pH of the environment that is polluted can all affect the rates and extent of biodegradation
Microorganisms can degrade LNAPLs under aerobic or anaerobic conditions, but in general the potential to degrade a hydrocarbon depends on a microorganism's ability to introduce oxygen into the LNAPL and with just a few reactions convert the substrate to a usable intermediate in an energy-yielding pathway. The main determinant of how oxygen is introduced is the type of LNAPL substrate that is presented to the microorganism. In general, alkane substrates are terminally oxidized to their complementary aldehyde, alcohol or fatty acid intermediate. These intermediates are then further oxidized to odd-chain alkanes like acetate and propionate by the β-oxidation pathway (Rosenberg and Ron, 1996). In order to effectively oxidize alkane substrates a microorganism must be able to transport the substrate of interest into the cell. Thus, alkane degradation is dependent on the length of the alkane. Longer alkanes being difficult to transport are therefore more difficult to degrade. Shorter alkanes are easier to transport and therefore are easier to degrade. This suggests that there may be specific transport systems for specific LNAPL substrates. While alkane degradation depends on specific or non-specific monooxygenases, aromatic LNAPL degradation depends on complex, sometimes multifunctional dioxygenase enzymes. Dioxygenases incorporate both atoms of molecular oxygen into the substrate forming cis-dihydrodiols. Dihydrodiols are then converted via various pathways to useable metabolic substrates (Rosenberg and Ron, 1996).

It is also possible for microbial populations to degrade LNAPL contamination to low molecular weight organic acids that could accumulate in the surrounding groundwater. This accumulation of organic acids can be attributed to the anaerobic conversion of monoaromatic hydrocarbons, such as benzene, toluene and xylene, to an oxidized intermediate state that has acidic properties, such as benzoic or toluic
acids (Cozzarelli, et al., 1990). In fact, mono-aromatic hydrocarbons have been shown to decrease over time, while the concentrations of the corresponding organic acids have been shown to increase (Cozzarelli, et al., 1994). Bacterial acid production from hydrocarbon substrate has also been shown to occur in aerobic environments. *Pseudomonas putida*, a propane oxidizing bacterium was shown to convert the common gasoline additive methyl-tert butyl ether to various derivative acids, such as methylacrylic acid and formic acid (Steffan et al., 1997).

Once organic acids have been produced they may alter chemical and physical soil characteristics. For instance, increasing content of organic acids may decrease the pH of the pore water and increase dissolution of native minerals causing an increase in dissolved solids and ions in the pore fluid of soil particles. These solids and ions can be periodically flushed from the soil pores and may over time cause changes in the electrophysical characteristics of the soil (Sauck, 1998).

Several studies have been conducted that focus on the effects of acids on resident soils. Cassidy et al. (2001) observed that when sediments were experimentally contaminated with LNAPL and supplemented with nutrients there was a concurrent increase in total dissolved solids and organic acids. While similar results were observed in both an aerobic and anaerobic reactors, there were higher concentrations of organic acids and dissolved solids found in the anaerobic reactors. The observed increase in dissolved solids was attributed primarily to dissolution of calcium ions from the LNAPL contaminated soil (Cassidy, et al., 2001).

This effect has also been studied on a physical level by directly observing the dissolution of soil particles. McMahon et al. (1995) observed increases in organic acid concentrations dependant on the amount of hydrocarbon in the sediment. Increased organic acid concentrations were also connected with increased concentrations of calcium, magnesium and iron in the groundwater. In addition the
physical effects of the mineral leaching were observed using scanning electron microscopy. Highly weathered quartz and feldspar grains were found within the area of organic acid rich ground water that corresponded with hydrocarbon impacted sediments (McMahon, *et al.*, 1995).

A more direct approach was taken to study this effect by Hiebert and Bennett (1992). In this work, cleaned and non-etched quartz fragments were visualized with microscopy and then submerged for 14 months in LNAPL contaminated ground water. They observed chemical etching of the quartz especially in the vicinity of attached bacterial cells. The chemical etching occurs as triangular pits on the surface of the observed quartz. Interestingly the low overall concentration of organic acids suggested that this etching should not have occurred. The authors attribute the etching to localized organic acid production by the attached bacteria (Hiebert and Bennett, 1992).

In addition to physical, chemical and geophysical changes, many biologically significant changes occur in the composition of resident microbial communities when a xenobiotic compound, such as LNAPL, is released into the soil subsurface. Often, when there is a sudden change in the nutrient ratio (as in the sudden addition of carbon as LNAPL) or when a toxic substance enters the ecosystem, only those organisms that are capable of utilizing the substrate or coping with the toxicity are able to survive. Ecological stressors such as these often lead to communities that are less diverse, as organisms capable of surviving the stress increase in numbers while those that are adversely affected by the stress decline in numbers (Liu *et al.*, 1997; Macnaughton *et al.*, 1999; Rosenberg and Ron, 1996; Song & Bartha, 1990). Changes in community structure may be a sharply delineated event in the subsurface because there appears to be patterned ecological succession in the microbial communities as nutrient conditions alter spatially. This implies a technique for
elucidating the distribution of different physiological types of microorganisms. By examining the concentrations of various terminal electron acceptors, such as oxygen, nitrates or sulfates in the aqueous phase at contaminated sites the distribution of different physiological types of microorganisms may be determined (Bekins et al., 1999).

In order to study changes in the composition of microbial communities several approaches may be used. Culture based methods such as plate counts and most probable number methods are reliable methods to enumerate different physiological types of culturable microorganisms. However, there are problems with using a solely culture-based approach for microbial community analysis. First, culturing methods can often give a misrepresentation of the structure of a community. This is primarily due to enrichment bias that occurs when plating an environmental sample onto a growth medium. Since specific phenotypes of bacteria are most often isolated from environmental samples by using enrichment, competition among the various species will be affected by the difference between these growth conditions and the growth conditions of the natural environment. This will cause organisms that can utilize these new resources the fastest to show increased numbers compared to those that demonstrate slow growth patterns on the same substrate (Dunbar et al., 1997).

Dunbar et al. (1997) have also suggested that direct plating onto a non-enrichment media may be a more general way to select for a broad variety of phenotypes, that is, more phenotypes were found on non-enrichment media than on enrichment media.

Even though there are problems associated with enrichment in culturing methods, Bekins et al. (1999) have shown that, if done properly, the cultivation of various physiological types of microorganisms can be used to characterize chemical gradients that exist in an aquifer during the process of bioremediation. They elegantly showed striking relationships between the presence of iron-reducing microorganisms
and the presence of methanogenic microorganisms. The work of Bekins et al. (1999) indicated that if sampling was conducted at small enough intervals, this technique was able to provide an accurate delineation of various electron-accepting zones present in the subsurface.

Another bias of culture methods is not that of enrichment, but that of selection. Many groups have estimated that out of all the microorganisms present in an environmental sample, only 0.1% to 10% are viable on common growth media (Dunbar, et al., 1997; Head et al., 1998; Macnaughton, et al., 1999; Muyzer et al., 1993). This indicates that not only are the results of plating skewed by enrichment bias, but they are also seriously misrepresentative of the total population of environmental microorganisms. In addition to these standard cultivational methods an automated system called Biolog® may also be used for community analysis. This method compares substrate utilization patterns on 95 different substrates and can provide insightful information for distinguishing various microbial communities. Model communities produced in laboratory settings can be easily distinguished with Biolog®, but it is less reproducible when used on environmentally isolated communities.

In order to accurately analyze microbial community structure less biased methods need to be utilized. There are several non-culture based methods that have been described to track changes in microbial community structure in situ. One method that is currently in use is phospholipid ester-linked fatty acid (PLFA) analysis. Under various environmental and physiological conditions microbes respond by altering the types and amounts of phospholipids in their membranes. This technique is useful because many genera of organisms have characteristic lipids in their membranes allowing for the determination of genera present via a lipid biomarker. While PLFA is excellent at comparing shifts that may occur in total
microbial communities, it lacks the ability to resolve shifts in individual groups of microorganisms. More recently developed methods of microbial community analysis have focused on the amplification and analysis of the DNA region encoding 16S rRNA. This DNA region has also been referred to as 16S rDNA (Acinas et al., 1997; Borneman & Triplett, 1997; Garcia-Martinez et al., 1999; Macnaughton, et al., 1999).

The 16S rDNA region is involved in the essential process of protein synthesis in microbial cells. Protein synthesis can only take place in the presence of ribosomes. This structure consists of proteins and ribosomal RNA (rRNA). In bacteria, the complete ribosome is referred to as the 70S ribosome. It consists of the 50S subunit, which contains both 23S and 5S rRNA along with 31 different ribosomal proteins, and the 30S subunit that consists of the 16S rRNA and 21 different ribosomal proteins. During the initiation of translation, the 30S subunit binds to the mRNA and then the 50S subunit binds to this complex. This complete complex then allows tRNA to couple with mRNA codons and translate the mRNA to proteins (Snyder & Champness, 1997). The 16S rRNA role is crucial to the process of protein synthesis. Because of the importance of its function, the 16S rDNA has a highly conserved sequence that makes it a perfect target for phylogenetic analysis and thus it can be used to provide a more accurate method for the description of microbial communities. However, before analysis can be done to characterize the 16S rDNA, it must be obtained from environmental samples and then amplified to working concentrations. Various strategies may be used to take environmental samples and will differ by the environment to be sampled. Once the environmental sample has been obtained, the nucleic acids must be isolated from the sample. In the case of air and water samples standard extraction protocols can be used to isolate DNA. However, in the case of soil samples there is often a coextraction of humic acids from the soil. These substances can interfere with DNA detection and quantification (Zhou, et al., 1996).
It has also been demonstrated that humic acid contamination can interfere with Taq DNA polymerase during polymerase chain reaction (PCR) (vanElsas & Smalla, 1997). Zhou et al. (1996) found that a general DNA extraction protocol using SDS - cell lysis treatment with high-salt and temperature treatments was effective for extracting DNA from most soils, but it was not as effective for Gram positive cells in high clay environments. After the DNA was recovered, the most effective method for purification before PCR amplification was to first purify the DNA on a gel and then run the DNA through a mini-purification column. The recovered DNA may then be subjected to PCR using primers that are specific for the 16S rDNA region. Once this DNA region has been amplified, it can be analyzed by various methods.

