

Vertical Profile of the Bacterial Communities in Biovented and non-Biovented Petroleum Contaminated Cores using Terminal Restriction Fragment (TRF) Pattern Analysis

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Abstract

Biosparging is a common method for the remediation of petroleum hydrocarbons in the soil subsurface. The addition of oxygen is thought to encourage the growth and activity of petroleum degrading bacteria in the contaminated soils. This study focused on the characterization of bacterial communities in a vertical profile through three different drilled soil cores that extended from just below the soil surface down to the aquifer (approx. 20 ft.) in the Guadalupe Dunes former oil field (Guadalupe, CA). The goal was to determine the effect biosparging had on the bacterial communities within a petroleum contaminated soil core. Two petroleum contaminated cores were taken before and after a two month long bioventing process. The third core was from an uncontaminated control area. Soil cores were homogenized at 6" intervals, resulting in over 30 samples per core. The process used to examine the samples involved a soil DNA extraction using the Mo Bio kit, PCR of the extracted DNA with eubacterial 16S rDNA primers (46F and 536R), digestion with restriction endonucleases, and detection of terminal restriction fragment patterns after capillary gel electrophoresis. TPH concentration in the two contaminated cores was highest at 12 to 13 ft below the surface. Bacterial numbers were low in these sand-dune soils, less than 10^4 CFU/g, and DNA was successfully isolated from only 30 of the 103 samples collected. There was no statistically significant effect of petroleum on the overall bacterial community structure. Additionally, the bioventing process did not have a detectable effect on the bacterial communities. 16S rDNA TRF patterns indicated that the bacterial phylotypes making up each sample were mostly unique to that sample and less than six percent of the phylotypes were present in ten or more of the thirty

samples. Although TPH did not appear to affect total community structure, TPH concentration was correlated with the abundance of eleven phylotypes that appeared in several of the samples. These data indicate that the subsurface communities changed rapidly with depth and thus may have obscured any TPH or bioremediation dependant changes in community structure.

Introduction

Aromatic and aliphatic hydrocarbons constitute some of the major environmental pollutants because of leakage and spills of both crude and refined petroleum products (Holliger, 1996). Petroleum hydrocarbon degradation by microorganisms is one of the best known ways to alleviate this problem. However, one of the problems encountered with aerobic biodegradation is a lack of sufficient oxygen to support the aerobic bacteria. One common solution to this problem is through the use of a technique known as bioventing. This method uses low rates of airflow through the contaminated soil in order to supply oxygen and is generally used as a long-term bioremediation method, lasting for months to years. This technique is effective because oxygen is a limiting factor for bacterial growth below the soil surface. The goal is to stimulate the growth of aerobic bacteria by providing oxygen to serve as a metabolic electron acceptor. The method is not only effective, but also inexpensive (DePaoli, 1996).

Terminal restriction fragment (TRF) patterns are a practical way to categorize bacterial communities. For several years, TRF patterns have been used to study microbial communities ranging from those found in rat feces (Kaplan, 2001), to termite guts (Lui, 1997), and soil (Clement, 1998). The method can be used to identify both species dominance and species richness within the samples (Clement, 1998). Generation of a TRF pattern involves extraction of DNA from a sample, PCR using a labeled primer, digestion with a restriction endonuclease, ethanol precipitation to clean up the digestion product, and use of a capillary gel electrophoresis system to generate the TRF pattern.

This pattern is then analyzed using a variety of statistical techniques. From the pattern, the different phylotypes of bacteria present in each sample can be identified.

This experiment was an attempt to test the theory that bioventing helps aerobic species of soil bacteria to flourish in areas where oxygen would otherwise have been unavailable and to observe the effect that petroleum hydrocarbon contamination has on the bacterial communities throughout a vertical core. Bacteria from three soil cores were compared and characterized using TRF patterns.

Materials and Methods

Extraction of bacterial DNA

Soil samples were obtained from three areas at the SBBV5X site at Unocal's Guadalupe dunes oil field. Two inch in diameter cores of soil were drilled and collected from a depth of two feet down to the aquifer. Each of the samples was then cored using an apparatus and method specifically developed for obtaining sterile one-inch cores. The samples were then each homogenized. The first set of samples (core 1) was taken at half-foot intervals in a core from 2.0 to 23.0 feet through a petroleum-contaminated area at the site. The control set of samples (core 2) was obtained from an uncontaminated area of the same site at half-foot intervals from 3.0 to 16.0 feet. The third set of samples (core 3) was taken at half-foot intervals from 4.0 to 24.5 feet through a contaminated core in an area that had just undergone a bioventing process for a period of two months.