One type of 16S rDNA analysis is referred to as denaturing gradient gel electrophoresis (DGGE). DGGE is based on electrophoretically separating 16S rDNA fragments on a polyacrylamide gel with a linearly increasing gradient of denaturants (Muyzer, et al., 1993). In DGGE separation is based on the migration of partially melted DNA. The mobility of this form of DNA is altered from the helical form that is normally run on gels. This is an excellent technique for distinguishing phylogenetic diversity within a microbial community. By analyzing DGGE gels with statistical methods an accurate estimation of the number of phylogenetically unique microbes can be made. This technique varies from other 16S rDNA techniques in that rather than focusing on the size of the fragments; it focuses instead on the denaturing behavior of the DNA (Moseneder et al., 1999).

While it may be possible to distinguish individual species of bacteria based on a band in a complex banding pattern, it is not possible to determine species evenness (relative amount of each species) in a complex community. Depending on the complexity of a certain community it may not even be possible to resolve species richness (variety of species), although by using more narrow gradients of denaturant,
this problem may be overcome (Muyzer, et al., 1993)

Another powerful method of 16S rDNA analysis that has recently been applied to resolve species richness and evenness is terminal restriction fragment length polymorphism (T-RFLP) analysis. When the 16S rDNA region is amplified using PCR, instead of using standard primers, one or both of the primers are labeled with a different fluorescent marker and then PCR is carried out (Liu, et al., 1997). The PCR products are digested with restriction enzymes leaving multiple fragments, with just the terminal ends marked with the fluorescent label. The terminal fragments will be of various lengths depending on the organism from which the 16S rDNA was derived. The exact lengths of these terminal fragments can then be determined by electrophoresis in an automatic DNA sequencer. This means that if all microorganisms are subjected to this process, then each should have its own genetic fingerprint. In addition to identification of individuals, T-RFLP can be used for community analysis by visual comparing the electropherograms produced by the sequence or by statistically analyzing gel images with computer software (Liu, et al., 1997). This technique is very sensitive and can distinguish between fragments that differ in length by 1-2 base pairs, and can also indicate the number of species within a communities and the size of each species with in that community.

While the 16S rDNA methods of analysis are very useful in their applications, another method is gaining popularity for microbial community analysis. Ribosomal DNA intergenic spacer region analysis (RISA) is based on the amplification of the 16S-23S spacer region (Acinas, et al., 1997; Garcia-Martinez, et al., 1999). The spacer region may have high length variability both within an operon and between different operons within a single cell (Acinas, et al., 1997; Garcia-Martinez, et al., 1999). Generally, the variability is due to various functional gene units that may be found in the spacer region. Most commonly found here are tRNA genes, usually one
or two per spacer region, but this varies between species. Also present in the spacer region are sequences that recognize various enzymes, including the boxA sequence that plays a role in transcriptional enzyme recognition and a ribonuclease III enzyme recognition site that helps in forming the mature ribosome of the bacterium (Garcia-Martinez, et al., 1999). Theses recognition sites are only conserved between closely phylogenetically related bacteria. The entire spacer region is not highly conserved. In fact, most of the region is subject to frequent insertion and deletion events. This would indicate that while there is a high degree of similarity in the spacer regions of closely related species, there also may be a quick divergence within some closely related strains due to insertion and deletion events (Garcia-Martinez, et al., 1999).

RISA has many advantages over other 16S rDNA methods. First, it is a very efficient method of community analysis because there is no reason to clone or enzymatically digest any of the products of the initial PCR. Second, the 16S-23S spacer region offers more size variability than does the amplification of just the 16S gene and third, by including more of the 16S gene within the area of amplification identification may be quickly done based on 16S sequence analysis (Acinas, et al., 1997; Garcia-Martinez, et al., 1999).

RISA has been used to analyze several different types of microbial communities. Borneman and Triplett (1997) used the RISA approach to distinguish populations shifts associated with deforestation in soil in the eastern Amazon rainforest (Borneman and Triplett, 1997). The study illustrated a clear difference in RISA banding patterns with hundreds of distinct bands that ranged from 0.4 to 1.4 kb. Fisher and Triplett (1999) also used an automated RISA (ARISA) approach to study diversity within freshwater bacterial communities. This method is very similar to the T-RFLP method described earlier in that it used a fluorescently labeled primer during the initial amplification of the 16S-23S spacer region. Once amplification was
accomplished, the electrophoresis was performed with an automated system that allowed for laser detection of the length of the DNA fragments. The ARISA approach showed a high reproducibility within a sample and also showed variations between freshwater sources. In addition to the techniques high reproducibility the ARISA technique could also be used to demonstrate the evenness and richness of each species within a sample (Fisher & Triplett, 1999).

III. MATERIALS AND METHODS

FIELD DATA COLLECTION

Study Site

Carson City Park is located in Carson City, Michigan. The park lies directly to the south of the former Crystal Refinery Site. The Crystal crude oil refinery was in operation from 1935 to the early 1990’s. There have been historical releases from underground tanks and pipelines that have impacted the ground water and sediments with LNAPL. Initial observation of hydrocarbon contamination was observed in Fish Creek, which borders the refinery and the park on the west, in 1945 by the Michigan Department of Environmental Quality.
Figure 1. Map of Carson City Park. This is a detailed site map of Carson City Park. The locations of vertical resistivity and monitoring wells are noted as well as elevation contour, free phase LNAPL thickness, residual phase LNAPL boundaries and the location of a drainage ditch that crosses the park. Biological samples were taken from VRP 1, 4, 5, 9, and 10.

This study focuses on the southern plume that is 228 m long and 82 m wide with a free phase thickness of 0.3 to 0.6 m and has an estimated volume of 167,000 liters. The dissolved phase of the plume consists primarily of the BTEX compounds benzene, toluene, ethyl benzene and xylene (Atekwana, et al., 1998). The site is geologically characterized by 4.5 to 6.1 m of fine to medium sands, which become gravel below the water table. These sediments are underlain with a 0.6 to 3 m clay aquitard unit. Water table depth varies from 0.6 m to 5.8 m depending on site location. Groundwater flow is 1.7 m/day flowing west-southwest towards Fish
Vertical Resistivity Probes

Vertical resistivity probes were installed at various locations within Carson City Park. The vertical resistivity probe is a semi-permanent probe composed of a 2” polyvinyl chloride (PVC) pipe that uses stainless steel screws placed every inch along the vertical axis as electrical contact points between the soil and the measuring electrode. The measurements are taken by using an internal slider that produces electrical contact between 4 of the screw contact points. An excitatory voltage is passed from the first electrode to the fourth electrode and the second and the third electrodes in the contact series monitor the potential change.

Sample Collection

Two-foot acetate liners and liner caps for direct push coring were disinfected in the laboratory prior to field sampling using approximately 250 ml of an 80% ethanol solution. The liners were exposed to the ethanol for 2 minutes and placed in a laminar flow hood until the ethanol had evaporated completely. The liners were then capped with 80 % ethanol disinfected caps that have also been dried in the laminar flow hood.

During coring the liners were uncapped and placed into a hand operated direct push, soil-coring rig. The two-foot core was taken and freshly ethanol disinfected caps were placed on the liners as soon as they were removed from the coring mechanism. These cores were then packed in ice and placed out of direct light for transport back to Western Michigan University, where they were placed at 4 degrees Celsius until analysis.
Soil Sub-sampling

The sterile cores were opened in a clean bench using an ethanol sanitized cutting knife. An approximately 1g sub-sample of soil was taken every six inches (15.25 cm) of depth from a five centimeter opening in the core and placed into pre-weighed gamma sterilized 15 ml polypropylene tubes for cell extraction from the soil. In addition to this sample for microbiological analysis, a sterile 1.5 ml microcentrifuge tube was filled with soil for each sample point and placed at -20°C until DNA extractions were performed. There was also 15 g of soil placed into a clean scintillation vial. This soil sub-sample was stored at 4°C until used for soil pH analysis or hydrocarbon extraction analysis.

Cell Extraction and Plating

Cells were extracted from the one-gram sub-sample using a modification of the cell extraction procedure described by van Elsas and Smalla (1997). A volume of 9.5 ml of 0.1% sodium pyrophosphate was added to the one gram of soil and the cells were extracted via mechanical shaking at 250 rpm for 30 minutes at 28°C. The samples were then centrifuged in a clinical centrifuge at ~ 900g for 11 minutes. Four 25 µl aliquots of supernatant were then taken from the top 3 ml of the tube and the appropriate 10-fold serial dilutions were made in the wells of a gamma sterilized 96-well microtiter plate for MPN determination or one 100 µl sample was taken from the top 3 ml of the tube and serially diluted and plated onto sterilized growth media for the colony forming unit determination.
Colony Forming Unit (CFU) Determination

An aliquot of extracted cells (100 µl) was serially diluted to the appropriate dilution. A 100 µl volume of each dilution was then plated onto two plates of 10% tryptic soy agar (1/10 TSA) or onto Oil Agar 2 (OA2) (Walker & Colwell, 1975). These plates were incubated at 28 degrees Celsius for 6 weeks. The number of colonies on each plate was counted and the number of CFU/g of soil was calculated. Those colonies that grew on OA2 were assumed to be capable of oil degradation, and those that grew on 10% TSA were assumed to account for the total heterotrophic fraction of soil bacteria.

Most Probable Number (MPN) Determination

Aliquots of extracted cells (25 µl) were placed into wells filled with 225 µl of either 10% Tryptic Soy Broth (10% TSB) or Bushnell-Haas (BH) medium (Becton Dickinson, Detroit, MI) supplemented with 2% NaCl and 6.25 µl of n-hexadecane. Serial dilutions were performed in each successive row from full strength to a final dilution of 10^{-10}; the twelfth row of each plate was left as an uninoculated control. The plates were then allowed to incubate for two weeks at room temperature. On the fourteenth day, 50 µl of filter sterilized p-iodonitrotetrazolium violet (3g/l) was added to each of the BH wells (Wrenn & Venosa, 1996). These plates were then incubated for 24 hours at room temperature after which the wells were visually scored for a red precipitate. Once each plate was scored, the numbers of positive wells were entered into a computer program (Klee, 1993) to determine the most probable number of bacteria per ml of original sample. The Klee (1993) program gives MPN values that are corrected for a positive bias that is present in published MPN charts (Salama et al., 1978). After this number was determined, the number of bacteria per gram of soil was calculated.
DNA Extractions from Soil

Total DNA was extracted from soil using the commercially available MoBio UltraClean Soil DNA Isolation Kit (Solona Beach, Ca). DNA was extracted by following the manufacturer's protocol and using 0.5 g of soil. Total DNA was stored at -20°C until needed for other applications.