DNA from five grams of each soil sample was then obtained by extracting five one gram aliquots and combining all five onto one spin filter to obtain more DNA than a standard extraction. The extractions were performed using a MoBio UltraClean[®] soil DNA extraction kit following manufacturer's protocol with the exception of the modified spin filter step. Success of each extraction was determined by measuring DNA concentration in the extraction product with a Spectramax spectrophotometer.

PCR Amplification

PCR was performed using 16S rDNA primers homologous to highly conserved regions on the 16S rRNA gene. The reverse primer K2R (5'- GTA TTA CCG CGG CTG CTG G-3'), and the forward primer Ba2F (5' GCY TAA CAC ATG CAA GTC GA-3'), which was fluorescently labeled with phosphamide dye, were used for each reaction. Reactions were carried out using 10µL of undiluted extraction product, 5 µL of 10x Buffer, 3 µL of 10 mM DNTP, 2 µL 20 µg/mL BSA, 7 µL 25 mM MgCl₂, 1 µL K2R, 1 µL Ba2FD4, 20.7 µL water, and 0.3 µL 5 U/µL TaqGold. Reaction temperatures and times were 96 °C for 10 min; 35 cycles of 94 °C for 1 min, 46.5 °C for 1 min, 72 °C for 2 min; and 72 °C for 10 min. All reactions were performed in triplicate and then combined using a MoBio Ultraclean[®] PCR Cleanup Kit following manufacturer's protocol. Amounts of DNA in each sample were again determined using a Spectramax spectrophotometer.

Enzyme Digest and TRF Pattern Generation

An enzyme digest was performed on each PCR cleanup product using the New England Biolabs restriction endonuclease *DpnII*. Each 40 µL digestion used 75 ng of DNA, 1 U of enzyme, and 4 µL of buffer. The samples were digested for 2 h at 37 °C and inactivated for 20 min at 65 °C. PCR product was also digested with New England BioLabs *HhaI* using 50 ng of DNA, 1 U of enzyme, 4 µL of buffer, and 0.2 µL of BSA following the same digestion conditions as the *DpnII*. Selected samples were digested with New England BioLabs *Taq^αI* using the same volumes and concentrations as with *HhaI*. The samples were digested for 3 h at 65 °C and inactivated for 20 min at 80 °C.

The digestion products were ethanol precipitated and resuspended in 20 μL of formamide and 0.5 μL of CEQ 600 base pair standard. Terminal restriction fragment profiles were obtained using a Beckman Coulter 2000X DNA Analysis system.

TPH Quantification

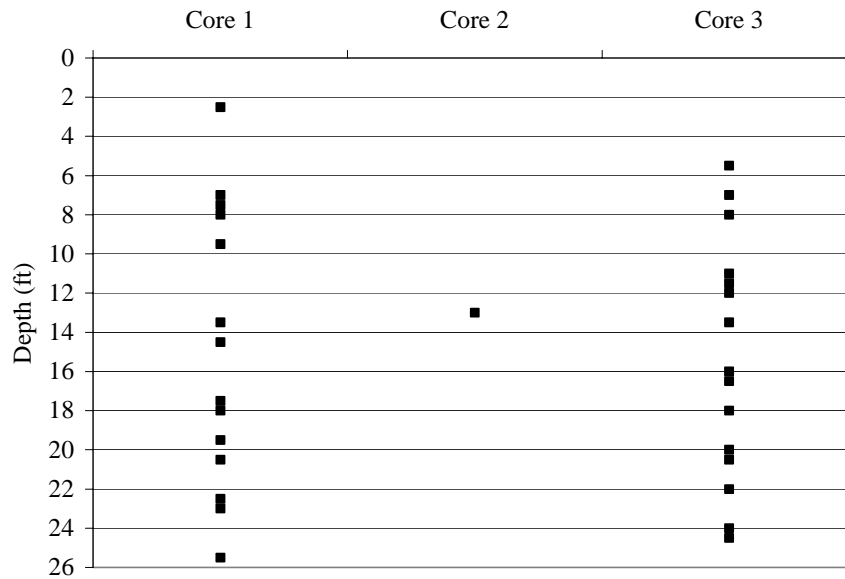
Selected samples were measured for total petroleum hydrocarbon (TPH) levels by Zymax Envirotechnology in San Luis Obispo, California.