Ribosomal DNA Intergenic Spacer Region Analysis (RISA)

Isolated soil DNA was subjected to polymerase chain reaction to amplify the region between the 16S and 23S rRNA genes. The region was amplified in 50 µl PCR mixtures at the following concentrations or volumes: 1 µl of soil DNA from MoBio DNA extractions, 38.5 µl of sterile water, 5 µl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂ and 0.01% gelatin), 1 µl of 10 mM dNTP mix, 2.5 U of REDTaq Genomic DNA Polymerase. The primers used were 1406F (TGYACACACCGCCCGT) (universal for 16S rDNA) and 23SR (GGGTTBCCCCATTCRG) (bacterial 23S rDNA) (Borneman and Triplett, 1997). Both primers were used at a final concentration of 0.5 µM. Reagents were mixed and an equal volume of sterile mineral oil was placed on the mixture. PCR was performed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus). Thermal cycling conditions were as follows: 94°C for 2 min, 32 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min with a final extension of 72°C for 5 min. Once PCR was complete, 10-25 µl of product was run on a 1.2% agarose gel with Tris-Borate-EDTA (TBE) buffer for 24 hours. All gels were run at 4°C.
Gel Visualization

After electrophoresis was complete, the gel was stained in a 0.5 µl/ml ethidium bromide bath with 0.5x TBE buffer for a minimum of 10 minutes. DNA was then visualized by placing the gel on a Fotodyne Foto/Prep Ultraviolet transilluminator (Hartland, WI) using the analytical UV setting. Photographs were then taken on Polaroid 667 black and white film using the Fotodyne FCR-10 camera, with ethidium bromide filter (Exposure time 1.0 sec, F-stop 4.5).

Soil Hydrocarbon Extraction

Aliphatic and aromatic hydrocarbons were extracted and analyzed using gas chromatography/mass spectrometry by a previously published protocol (Means, 1998). This data was generously provided by Min Sun and Dr. Jay Means.

Data Analysis and Zones

Data was analyzed where appropriate using a chi-squared test with 1 degree of freedom. Microbiological data was grouped into zones for comparison. These zones were defined as a greater than 10% increase in percentage of alkane degrader over 2 sample points.

SOIL IMAGING

Sampling

Soil samples were collected on 6-7-00 from VRP site 5 and VRP site 9. Samples were collected as previously stated and a one gram sub-sample was aseptically collected from 10 cm below the water table at each site (Hiebert and
Bennett, 1992). This sample was then placed in 5 ml of sterile 0.1 M phosphate buffer.

**Treatment and Microscopy**

Samples were prepared for scanning electron microscopy (SEM) using an ethanol dehydration series of increasing concentrations of ethanol (10%, 30%, 50%, 75% and 100%) for 10 minutes at each step. This was done to ensure that any microbes attached to the soil particle remained visible. Upon completion of the dehydration series, the samples were affixed to a silver SEM stub using a press-on adhesive and gold-coated using a Polaron Autocoater. The samples were then observed and photographed under various magnifications using an ISI DS 130 Scanning Electron Microscope with an EDS x-ray diffraction spectrometer (Kevex-Moran).

**LAB DATA COLLECTION**

**Soil Collection and Sterilization**

In addition to field investigations, a separate laboratory column experiment was conducted to attempt to reproduce field conditions in a controlled setting. Soil was collected from approximately 15 cm below the surface between VRP 4 and VRP 5 at Carson City Park. The soil was loaded into 5 gallon buckets, sealed and then transported to Western Michigan University for homogenization. Homogenization was completed using a large concrete mixer. 25 gallons of soil were placed into the mixer and mixed for approximately 10 minutes. Then 2-3 gallons of soil were taken out and sifted to ensure that no large organic particles remained. At this time 2-3 more gallons of soil was introduced into the mixer and again
homogenized for 10 minutes. This process was repeated for all soil collected. Once homogenized the soil was placed into large aluminum pans and autoclaved for 3 cycles with the following conditions: 121°C, 15 PSI for 60 minutes. After sterilization, soil was aseptically sampled in a random fashion. These samples were then subjected to the above Cell Extraction and Colony Forming Unit Determination using 1/10 TSA to ensure that the soil was sterile.

**Columns**

Columns were constructed of 30.48 cm (12 inch) ID polyvinyl chloride (PVC) tubing cut into 80 cm (36 cm) cylinders. These tubes were affixed to a PVC base using PCV cement. The columns were equipped with fluid and gas sampling ports that were 2.54 cm width and extended for 30 cm. These ports were then covered internally with a rubber septum, filled with a silicone-based calk, and finally covered again on the exterior of the column with a rubber septum. Opposite the sampling ports was the vertical array of electrodes for the geoelectrical measurements. Two and one-half inch long, 1/8 inch diameter stainless steel screws serve as the electrodes. Thirty-two electrodes (screws) were placed every 2 cm for collection of a 1 cm Wenner array resistivity survey. The base of the columns was equipped with two brass ball valves, one for fluid input and one for outflow. Another brass elbow fitting was used and connected to a clear tube along the side of the column to serve as a manometer. Finally, a 1 inch PVC monitoring well was affixed to the inside of the columns for fluid level monitoring and water sampling. The monitoring well was screened at a depth of 65 cm from the surface.

The columns were filled completely with sterile 25% Bushnell Haas (25% BH) solution that served as a medium for calibration of the geoelectrical measurements.
Soil Sample Cores

Soil cores were made by vertically slicing a plastic well bailer. These bailers were then reinforced with circular plastic spacers placed vertically along the inside of the sample cores approximately every 3 cm. Plastic ties were then used to hold the pieces of the bailers together. Once this was completed the sample cores were wrapped in a fine weave synthetic mesh. Once completely wrapped with mesh the sample cores were again bound with plastic ties every 5 cm. Sample cores were then filled with pre-sterilized soil, wrapped in foil and then sterilized again. Cores were then introduced to the columns when the columns were filled.

Column Filling

Each column was drained of 25% BH used for calibration and filled with approximately 10 l of fresh sterile 25% BH. Then 5 soil-sampling cores were placed inside of each column. The columns were aseptically filled with soil using a large funnel to evenly distribute the soil amongst the soil-sampling cores. The columns were alternately filled with water and soil until each 75 cm column was full and 100% saturated with 25% BH. Columns were then left to electrically equilibrate for 10 days at which time they were subjected to various experimental treatments. During the course of monitoring the columns prior to the experiment, it became apparent that the uninoculated columns (1-4) were in fact contaminated with unintentionally introduced bacterial cells. Multiple rinses with a 1 ppm, 10 ppm and 100 ppm solution of sodium azide were executed to sterilize the columns. While this served to reduce the numbers of live cells, it did not sterilize the columns. The experiment was carried out with the knowledge that there would be background bacteria in the uninoculated columns.
**Column Treatments**

The columns were subjected to the following experimental conditions in duplicate: Columns 1 and 2 were filled with sterile soil and sterile 25% BH; columns 3 and 4 were filled with sterile soil, sterile 25% BH and diesel fuel; columns 5 and 6 were filled with sterile soil, 25% BH and extracted bacterial cells; columns 7 and 8 were filled with sterile soil, 25% BH, diesel and extracted bacterial cells.

**Column Inoculation and Contamination**

Soil was collected from the contaminated zone at VRP 4 site as previously described. Sterile 25% BH was used to do a shaking extraction with the ratio of 1 g of soil per 9.5 ml of 25% BH solution as in the above Cell Extraction Procedure. The mixtures were allowed to settle and then the fluid phase was aseptically siphoned away from the solid phase and collected in sterile glass carboys. The inoculation fluid was then used to completely saturate the columns that were to be inoculated with extracted bacterial cells. The remaining columns were then filled with sterile 25% BH until saturated. Once completely saturated, those columns that received the diesel treatment had 4 l of diesel layered onto the water, creating a free phase of diesel on the water. An equal volume of water was drained from each column to a final water level of 45 cm. This was considered day one of the experiment.

**Sampling**

The soil sampling was performed by extracting one soil core per column, per sampling event. The soil core was then cut open and soil samples were aseptically collected every 5 cm from 15 cm depth to 65 cm depth. The samples were then placed into gamma sterilized 15 ml polypropylene tubes.
Sub-sampling

Each sample was then placed in a sterile hood and a 5 g sub-sample was placed into a scintillation vial for chemical analysis and a 3 g sub-sample was placed into a sterile 1.5 ml microcentrifuge tube and placed at -20°C for RISA analysis.

Cell Extraction and MPN analysis

Cells were extracted and MPN analysis was conducted as previously described in Cell Extraction and Most Probable Number Determination sections above.

IV. RESULTS

SITE CHARACTERIZATION

In order to properly investigate shifts in microbial communities at the Carson City Park it was important to follow a systematic analysis of the area. Extensive mapping and characterization of the site led to the development of a site map that drove all further investigations (Figure 1). Within Carson City Park, five VRP locations were chosen as sampling points. Soil samples were taken for microbiological analysis from VRP 1, 4, 5, 9 and 10. Sites 1, 4 and 5 were chosen to represent soil that had free phase LNAPL. VRP 10 was chosen as a representative of the dissolved phase of the contaminant plume, while VRP site 9 was chosen as the negative control due to its distance from the plume and its isolation from the plume due to a drainage ditch in the south-east corner of the park (Figure 1).
METHODOLOGY COMPARISON

Initially two different methods of sampling and enumeration were compared to determine which would be the most useful in a long-term study of the soil microbial communities in Carson City Park. The two methods that were compared were a colony forming unit enumeration and a most probable number enumeration.

Results from both methods showed similar profiles of community structure at various VRP locations. CFU values ranged from $4 \times 10^2$ to $8.25 \times 10^5$ while MPN values ranged from $2 \times 10^2$ to $1 \times 10^7$. VRP site 5 was found to have 118% of the heterotrophic community composed of alkane degraders at 240 cm depth using the colony count method. A value over 100% was observed, indicating the drawbacks to culture based community analysis. In this instance our heterotrophic community was composed of 100% alkane degraders, but there was also an additional number of alkane degraders present on the alkane plates. This demonstrates that even a general growth medium such as 1/10 TSA may not be general enough to cover all of the specific nutritional requirements of specific bacterial types, like alkane degraders. The MPN analysis also indicated that 100% of the heterotrophic community was composed of alkane degraders at a depth of 240 cm (Figure 2). Results from both methods were found to be similar at other sites as well.