Results

This experiment was an attempt to identify species richness and species dominance in each sample by identification of the bacterial phylotypes present in three soil cores. The goal was to see if the bioventing process that one core underwent (core 3) shifted the bacterial communities within this core. This was observed by comparing core 3 to a core that was sampled in the same area, but before the bioventing process began (core 1). To obtain the TRF patterns, the DNA was extracted from each sample, PCR was performed using a labeled primer, PCR product was digested with a restriction endonuclease, and a TRF pattern was produced and analyzed.

Thirty samples of the one hundred and three original samples had high enough levels of extracted bacterial DNA to create TRF patterns. Of those thirty samples, fourteen were from core 1, one sample was from core 2 and fifteen samples were from core 3 (Figure 1).

Figure 1: Profile of the depths of successful DNA extractions and subsequent TRF pattern generation



A large majority of the TRF peaks were unique to a small number of samples (Figure 2). Ninety four percent of the TRF peaks were found in ten or fewer of the thirty TRF patterns. Only six percent of the TRF peaks were found in more than ten samples. This indicates that the bacterial community in each sample was drastically different from all the other samples, including those samples that were in close proximity.

TPH levels correlated closely with depth. Levels in both cores peaked at around thirteen feet (Figure 3). There were very low levels of TPH at depths smaller than ten feet and higher than twenty feet.

Figure 2: Percent of Total TRF Peaks that are Unique to a Few Samples or are Found in a Large Number of Samples

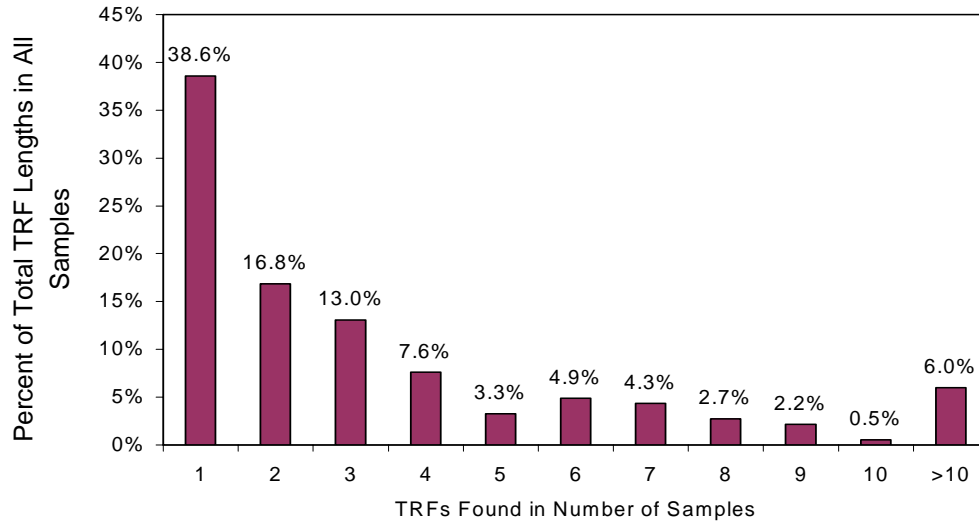
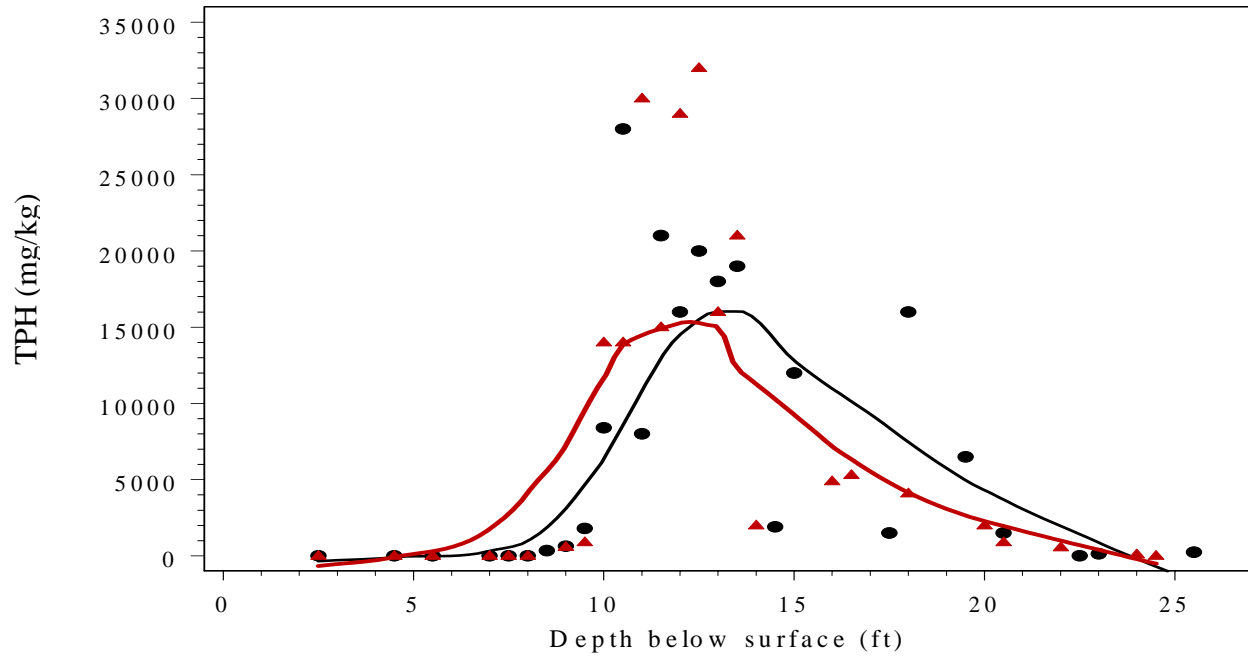