The similarity of the results was independent of contamination as it was also observed at VRP site 9 that was not contaminated with LNAPL. The percentage of the community composed of alkane degraders ranged from 0% to 0.13% with the CFU analysis, while ranging from 0.03% to near 80% (at one depth only) using the MPN analysis.
Figure 2. Comparison of Results from Different Enumeration Techniques. This figure illustrates results from enumerations of bacterial communities using two different techniques. (A) Represents colony forming units per gram of soil in May of 1999. (B) Represents most probable number per gram of soil in June of 2000. The solid line with solid square represents alkane degrading microorganisms. The solid line with the open triangle represents the heterotrophic organisms. The dashed line represents the depth of the water table on the day of sampling.

These initial comparisons demonstrated that both enumeration methods were accurate and robust. The MPN method was chosen for all future analysis because the resolution of smaller depth intervals was possible in sampling and the because of the relative ease and speed with which samples could be processed using this technique.

SPATIAL COMPARISONS

In addition to seasonal comparisons, spatial differences were analyzed. Samples were analyzed to detect any dependence on hydrocarbon as a source of
community change.

The contaminated sites VRP 1, 4, 5 and 10 were compared within Carson City Park (Figure 1).

In general, microbial measurements at VRP 1 were erratic but there were 3 distinct zones of increased and decreased fractions of alkane degrading bacteria out of the total community (Figure 3A). Zone I showed an increase in the ratio of alkane degraders to total heterotrophs occurring from 15 cm to 275 cm depth with ratio values ranging from 0.8% to 100% of the community within this zone. There actually appear to be milder fluctuations in this ratio within zone I. Zone Ia appears from 15 cm to 80 cm and indicates an average of 10.9% of the total heterotrophs being represented by the alkane degraders with ratio values ranging from 0.84% to 19.6% of the community. Zone Ib was found from 90 cm to 170 cm in depth and the alkane degraders represent an average of 5.4% of the community. The range of ratios of alkane degraders in this area fell between 1.3% and 13.5%. Zone Ic was found between 180 cm and 275 cm depth. In this range the alkane degraders represented an average of 33.4% of the community. The percentages of alkane degraders represented between 10% and 100% within this zone. The second zone (zone II) was indicated by a decrease in the ratio of alkane degraders to total heterotrophs and was found from 275 cm depth to 340 cm depth. Ratios ranged from 0.28% to 2.63% of the total community being composed of alkane degraders.

The third zone was indicated by an increase in the ratio of alkane degraders and occurred below the water table from 350 cm to 475 cm depth. Fractions of alkane degraders ranged from 2.8% to 100.0% of the heterotrophic population at this depth. Interestingly, the highest ratios of alkane degraders within zone Ic, from 180 cm to 275 cm, corresponds to the point where the geophysical readings begin to slowly taper off from near 1200 Ωm to nearly 800 Ωm by the end of this zone. The
third zone (zone III) within the VRP 1 community profile is also concurrent with a drop in resistivity from 85 Ωm to 50 Ωm (Figure 3A).

![Apparent Resistivity (Ωm)](image)

**Figure 3.** Depth Profiles of VRP 1 and VRP 4 Showing Bacterial Numbers and Electrical Resistivities. (A) VRP 1 Soil (B) VRP 4 Soil. Solid black line with squares represents MPN/g soil of alkane degraders, solid black line with open triangles represents MPN/g of total heterotrophs, solid grey line with circles represents apparent resistivity (Ωm), and dashed line represents water table on day of sampling.

VRP site 4 is also within the free phase of contaminants at Carson City Park. Within VRP 4 three distinct community zones were noticed (Figure 3B). However, in this case, Zone I was a zone in which alkane degraders represented only a small fraction of the total community. Zone I was found from 15 cm to 90 cm depth. Alkane degraders ranged from 0.9% to 26.4% of the community within this range. Zone 2 was found between 100 cm and 200 cm in depth and alkane degraders accounted for 41.3% of the community, with ratio values ranging from 3.8% to 55.5% of the community. The apparent resistivity within zone 2 showed a strong decrease.
from 1000 Ωm to 80 Ωm. Zone 3 was found in the saturated zone from 210 cm to 230 cm depth. Alkane degraders accounted for 3.8% of the community in this zone.

VRP site 5 is also located within the region of free phase LNAPL at Carson City Park. 3 distinct zones were observed within the community profile at this site (Figure 4A) as well. Zone I was from 15 cm to 60 cm in depth and demonstrated fractions of alkane degraders from 7% to 40% with the alkane degraders accounting for an average of 17.7% of the total heterotrophic community. Zone II showed a decrease in the fraction of alkane degraders from depths of 90 cm to depths of 230 cm, with ratio values ranging from 0.01% to 2.4%.

Figure 4. Depth Profiles of VRP 5 and VRP 10 Showing Bacterial Numbers and Electrical Resistivities. (A) VRP 5 Soil (B) VRP 10 Soil. Solid black line with squares represents MPN/g of alkane degraders, solid black line with empty triangles represents MPN/g of total heterotrophs, solid grey line with circles represents apparent resistivity (Ωm), and dashed line represents water table on day of sampling.
The alkane degraders accounted for an average of 0.46% of the heterotrophic community in zone 2. Zone III showed an increase in the fraction of the community with ratio values ranging from 3% to 100% within the zone from 245 cm to 305 cm. Alkane degraders at this level accounted for an average of 46.4% of the total heterotrophic community. The apparent resistivity at VRP 5 showed a steady decrease from 150 cm to 250 cm, dropping from 800 Ωm to near 100 Ωm at the bottom of the zone. In zone III there was a small drop in resistivity from 100 Ωm to 80 Ωm, but the resistivity profile at this point also showed a sharp Wenner shoulder indicating rapid changes from resistive to conductive soil within this region (noticeable as a zigzag pattern in the resistivity profile near 250 cm depth).

VRP site 10 is located in the area of residual phase LNAPL at Carson City Park. It also differs from the other sites in that there are 5 zones of community variation within this site (Figure 4B). Zone I occurs from 15 cm to 155 cm in depth and the alkane degraders represent an average of 21.1% of the community with ratio values of alkane degraders ranging from 2.2% to 74.2% of the community. Zone II shows a decrease in the ratio of alkane degraders from 165 cm and 215 cm in depth. The alkane degraders account for an average of 2.62% of the population in this zone which has ratio values of alkane degraders ranging from 0.5% to 5.6% of the heterotrophic community. The third zone (zone III) is found between 240 cm and 290 cm in depth. The community is composed of an average of 72.1% alkane degraders with percent values of alkane degraders ranging from 21.0% to 160.0%. It is interesting to note that within zone III there is a narrow zone where a sharp drop in resistivity from 150 Ωm to 100 Ωm occurs corresponding exactly to the highest ratios of alkane degraders. Zone IV extends from 300 cm to 460 cm in depth. Alkane degraders account for 2.6% of the community in this zone with percent values of alkane degraders ranging from 0.4% to 7.4%. Also within this zone, resistivity
increases above the water table from 200 $\Omega$m to 500 $\Omega$m. Zone V is found from 470 cm to 490 cm and represents an increase in the ratio of alkane degraders that accounts for an average of 9.3% of the total heterotrophic community. The ratio of alkane degraders within zone V ranges from 5.1% to 13.5% of the community.

VRP site 9 is located in an uncontaminated area of Carson City Park. There are 2 different zones within the microbial community profiles at this site (Figure 5). The first zone is found from 15 cm to 110 cm. Alkane degraders make up an average of 9.0% of the community at this site, with percentage values of alkane degraders ranging from 1% to 26.3% of the community. Zone two is found from 120 cm to 275 cm in depth with alkane degraders accounting for an average of 0.5% of the community. The percentage values of alkane degraders range from 0.2% to 1.0%. The apparent resistivity never shows a sloping decrease and stays very close to its 355 $\Omega$m average from just beneath the top soil to the saturation point of the soil near 250 cm.

When looking at a community profile from a contaminated site and a profile from a non-contaminated site, there are obvious differences (Figure 4A and Figure 5). The contaminated site, exemplified by VRP 5, shows zones of increased and decreased percentages of degraders, while the non-contaminated site, in this case VRP 9, shows only two zones. These zones indicate increases and decreases in ratio of alkane degraders. Nevertheless, it is important to note that when the ratio of alkane degraders increases at a contaminated site the ratio is on average 40% of the total community, while in a non-contaminated well alkane degraders make up only an average of 9.0% of the community. Notice also that the lines representing the two populations never converge.
It is also interesting to note that the apparent resistivity measurements in a non-contaminated well stay relatively consistent with depth, while a contaminated well shows extremely high values that gradually decrease as they traverse the contaminated zone.

In summary, it has been observed that there are fluctuations in the structure of microbial populations coincident with the presence of LNAPL and concurrent with regions of anomalously low resistivity.
To determine whether the observed changes in the ratio of alkane degraders to total heterotrophs were connected to the presence or absence of hydrocarbons a comparison of these factors was made. The relationship between community structure and hydrocarbon concentration was exemplified at VRP site 4 where hydrocarbons were detected with GC/MS from depths of 150 cm to 200 cm and hydrocarbon staining of the soil was visually observed at a depth of 100 cm. Increases in the ratio of alkane degraders to total heterotrophs were noted just below 100 cm depth and stayed near 50% of the community until a peak in absolute numbers of $3.72 \times 10^5$ occurred at 200 cm. There were decreases detected in the ratio of alkane degraders at 150 cm, possible due to hydrocarbon toxicity and from 215 cm to the last sample, probably due to the anaerobic nature of the sediments at these depths (Figure 6). These data indicate that the structure of the microbial community is shifting in response to the LNAPL stress to a community that is more adapted to the utilization of the LNAPL carbon source. This phenomenon was also observed at other free phase contaminated sites and dissolved phase contaminated sites as described below.

At the free phase LNAPL site VRP 1 aromatic and aliphatic hydrocarbons were first detected at a depth of 304 cm. There were two depths at which the ratio of alkane degraders increased. One of these areas was seen at 180 cm depth and the other was seen at 350 cm depth.
At the free phase LNAPL site VRP 5 aliphatic and aromatic hydrocarbons were detected with GC/MS at 220 cm, while visual inspection indicated that the hydrocarbon impacted zone began at 140 cm depth. The percentage of alkane degraders was the highest near 240 cm depth where they represented 100% of the total heterotrophic bacterial community.

At the dissolved phase site VRP 10 aliphatic and aromatic hydrocarbons were detected starting at a depth of 365 cm with GC/MS, and at a depth of 280 cm with
visual inspection. At 275 cm depth the alkane degraders accounted for 1.6 times the total heterotrophic community.

At the negative control site VRP 9 there were no hydrocarbons detected with GC/MS or with visual inspection at any depth. The percentage of alkane degraders never exceeded 18% of the community. This peak was observed at a depth of 80 cm (Figure 7). However, it is important to note that the lines representing the alkane degrading population and the heterotrophic population never converge as is observed in contaminated sediments.

![Graph](image)

**Figure 7.** Non-Contaminated Control Site VRP 9 Shows No Change in Percentage of Alkane Degrading Bacteria. VRP 9 Soil (June 2001). The dotted line represents water table on day of sampling. The dashed line with solid squares represents alkane degrading bacteria. The solid line with empty triangles represents the heterotrophic bacteria. The solid line with solid circles represents the aliphatic hydrocarbons. The dashed line with open triangles represents the aromatic hydrocarbons.
SEASONAL COMPARISONS

A more detailed analysis was done on VRP site 5 to compare samples taken from multiple months. In addition to sampling for microbial communities, direct current resistivity measurements were made. It was observed that there was a similar pattern of the community profile from VRP 5 from month to month with some small variations (Figure 8). It was also noted that these zones showed connections with resistivity profile changes. In a typical resistivity profile it is expected that the soil will be more resistive near the surface and should gradually decrease in resistivity until the soil becomes saturated with groundwater, at which point the resistivity should show relatively low fluctuation. Hydrocarbon should form a resistive layer just above the water table, but actual observations do not indicate this is the norm.

Once the samples and measurements were analyzed individually, these factors were compared with each other.

The April 2000 sample of VRP 5 demonstrates a key characteristic of this site. It was observed that there were three different zones of community composition within the depth profile of VRP 5 (Figure 8A). The first zone was found from 15 cm to 30 cm and was observed as an increase in the ratio of alkane degraders to total heterotrophs within this region. Geophysically this zone is characterized by high apparent resistivities near 10,000 Ωm.

The next zone (zone II) was observed immediately following this zone and was indicated by a decrease in the ratio of alkane degraders to total heterotrophs in the community. In zone II the alkane degraders never reached more than 1.5% of the total heterotrophs. Zone II extended from 45 cm to 215 cm in depth and geophysically was characterized by a steady decrease in apparent resistivity from
Figure 9. Comparisons of Monthly Samples for VRP 5. Black line with open triangle represents heterotrophs, black line with closed square represents alkane degraders, dashed line represents water table on date of sampling and grey line with circles represents the apparent resistivity profile on the day of sampling. (A) April 2000 sample (B) June 2000 sample (C) July 2000 sample (D) August 2000 sample (E) September 2000 sample.
The third zone (zone III) was observed starting at 230 cm depth and continuing to 335 cm depth. Zone III was characterized by an increase in the ratio of alkane degraders to total heterotrophs with ratios of alkane degraders ranging from 5% to 80% of the heterotrophic populations. Interestingly, as the geophysical measurements do not indicate a resistive layer where the soil is saturated with free phase product, instead resistivity values decrease to 50 Ωm, below the 75 Ωm recorded for the water-saturated sediment.

The June 2000 sample from VRP 5 showed a very similar pattern of increasing and decreasing ratios of alkane degraders with the heterotrophic organisms of the community (Figure 8B). Zone I was from 15 cm to 60 cm in depth and demonstrated fractions of alkane degraders from 7% to 40%. Zone II showed a decrease in the fraction of alkane degraders from depths of 90 cm to depths of 230 cm, with fractions ranging from 0.01% to 2.4%. Zone III showed an increase in the percentage of the community represented by alkane degraders with fractions ranging from 3% to 100% within the zone from 245 cm to 305 cm. Geophysically, these zones showed very similar patterns to the April samples again corresponding to the biological zones (Figure 8B).

The July 2000 sample indicated a similar pattern of increasing and decreasing ratios of alkane degraders to total heterotrophs also indicated 3 distinct community zones (Figure 8C). Zone I was observed between 15 cm and 60 cm depth with percentages of alkane degraders ranging from 5% to 90% of the heterotrophic populations. Zone II ranged from 90 cm to 215 cm and percentages of the community represented by the alkane degraders ranged from 0.01% to 1.25%. The Zone III began at 245 cm and ended at 290 cm and had percentages of alkane degraders ranging from 3.0% to 40.0%. The geophysical measurements for the July samples again followed the zonation of the bacterial community profiles. High apparent
resistivities were observed in zone I, steadily decreasing apparent resistivities were observed through zone II and the lowest resistivities were observed in zone III (Figure 8C).

The August 2000 sample demonstrated the same three zones (Figure 8D). Zone I ranged from 15 cm to 90 cm in depth and the percentages of alkane degraders within this zone were between 4.0% to 15.0%. Zone II ranged from 105 cm to 230 cm depth and percentage values for alkane degraders were between 0.01% and 1.00%. Zone III (260 cm to 335 cm) demonstrated an increase in the fraction of the community represented by alkane degraders, but ratio values had a high degree of variance and ranged from 0.05% to 26.0% of the heterotrophic population (Figure 8D). Similar geophysical results were observed with zone I, a steady decline of resistivity values were observed in zone II and the lowest resistivity values were observed at the beginning of zone III.

The sample from September 2000 again demonstrated a three zone profile (Figure 8E). Zone I was found between 15 cm and 105 cm depth and had alkane degraders’ percentage values ranging from 0.1% to 230%. We believe that the large increase in the ability of the community to degrade during September may be connected with leaf litter being deposited on the ground causing an increase in overall carbon for the bacterial communities closer to the surface. Zone II was found between 120 cm and 245 cm and had alkane degrading fractions ranging from 0.001% to 2.21%. Zone III was found between the depths of 285 cm and 370 cm and the alkane degraders represented percentages of the total heterotrophic population ranging from 7.0% to 135.1% (Figure 8E). Again, geophysical results seemed to follow the same 3 zones with high resistivity values in zone I, steadily decreasing values in zone II, and low values near the water table where free phase LNAPL is present in zone III.
These data suggest that there are small shifts in community structure from month to month, but the overall patterns of the population fractions remain the same. The same 3 zone profile is seen in each month, with the absolute values varying, but the overall community structure changing very little. Interestingly, we see a very consistent geophysical profile for each site studied. This supports the hypothesis that the geophysical profiles of the sediment respond to changes in microbial community structure and the chemical alterations that those changes effect. Because this is an aged site, many of these changes have already occurred, thus there are only very minor alterations in the geophysical profiles resulting mainly from water recharge events (i.e. rain). It is important to also note that the resistivity of the free phase LNAPL region of the soil is consistently lower than the apparent resistivity of the groundwater saturated sediments, indicating a more electrically conductive fluid is occupying the pore space of these sediments.

MOLECULAR ANALYSIS

In addition to MPN analysis a RISA analysis was done to study community structure at various times and sites. VRP 5 exemplifies the free phase contaminated sites and the June and August samples were chosen to attempt to account for any seasonal differences in community structure (i.e. from summer to fall) (Figures 9 and 10). Interestingly, three different zones were detected within the RISA profiles for each month’s samples.
Figure 9. Monthly RISA Profiles of VRP 5. RISA profiles from June 2000 (A) and August 2000 (B). Lanes represent descending vertical profiles from left to right. Marker at the right is a 1 KB marker sizes indicated are in base pairs.

This RISA analysis indicates zonation via banding patterns. Bands of the same length shared across lanes indicate that there are common community members, and band intensity indicates whether a particular member is dominant in that community.
Figure 10. Breakdown of RISA Profiles from June and August Samples of VRP 5. (A) June 2000 RISA, non-impacted sand (1 = 30 cm, 2 = 45 cm, 3 = 60 cm, 4 = 90 cm, 5 = 105 cm) (B) June 2000 RISA, impacted-sand (1 = 120 cm, 2 = 150 cm, 3 = 165 cm, 4 = 195 cm, 5 = 210 cm) (C) June 2000 RISA, impacted water (1 = 240 cm, 2 = 255 cm 3 = 270 cm, 4 = 285 cm, 5 = 300 cm) (D) August 2000 RISA, non-impacted sand (1 = 15 cm, 2 = 30 cm, 3 = 45 cm, 4 = 60 cm, 5 = 75 cm, 6 = 90 cm, 7 = 105 cm 8 = 120 cm)(E) August 2000 RISA, impacted sand (1 = 150 cm, 2 = 165 cm, 3 = 195 cm, 4 = 210 cm, 5 = 225 cm, 6 = 255 cm) (F) August 2000 RISA, impacted water (1 = 270 cm, 2 = 300 cm, 3 = 315 cm). The 1 KB marker bands that are shown are the 1018 bp and the 506 bp bands. White arrows indicate bands that are shared in both June and August samples. Striped arrows indicate unique bands.

The zones that are observed in the RISA patterns also seem to correspond closely with the various conditions that are found within the soil, in much the same way that the MPN profiles do. That is to say that the most diversity is observed in the non-hydrocarbon impacted sand (indicated by numerous non-intense bands), while the LNAPL impacted regions (residual-phase and free-phase LNAPL) show decreased diversity (fewer bands), but an increase in dominant members (more
intense bands). There is a clear difference between the communities found in the impacted sand and those found in the impacted water, as observed by the differences in band location between these two environments, but the most differences are observed when comparing non-impacted sand with impacted sand or water (Figure 10).

There are definite similarities between the June and August sample. For instance, in the non impacted zone there are few prominent bands, however both have an ~900 base pair (bp) band and ~600 bp band at several depths (Figure 10A and 10D).

The RISA analysis of the LNAPL impacted sand also indicates similarities in the structure of the microbial communities in June and August. For instance, both June and August samples share a set of prominent bands near 800 bp and they also share bands of ~750 bp, ~725bp and ~650 bp at various depths. However, prominent bands near 775 and 700 bp in length seem to be unique to the August sample (Figure 10B and 10E).

It seems that diversity within the impacted water is higher than in the impacted sand in both the June and August samples. The samples share prominent bands at 1000 bp, 900 bp, as well as a pair of bands at 750 bp and 600 bp respectively (Figure 9 and 10). Both months also share a band near 490 that only appears in the impacted water region. The June sample shows unique bands at 580 bp and 530 bp (Figure 10C and 10F). The higher diversity in the impacted water indicates that there may be less interaction between microbes and LNAPL in the water. This lack of interaction allows more diversity, but still shows less diversity than the non-impacted sand.