Figure 3: Total petroleum hydrocarbon levels at various depths throughout cores one and two



There were no clear community differences between core 1 and core 3 in either number of species present (Figure 3) or species dominance (Figure 4). There were no community differences that were related to either depth or TPH.

Figure 4: Simpson's Dominance Indicating if Certain Species (TRF Peaks) are More Dominant than Others.

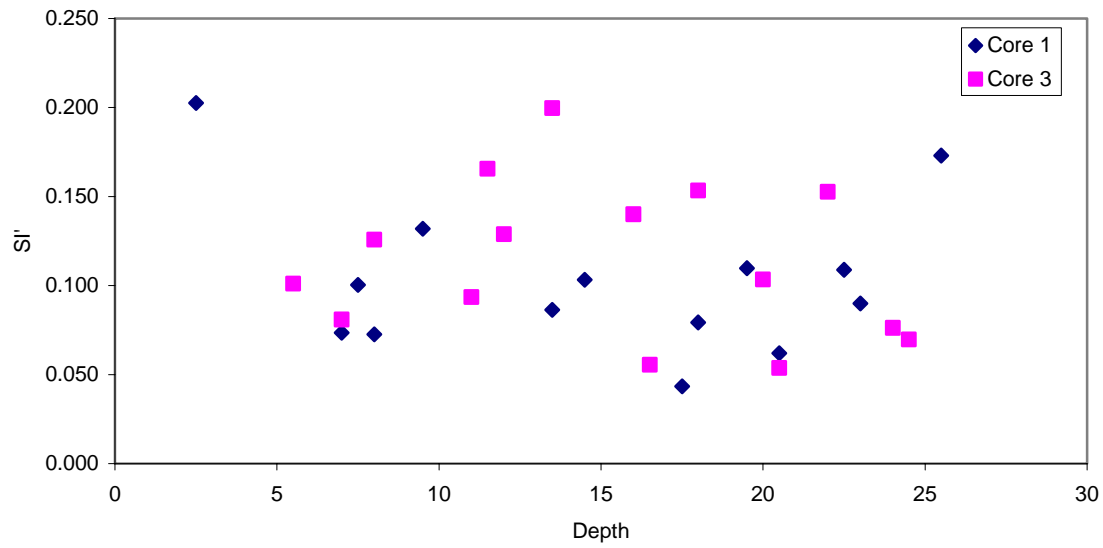
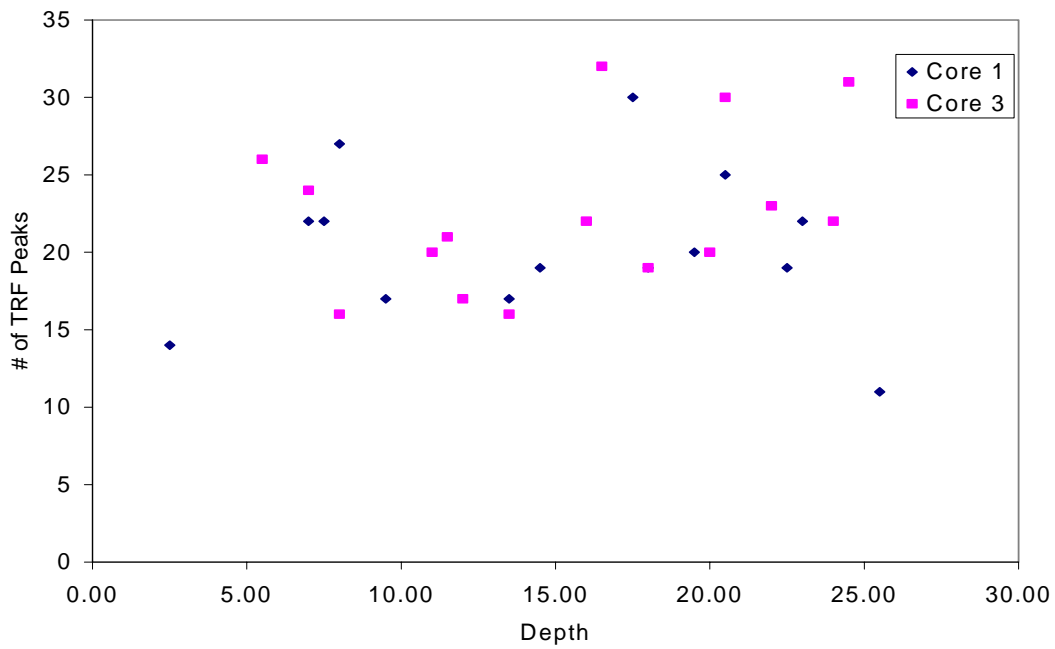


Figure 4: Number of TRF peaks indicating the number of species detected in each sample



A small subset of the most common peaks generated after digestion with *DpnII* were found in ten or more of the samples. These peaks occurred in both areas with TPH and areas without TPH. The most likely organisms for each of the peaks (base pairs) are shown in Table 1.

Table 1: Database matches for the most common *DpnII* peaks

| <i>DpnII</i> Peak | Peak Found in # of Samples | Database Match |
|-------------------|----------------------------|--|
| 230 | 20 | <i>Pseudomonas</i> or <i>Actinomyces</i> |
| 488 | 18 | <i>Actinomyces</i> |
| 461 | 15 | <i>Actinomyces</i> |
| 85 | 14 | <i>Shewanella</i> or <i>Aeromonas</i> |
| 464 | 13 | <i>Streptomyces</i> or <i>Actinomyces</i> |
| 154 | 13 | <i>Pseudomonas</i> , <i>Azoarcus</i> , or <i>Thauera</i> |
| 456 | 13 | <i>Frankia</i> |
| 81 | 12 | <i>Corynebacteria</i> |
| 84 | 11 | <i>Shewanella</i> |
| 204 | 11 | <i>Bartenella</i> , <i>Bradyrhizobium</i> , or <i>Alpha-proteobacteium</i> |
| 228 | 11 | <i>Pseudomonas</i> |
| 470 | 10 | <i>Actinomyces</i> |

The peaks that were statistically more abundant in high TPH ($P < 0.001$, F test) and the peaks that were statistically more abundant in low TPH levels ($P < 0.001$, F test) were matched to possible corresponding organisms (Table 2).