In addition to these similarities between months it was also observed that the RISA profile for each month corresponds to the profile of the resistivity for that
month (Figure 11). Therefore, the transition between the non-impacted zone and LNAPL impacted soil is coincident with the beginning of the anomalous resistivity zone and with the shift in the RISA profile from a very diverse community with few prominent members to a less diverse community with several dominant members.

The three zones in VRP 5 the RISA profiles can be contrasted with only two obvious zones found in the VRP 9 RISA profiles (Figure 12). These zones fall within the same depths as observed with the MPN analysis.

VRP 9 RISA analysis (Figure 12B) indicates that there are several common bands through out the different depths. In the non-impacted sand of VRP 9, there are bands at 980 bp, 800 bp, 750 bp, 600 bp, and 490 bp. Not only are these prominent bands observed in the non-impacted sand of VRP 9, but they are also present at certain depths in the non-contaminated water of VRP 9. The most predominant member of VRP 9 has a RISA band that falls near 490 bp. This band is seen with various intensities at each sampled depth.

VRP 9 is contrasted with VRP 5 (Figure 12A) in which there is three distinct zones. The non-impacted sand zone of VRP 5 has few distinct bands, but it does share bands at 980 bp and 490 bp with both the non-impacted sand of VRP 9 and with the impacted sand and water of VRP 5. However there are 3 dominate band pairs in the impacted sand at 850/800 bp, 750/700 bp and 600/550 bp.
Figure 11. Apparent Resistivity Profile for VRP 5 Compared to RISA Profile from VRP 5. Notice that the start of the decrease in resistivity (black arrow) corresponds with the LNAPL impacted soil and the RISA profile parallels those changes. The small white arrows indicate prominent community members, while the long striped arrows represent members that are common to multiple depths.

This indicates that there are not only more prominent members in the contaminated soil, but that the members that are present are different from those in the non-contaminated soil. This is expected due to the selective pressure that the LNAPL contamination places on the community.
Contrast can also be drawn between the impacted water of VRP 5 and the non-impacted water of VRP 9. VRP 5 has unique bands at 900 bp, 760 bp and 525 bp, but shares bands with VRP 9 at 750 bp, 600 bp and 490 bp. It is also interesting to note, that while the non-impacted water of VRP 5 is very diverse from depth to depth, the impacted water of VRP 9 shows a very consistent RISA profile at almost every depth, suggesting homogeneity within the environment (Figure 12).

So in summary, the microbial communities from Carson City Park non-impacted water and sediment show more species richness and evenness than LNAPL impacted water and sediment, which show decreased species richness and decreases evenness of species.

MICROSCOPIC STUDIES

In addition to studying the composition of the microbial communities at various sites, it was also important to investigate potential geological changes that
may result from the action of impacted microbial communities. In order to investigate the hypothesis that minerals in contaminated soil are weathered at an increase rate due to organic acid production by LNAPL degrading microbes, soil grains from a contaminated and a non-contaminated well were compared.

VRP 5 was chosen as the contaminated study site and soil grains were imaged and observed for characteristic feldspar etching patterns (Figure 13).

The low power image (Figure 13A) shows several etched feldspar grains as evidenced by the jagged, rough nature of a majority of the grains in this typical field. The medium power image indicates an etched nature to this particular soil grain. Notice that there are round weathered pits and several “V” shaped etched areas on the particle (Figure 13B). When particles were observed on high power, “V” shaped etching in cleavage patterns (Figure 13C), round pits and in some instances attached bacteria were observed (Figure 13D). 161 etched grains were observed under various magnifications to check for characteristic acid etching/weathering of the feldspar soil particles, and 72.0% of those soil particles were found to have characteristic etching.

Several other grains were observed from VRP 5 soil that had a very “severe etching pattern”, as if they were exposed to a more concentrated acid. In our study we routinely observed this severe etching pattern (Figure 14A) and was found to be remarkably similar to data published by Berner and Holdren (1979) (Figure 14 B and C). Weathering results in deposits of clays on the surface of the feldspars and many soil particles observed carried this motif (Berner & Jr., 1979; Holdren & Berner, 1979).
Figure 13. VRP 5 Feldspar Soil Grain Images. (A) Low power (100x) image of a typical field of soil particles (bar = 200 µm) (B) Medium power (500x) image of a typical VRP 5 soil particle (bar = 100 µm) (C) High power (5000x) image of VRP 5 soil particle (bar = 2 µm) (D) High power (5000x) image of VRP 5 soil particle (bar = 2 µm). Small arrows indicate characteristic etching, while the large arrow indicates a potential diplococci bacterial cell pair.
Soil taken from a non-contaminated site, VRP 9, was also analyzed. VRP 9 demonstrated minimal etching, of 244 soil particles observed only 4 (1.6%) showed visible etching. Under low magnification most of the observed particles had a smooth appearance and lacked the clay layer associated with many of the VRP 5 soil particles observed (Figure 15A). Closer examination of these particles revealed that they were generally smooth or, if they were etched, the etching was much less severe (Figure 15B). Higher magnification images revealed that there was indeed minimal etching showing only small numbers of etch pits (Figure 15C) or no etching on smooth fracture lines (Figure 15D). A Chi-Squared analysis was conducted to test the hypothesis that the presence of LNAPL degrading bacteria had no effect on the etching of sediments in Carson City Park. 244 soil particles observed from VRP 9 and four were etched, 161 soil particles were observed from VRP 5 and 116 were found to be etched. The critical value for a Chi-Squared test with 1 degree of freedom is 3.84, the calculated Chi-Squared value for this data set was 230.6 (p< 0.0001). The
null hypothesis is rejected and it was concluded that hydrocarbon degrading bacteria affect the etching of soil particles.

Figure 15. VRP 9 Feldspar Soil Grain Images. (A) Low power (100x) image of a typical field of soil particles (B) Medium power (500x) image of a typical VRP 9 soil particle (C) High power (5000x) image of VRP 9 soil particle (D) High power (5000x) image of VRP 9 soil particle.

SOIL COLUMN EXPERIMENTS

Soil columns were designed and implemented in order to elucidate the relationship between four parameters, LNAPL contamination, geochemical parameters, microbial communities and electrical resistivity. Samples were taken and
processed using MPN analysis and RISA analysis.

The uninoculated columns received two treatments, VRC 1 and 2 were not contaminated with diesel fuel, while VRC 3 and 4 were contaminated with diesel fuel. The water table in all columns was at 45 cm. It was attempted to keep the uninoculated columns sterile, but our analysis indicated that sterile conditions were not achieved (see below).

VRC 1 and 2 demonstrate that as time progresses there is an increase in the ratio of alkane degraders to total heterotrophs in the community profiles, but there is no consistently noticeable zonation occurring (Table 1). The highest ratio of alkane degraders was found near the water table, with the highest values observed in the 12-27-01 and 2-2-02 samples, with the highest ratio being 55.36% from the 2-2-02 sample in VRC 1.

VRC 3 and 4 demonstrate that even in columns not inoculated with Carson City Park bacterial communities there seems to be a tendency towards zonation within contaminated columns. While the 12-27-02 samples from VRC 3 and 4 seem quite similar in its zonation patterns, both having a zone of higher percentages of alkane degraders at 35 cm and 40 cm, the third sample shows lower absolute values and the same zonation does not seem to carry through. However, there seems to be a difference between columns 3 and 4 in that column 3 shows a zone of increased percent values of alkane degrades from 55 to 65 cm in both the second and third samples, while there is no similar peak in the second and third samples of VRC 4. In addition to looking at the MPN analysis for the uninoculated columns the RISA profiles were also analyzed (Figure 16).
Columns one and two show relatively consistent RISA profiles across not only time, but also between replicates. There is one predominant band which all samples from all times share at ~625 bp. Column 1 shows a region of prominent bands between ~750 bp and ~625 bp, while column 2 shows a different region of intense bands from ~950 bp to ~800 bp. These bands were the most prominent in the 11-8-01 sample, with 4 bands being easily identified, but were less intense in the second and third samples where there were only 2 dominant bands (Figure 16A and

### Table 1

Percentage of Alkane Degraders in Uninoculated Columns

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>VRC 1</th>
<th>VRC 2</th>
<th>VRC 3</th>
<th>VRC 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.21%</td>
<td>0.48%</td>
<td>3.80%</td>
<td>0.13%</td>
</tr>
<tr>
<td>20</td>
<td>0.06%</td>
<td>2.63%</td>
<td>7.72%</td>
<td>0.13%</td>
</tr>
<tr>
<td>25</td>
<td>0.17%</td>
<td>0.48%</td>
<td>6.25%</td>
<td>0.02%</td>
</tr>
<tr>
<td>30</td>
<td>0.22%</td>
<td>1.00%</td>
<td>6.25%</td>
<td>0.06%</td>
</tr>
<tr>
<td>35</td>
<td>0.75%</td>
<td>0.82%</td>
<td>55.36%</td>
<td>0.00%</td>
</tr>
<tr>
<td>40</td>
<td>0.01%</td>
<td>10.00%</td>
<td>1.23%</td>
<td>0.01%</td>
</tr>
<tr>
<td>45</td>
<td>3.80%</td>
<td>28.40%</td>
<td>1.23%</td>
<td>0.55%</td>
</tr>
<tr>
<td>50</td>
<td>19.53%</td>
<td>29.81%</td>
<td>13.47%</td>
<td>0.11%</td>
</tr>
<tr>
<td>55</td>
<td>1.95%</td>
<td>13.38%</td>
<td>1.00%</td>
<td>0.25%</td>
</tr>
<tr>
<td>60</td>
<td>1.00%</td>
<td>2.87%</td>
<td>1.95%</td>
<td>0.06%</td>
</tr>
<tr>
<td>65</td>
<td>1.00%</td>
<td>4.52%</td>
<td>1.35%</td>
<td>1.00%</td>
</tr>
</tbody>
</table>
In the 12-27-01 sample, columns 1 and 2 lost the band at 750 bp and 800 bp, but a band near 650 bp began to intensify. Interestingly, both VRC 3 and 4 developed intense bands around 900 bp. In column 3 this band were observed in the 15 cm and 20 cm samples, while this band was only present in the 15 cm sample from VRC 4. The third sample continued the trend of decreased diversity over time. The band that was present at 900 bp became more intense and was observed at 20 cm in column 4 where it had not previously been observed (Figure 16C and 16D).

Figure 16. RISA Profiles of Uninoculated Columns. (A) RISA profiles of VRC 1 (B) VRC 2 (C) VRC 3 (D) VRC 4. Each box denotes a different sample (11-8-01, 12-27-02, 2-2-02) arranged chronologically from left to right across the pictures. Samples are arranged by depth starting at 15 cm to the left of each box and going to 65 in 15 cm increments.
The columns that were inoculated with bacterial communities from Carson City Park were treated in a similar fashion. Columns 5 and 6 received bacteria, but no diesel fuel, and columns 7 and 8 received bacteria and diesel fuel. The water table on these columns was 45 cm also.

In columns 5 and 6, the 11-8-01 samples indicated a very low percentage of alkane degraders throughout the depth of the columns (Table 2). The 12-27-01 sample showed some shifting in the MPN profiles in that the absolute percent values are higher and a zone is forming from 55 cm to 65 cm. The alkane degraders account for an average of 14.36% of the community in this zone in VRC 5 and they account for 10.9% of the community. The 2-2-02 sample showed only small changes from the 12-27-02 sample. The percent values of the alkane degraders increase, but the zonation at the bottom between 55 cm and 65 cm was similar. In VRC 5, the average percentage of alkane degraders in this zone actually decreased slightly accounting for only 9.5% of the community in the third sample.

VRC 6 showed a similar decrease with alkane degraders in the third sample accounting for only 8.9% of the community.

In columns 7 and 8 there was a higher percentage of alkane degraders in the 11-08-01 sample than in any of the other columns. However the ratio values were still low, with the maximum value of 10% found in the 11-08-01 sample from VRC 7 (Table 2). The 12-27-014 sample showed a very clear pattern of zonation occurring in both columns 7 and 8. Column 7 developed a zone of increased percentage of alkane degraders out of total heterotrophic organisms form 45 cm to 65 cm depth. The alkane degraders accounted for 1.2x (120%) the number of heterotrophic bacteria within this zone. From 15 cm to 40 cm in depth, the alkane degraders accounted for only 0.46% of the total community.
This trend was also observed in the 12-27-01 sample from VRC 8, but the zone of increased ratio of alkane degraders ranged from 50 cm to 65 cm depth and alkane degraders accounted for an average of 76.1% of the heterotrophic bacteria.

The third samples in columns 7 and 8 showed an increase in the percentages of alkane degraders. The new zone of increased percentage of alkane degraders near the top of the columns (zone 1) ranged from 15 to 20 cm in VRC 7 but was only found at 20 cm in VRC 8. In VRC 7 the average percentage of alkane degraders was 24.1%, while in VRC 8 the alkane degraders in this zone represented 18.06% of the

### Table 2

Percentage of Alkane Degraders in Inoculated Columns

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>VRC 5</th>
<th>VRC 6</th>
<th>VRC 7</th>
<th>VRC 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.18%</td>
<td>0.24%</td>
<td>0.43%</td>
<td>0.10%</td>
</tr>
<tr>
<td>20</td>
<td>0.13%</td>
<td>1.00%</td>
<td>0.26%</td>
<td>1.60%</td>
</tr>
<tr>
<td>25</td>
<td>0.07%</td>
<td>0.82%</td>
<td>13.47%</td>
<td>0.08%</td>
</tr>
<tr>
<td>30</td>
<td>0.01%</td>
<td>0.96%</td>
<td>10.00%</td>
<td>1.00%</td>
</tr>
<tr>
<td>35</td>
<td>0.00%</td>
<td>4.11%</td>
<td>0.24%</td>
<td>0.12%</td>
</tr>
<tr>
<td>40</td>
<td>0.00%</td>
<td>1.26%</td>
<td>0.18%</td>
<td>0.11%</td>
</tr>
<tr>
<td>45</td>
<td>0.22%</td>
<td>1.35%</td>
<td>1.00%</td>
<td>0.02%</td>
</tr>
<tr>
<td>50</td>
<td>0.00%</td>
<td>3.52%</td>
<td>2.43%</td>
<td>0.11%</td>
</tr>
<tr>
<td>55</td>
<td>0.06%</td>
<td>3.80%</td>
<td>13.38%</td>
<td>1.00%</td>
</tr>
<tr>
<td>60</td>
<td>0.02%</td>
<td>38.01%</td>
<td>10.00%</td>
<td>0.06%</td>
</tr>
<tr>
<td>65</td>
<td>0.00%</td>
<td>1.21%</td>
<td>5.12%</td>
<td>5.54%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Depth (cm)</th>
<th>VRC 7</th>
<th>VRC 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1.06%</td>
<td>1.00%</td>
</tr>
<tr>
<td>20</td>
<td>0.22%</td>
<td>0.38%</td>
</tr>
<tr>
<td>25</td>
<td>0.38%</td>
<td>3.47%</td>
</tr>
<tr>
<td>30</td>
<td>1.00%</td>
<td>0.05%</td>
</tr>
<tr>
<td>35</td>
<td>1.81%</td>
<td>0.13%</td>
</tr>
<tr>
<td>40</td>
<td>3.35%</td>
<td>1.00%</td>
</tr>
<tr>
<td>45</td>
<td>4.11%</td>
<td>5.82%</td>
</tr>
<tr>
<td>50</td>
<td>10.00%</td>
<td>0.85%</td>
</tr>
<tr>
<td>55</td>
<td>4.11%</td>
<td>7.42%</td>
</tr>
<tr>
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<td>1.70%</td>
<td>7.42%</td>
</tr>
<tr>
<td>65</td>
<td>2.43%</td>
<td>3.80%</td>
</tr>
</tbody>
</table>
heterotrophic community. The next zone in both of the columns was a zone of decreased fractions of alkane degraders from 25 cm to 30 cm in both columns. In VRC 7 and 8 the alkane degraders accounted for 0.28% and 0.17% of the heterotrophic community respectively. The third zone in each column ranged from 35 cm to 65 cm. In VRC 7, the alkane degraders accounted for 83.8% of the total heterotrophic population with percent values in this zone ranging from 1.4% to 380% (3.8X). VRC 8 showed the average percentage of alkane degraders in this zone to be 123.4%, or 1.23 times greater, than the heterotrophic populations within this zone. The percent values in this zone had a high degree of variability ranging from 0.38% to 742.5% of the community within this zone being able to degrade alkane.

The RISA analysis for columns 5 and 6 showed very similar dominant banding patterns through all three samples. The main change occurred in the 12-27-01 sample in VRC 6 at a depth of 45 cm where there was the sudden emergence of a dominant band at 675 bp. This band was not dominant in the third sample at the same depth (Figure 17A and 17B)

The RISA analysis of the communities in columns 7 and 8 showed a shift towards less diversity over time. In sample one, dominant bands were found near 800 bp, 750 bp, 650 bp and 625 bp in both column 7 and column 8. However, the larger bands were more dominant in column 7 than in column 8 (Figure 17C and 17D).

In the 12-27-01 a new band of 900 bp appeared at 15 cm in column 7 and at 15 cm and 25 cm in column 8. These bands continued to intensify into the third sample, where they were present in both columns at 15 cm and 25 cm.

In summary, we have demonstrated that the alteration of microbial communities in an LNAPL contaminated zone is a slow, stepwise process. LNAPL contamination puts a selective pressure on the microbial communities, causing them to adapt to a composition that is more capable of the utilization of an alkane as a
carbon source. This is apparent in the MPN analysis by the increases in the ratio of alkane degraders. Gradual shifts in microbial community structure have also been demonstrated with the more robust RISA analysis that shows a decrease in overall species richness and a concurrent decrease in species evenness.

Figure 17. RISA Profiles of Inoculated Columns. (A) RISA profiles of VRC 5 (B) VRC 6 (C) VRC 7 (D) VRC 8. Each box denotes a different sample (11-8-01, 12-27-02, 2-2-02) arranged chronologically from left to right across the pictures. Samples are arranged by depth starting at 15 cm to the left of each box and going to 65 in 15 cm increments.

These column experiments have also demonstrated that the communities in the soil at Carson City Park are “pre-adapted” to LNAPL stress as evidenced by the more rapid zonation and ratio shifting that occurred in the inoculated columns.
V. DISCUSSION

Prior to the work described here, there has been little or no work done to investigate the effects of microbial communities on geophysical properties of LNAPL contaminated sediments. While almost nothing is known about the relation between geophysics and microbiology, initial studies performed in the lab in small scale bioreactors over short periods of time (120 days) have indicated that microbial communities under both aerobic and anaerobic conditions can cause an increase in the dissolution rates of calcium ions from calcium silicate sands (Cassidy, et al., 2001). In addition to increased ion content of pore water it was also demonstrated that there was a concurrent increase in the concentration of volatile organic acids (VOA) and a concurrent decrease in the surface tension of the pore water. Throughout the experiment it was also noted that there was a steady increase in electrical conductance (inverse resistance). These results are contrasted sharply with the sterilized control reactors, in which none of the above changes were noted. This indicates that microbial activity is the primary driving force behind not only geochemical changes, but also geophysical changes (Cassidy, et al., 2001).

Volatile organic acid production is known to be a result of LNAPL degradation and is known to take place primarily, but not exclusively under anaerobic conditions (Cozzarelli, et al., 1994; Cozzarelli, et al., 1990; McMahon and Chapelle, 1991; McMahon, et al., 1995; Thorn & Aiken, 1998). These organic acids are known to accumulate as petroleum products are degraded within contaminated sediments (Cozzarelli, et al., 1994; Cozzarelli, et al., 1990). Organic acids are known to accelerate the dissolution of feldspars by complexing the metals on the surface of the minerals which prevents oxidation and thus speeds the process of mineral leeching (Berner & George R. Holdren, 1977; Berner and Jr., 1979; Hiebert and Bennett, 1992;
Nevertheless, the implications for monitoring this information have never been applied to a model indicating how it should affect the electrical properties of the system.