Table 2: *DpnII* peaks related to TPH presence and the organismal identification(s) for each peak

| Base Pairs (<i>DpnII</i>) | % TPH High (>1,000ppm) | % TPH Low (<1,000ppm) | Average Area in High TPH (>1,000ppm) | Average Area in Low TPH (<1,000ppm) | Database Match |
|-----------------------------|------------------------|-----------------------|--------------------------------------|-------------------------------------|---|
| 236 | 47% | 13% | 61269 | 3616 | Unidentified Clones |
| 460 | 33% | 7% | 13351 | 901 | Microspora, Nonomuraea, Streptomyces, or Actinomycete |
| 202 | 33% | 7% | 12375 | 1287 | Rhizobium, Alpha-proteobacterium, Achromobacter, Agrobacterium, or Paracoccus |
| 463 | 33% | 13% | 19664 | 3067 | Microspora |
| 151 | 33% | 13% | 21728 | 4701 | Pseudomonas, Nitrospira, Azoarcus, Bradyrhizobium, or Nitrosomonas |
| 488 | 33% | 87% | 8234 | 60270 | Actinomycete |
| 154 | 27% | 60% | 12222 | 68509 | Pseudomonas or Nitrosospira |
| 318 | 7% | 47% | 1710 | 24617 | Pseudomonas |
| 435 | 7% | 40% | 867 | 15150 | Alpha-proteobacterium or Pseudanabaena |
| 343 | 7% | 40% | 2940 | 23826 | Unidentified Clones |
| 131 | 7% | 33% | 853 | 19975 | Pseudomonas |

Two of the samples in the core 3 (11.0' and 11.5') were examined further by digestion with *HhaI* or both *HhaI* and *Taq^oI* along with the *DpnII* digestion. The 11.0' sample had a large dominant peak at 236 base pairs when digested with *DpnII*, one at 139 base pairs when digested with *HhaI*, and one at 229 base pairs when digested with *Taq^oI*. The 11.5' sample had a large dominant peak at 206 base pairs when digested with *DpnII*

and one at 174 base pairs when digested with *Hha*I. These results indicated that the main bacterial species present in the two samples was likely an uncultured benzene decomposing species.

Discussion

The results of the study were not ideal because of problems in recovery of adequate amounts of DNA. This was possible due to the samples having been stored in the freezer for over two years before any of the research was begun. It is possible that some of the DNA was degraded over this time. It is also possible that there were just low levels of bacteria in the soil to start with. Plate counts that were performed on a few of the stored samples indicated that there were less than 10^4 CFU/g present in these samples. Other problems resulted from the uniqueness of the samples. The samples all had very different TRF patterns, even those that were very near to each other. Another limiting factor in the study was that the database matching was done using mainly a single enzyme digestion. Other enzymes were not used because there was not enough DNA due to the problems encountered in obtaining DNA from this particular soil.

Despite the problems encountered, this project resulted in some unexpected conclusions. There were no significant differences seen in either species richness or species dominance between core 1 and core 3. There were also few statistically significant differences between the areas with high TPH levels and those with low TPH levels. In fact, each sample was unique in that most of the TRF peaks were only found in one or two samples.

The major peaks that were identified after digestion with *DpnII* corresponded to *Actinomycetes* or *Pseudomonads*. Other likely organisms for these significant peaks included *Shewanella*, *Aeromonas*, *Frankia*, *Corenybacteria*, *Streptomyces*, *Bartanella*, *Bradyrhizobium*, and bacteria in the Alpha-proteobacterium group. There were a small number of peaks that were more abundant at high TPH levels and some that were more

abundant at low TPH levels. These peaks were also matched to likely organisms with the *Pseudomonads* and the *Actinomycetes* dominating the communities. These results are significant because *Pseudomonas*, *Actinomyces*, and *Shewanella* are all known to be involved in petroleum hydrocarbon degradation

Samples from the top of the TPH zone in core 3 were analyzed after use of the additional restriction endonucleases *Taq*^oI and *Hha*I and identified as uncultured benzene decomposing species. These results are significant because they indicate that some of the major oil degrading species in the subsurface are as yet unidentified. These species appear to make up a large part of the bacterial communities in the samples in areas with high TPH levels and therefore should be further studied and identified.

References

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