Until recently it was commonly believed that LNAPL spills would result in a resistive layer in the soil when conducting direct current resistivity measurements (DeRyck, et al., 1993). In 1997, a previously unknown LNAPL plume was discovered using purely geophysical measurements (Bermejo et al., 1997). This new plume was noticed based on a highly conductive shadow zone observed while conducting a ground penetrating radar survey at Wurtsmith Air Force Base in Oscoda, Michigan. This plume has since been characterized with multiple geophysical methods, and it has been demonstrated that aged, free-phase, LNAPL actually creates an electrically conductive rather than a resistive layer in the subsurface (Atekwana, et al., 1998; Sauck, 1998; Sauck, 2000; Sauck, et al., 1998). The modeling of this plume as well as other plumes (Sauck, 2000) has since demonstrated that the resistive model for the geoelectrical response of LNAPL contaminated soil is flawed, and a new model must be generated to account for the anomalously conductive groundwater that is observed in aged LNAPL contaminated sites.

The work presented in this study indicates that there is a definite effect of LNAPL contamination on the microbial community structure in the impacted sediments. LNAPL causes a concentration dependent shifting of the structure of a microbial community from having a low ratio of alkane degrading microbes to a community that has a high ratio of alkane degrading microorganisms. In some instances it was even observed that the ratio of alkane degraders increased to over 1x of the total heterotrophic population. This effect is probably attributed to the fact that the community was so adapted to LNAPL stress that when it was placed in a general
growth medium (10% TSB) there were actually fewer organisms adapted to this simple carbon source then to the more complex alkane source. There is an obvious LNAPL concentration effect on the structure of the community as well. Most often community profiles indicated that there was a large ratio of alkane degrading organisms near the surface, which is attributed to the adaptation of humic acid degradation, which is similar in structure to alkanes. Then, as cells were enumerated into the subsurface it was generally observed that there was a decrease in the ratio of alkane degrading organisms to heterotrophic organisms. However, as the concentration of the LNAPL gradually increased in the pore water, the ratio of alkane degraders increased in one of two ways; either there was a very noticeable decline in the heterotrophic population due to hydrocarbon toxicity, or there was an increase in the background of organisms that were capable of alkane degradation. In either case, as the concentration of LNAPL increased, the ratio of alkane degrading organisms increased simultaneously.

Shifting of the community to a higher percentage of alkane degrading organisms was indirectly detected using direct current vertical resistivity probes. Vertical resistivity profiles indicate areas of anomalously low resistivities both in the saturated zone and in the vadose zone, when these measurements are compared to a non-LNAPL contaminated background site. This difference is attributed to enhanced mineral dissolution caused by the LNAPL-degradation of the shifted microbial community.

Evidence of mineral dissolution in LNAPL impacted sediments at Carson City Park was obtained by scanning electron microscopy in our study. Scanning electron microscopy has previously been used in a variety of analytical techniques to analyze weathering of minerals from both native and artificial environments (Berner and George R. Holdren, 1977; Berner and Jr., 1979; Hiebert and Bennett, 1992; Hiebert,
et al., 1995; Holdren and Berner, 1979; McMahon and Chapelle, 1991; McMahon, et al., 1995).

Our studies have indicated that there is enhanced mineral dissolution at LNAPL contaminated sites, when compared to non-contaminated control sites. This is indicated by the increased number of characteristically etched feldspar grains taken from contaminated sites. Enhanced dissolution is further evidenced by the deposition of aluminosilicate “clay” minerals on the surface of native feldspar particles (Berner and Jr., 1979; Holdren and Berner, 1979).

Other studies have been done that have very accurately defined the subsurface microbiology of an LNAPL contaminated site in Bemidji, MN. This aquifer was contaminated with crude oil in 1979 when a pipeline burst, all but 30% of the 10,500 barrels of spilled oil was physically removed from the site. As it exists now the contaminated aquifer is characterized by a 1 m thick, and 80 m long crude oil plume with a ~6500 m² spray zone 180 m to the west-southwest of the spill (Cozzarelli, et al., 1994; Cozzarelli, et al., 1990; Thorn and Aiken, 1998). Within this total area of contamination there are 5 distinct geochemical zones. Zone I is represented by oxygenated and uncontaminated groundwater upgradient of the spill. Zone II is marked by an increased concentration of dissolved organic carbon (i.e. dissolved phase LNAPL). Zone III is represented by anaerobic groundwater that is in physical contact with the free phase product. Zone IV is the groundwater that directly surrounds the anaerobic zone III and is often labeled the “transition zone”. Zone V represents the oxygenated groundwater down gradient of the free phase product (Bekins, et al., 1999; Thorn and Aiken, 1998).

Within these various zones, biodegradative processes have been found responsible for the production of non volatile organic acids (Cozzarelli, et al., 1994; Cozzarelli, et al., 1990; McMahon and Chapelle, 1991; McMahon, et al., 1995; Thorn
and Aiken, 1998). These acids have been found to change type and amounts over time and space as degradation occurs in zones of iron reduction and methanogenesis. It has also been demonstrated that as the various electron acceptors are used up, there is an accumulation of organic acids in the groundwater near the petroleum plume (Cozzarelli, et al., 1994; Cozzarelli, et al., 1990).

Thorn and Aiken (1998) have also demonstrated that as a result of biodegradation a plume of dissolved organic carbon consisting primarily of non-volatile organic acids has developed down gradient of the plume in the spray zone (Thorn and Aiken, 1998).

In addition to these geochemical observations Bekins et al. (1999) used a most probable number method to delineate the spatial distribution of some major physiological types within and around the central contaminant plume (Zone III). Both free living and attached aerobes, denitrifiers, iron-reducers, heterotrophic fermenters, sulfate-reducers and methanogens were sampled and a physiological map was created (Bekins, et al., 1999). Small methanogen populations were found to be connected with high numbers of iron reducers, while low numbers of iron-reducers were found to be connected with higher numbers of methanogens, aerobes, sulfate-reducers or fermentative bacteria (Bekins, et al., 1999).

Additionally, Rooney-Varga et al. (1999) found that anaerobic benzene degradation under iron-reducing conditions is connected with high numbers of Geobacter spp. This study used DGGE and MPN-PCR to show that Geobacter spp. was present in significantly higher numbers where benzene oxidation was occurring in an iron reduction zone. This finding is interesting because Geobacter are the only pure culture species that can completely mineralize aromatic benzene compounds under iron-reduced conditions (Rooney-Varga et al., 1999). In the future the work presented in this thesis could be broadened to include analysis for various electron
zones within the soil using PCR probes designed for metal reduction genes and hydrocarbon degradation genes (Chandler & Brockman, 1996).

Initial molecular investigations however, have provided useful information on the general structure of the extractable communities in Carson City Park soils. There are various methods that have been used to analyze microbial communities in hydrocarbon contaminated sites (Dojka et al., 1998; Macnaughton, et al., 1999). Additionally, there are several other methods that have been used to analyze the structure of the microbial communities in non-contaminated soil (Acinas, et al., 1997; Liu, et al., 1997; Moseneder, et al., 1999; Ritchie et al., 2000; Roling et al., 2000). We chose to utilize rDNA intergenic spacer region analysis (RISA) for several reasons. It does not require the use of any special apparatus, is a very simple procedure and it eliminates the need for enzymatic digestion after amplification of the region (Garcia-Martinez, et al., 1999). Clearly there are more 16S gene sequences known, but as more genomes become completely sequenced RISA will become more and more useful as a tool, not just to delineate the general structure of a community, but to identify individual members of that community using band sequences (Ranjard et al., 2000; Ranjard et al., 2000). Additionally as automated approaches are developed the RISA approach will be useful in making quantitative measurements of species richness and evenness, where a standard RISA approach can only show you a qualitative estimate of these parameters (Borneman and Triplett, 1997; Fisher and Triplett, 1999).

Additionally, RISA has also been used to demonstrate that there may be differences in community analysis profiles depending on the DNA extraction technique that is used with the soil (Martin-Laurent et al., 2001). RISA analysis proved a useful tool in demonstrating total community response to LNAPL contamination and was also used to demonstrate that shifting of
community structure can be shown observationally to parallel the anomalous geophysical response observed at Carson City Park and also supported the zonation effect that was observed with the MPN approach to microbial community analysis.

In conclusion, we have demonstrated evidence to support the hypothesis that there are changes that occur in the structure of a microbial community due to the presence of LNAPL contamination. It was observed that the percentage of alkane degraders near the water table increases in response to the presence of LNAPL. We observed three distinct microbial zones at sites 1, 4 and 5. VRP sites 1 and 5 were found to have a zone of increased alkane degradation near the surface, probably due to the adaptation of the community at shallow depths to humic acid degradation, which are similar in structure to many hydrocarbons. A second zone, indicated by a low percentage of alkane degraders, was observed between Zone 1 and the water table. The third zone was observed near the capillary fringe and into the saturated sand in the groundwater and consisted of high percentages of alkane degrading bacteria.

VRP site 4 was found to have low percentages of alkane degraders near the surface and in the saturated sediments, but was found to have a large zone II that had high percentages of alkane degrading bacteria. The difference between VRP sites 1 and 5 and VRP site 4 is in the low topographic position of site 4 and the relatively large impacted zone (Figure 3).

VRP site 10 responded to the dissolved phase LNAPL in a 5 zone pattern, but a zone of high percentages of alkane degraders was observed near the water where hydrocarbons were dissolved.

The contaminated sites are contrasted by non-contaminated, site 9 which showed only 2 zones. Zone I showed slightly increased percentages of alkane degraders near the surface and Zone II showing low percentages of alkane degraders
as the soil is saturated with water.

RISA profiles showed corresponding shifts in community structure, with non-LNAPL-impacted sand demonstrating a high degree of diversity and few prominent members, while impacted sand and water show a lower degree of diversity and more dominant members. Several of the dominant members were common to several depths, but none were as consistent as non-contaminated sand, which showed very few changes in structure from depth to depth.

In addition to demonstrating community shifts in response to LNAPL we have shown that community profiles and zonation parallel anomalous geophysical measurements. Percentages of alkane degraders were found highest where apparent resistivities were lowest. This indicates a connection between resistivities and community structure. This conclusion was again supported by RISA profiles that indicate a decreased diversity corresponding to decreased apparent resistivities. This suggests that as the community adapts to degrade LNAPL it affects the physical properties of the system.

To confirm this hypothesis the physical structure of soil particles were compared using SEM. These experiments indicated that there was a higher degree of etching in contaminated sediments than in non-contaminated sediments.

When taken as a whole, the data from this work support the hypothesis that changes in microbial community structure brought about by LNAPL contamination are connected to an increase in mineral weathering. Thus, they are connected to anomalous geoelectrical signatures that are observed in the contaminated soils found in Carson City Park, Carson City, MI.


