Terminal Restriction Fragment (TRF) Analysis of Fungal Community Diversity in Petroleum Contaminated Soil

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Abstract

Analysis of a fungal community in a pilot scale petroleum-degradation land treatment unit (LTU) was performed by monitoring their individual genetic composition over a period of 98 days. The goal of the LTU was to determine the best environment for promoting efficient biodegradation of the hydrocarbons in the soil. The contaminated soil was divided into four cells with each one being uniquely manipulated for different growth conditions. This study will concentrate on cell three, which was amended with dextrose. Soil samples were taken from this cell at various time intervals. DNA isolated from the soil was amplified by PCR. Both primers used in the reaction were homologous to conserved regions in the fungal 18S ribosomal gene and one was fluorescently labeled. The PCR amplicons were then digested with three different restriction enzymes, *Msp*I, *Hae*III, and *Dpn*II. The fluorescently labeled terminal restriction fragments (TRFs) that resulted were separated by electrophoresis and detected by laser induced fluorescence on an automated gene sequencer. The differences in the ribosomal DNA sequences of assorted organisms created various TRF patterns (TRFPs) depending on the enzyme and sample used. TRFPs were compared to each other to visualize the changes in the fungal community with respect to time, total petroleum hydrocarbon (TPH) levels, and other conditions. Although specific identification of fungal species wasn't very accurate, this method does provide an analysis of the entire fungal population and not just those that can be cultured.

I. Introduction

Over the past century the manufacture and distribution of petroleum and chemical products has resulted in hydrocarbon contamination becoming a major environmental problem. Hydrocarbons have leaked from underground storage tanks into soil and groundwater and from huge tankers that have polluted the oceans. It is these huge marine oil spills that attract most of the public attention but in fact the majority of environmental contaminants originate from much smaller leaks. For example, it is estimated that more than 250,000 tanks are leaking at service stations in the United States (1).

In soil and other environments there is a naturally occurring process between the microflora population and carbon bearing molecules, including hydrocarbons from petroleum. This bioremediation process utilizes these complex molecules as a source of carbon and other nutrients. Over time they are eventually degraded into simple compound such as CO_2 and H_2O (2). Bacteria are often the dominant hydrocarbon degraders in aquatic systems and fungi tend to have a more significant role in soil (1).

The contaminated site investigated in this project is located in Guadalupe, CA. The site occupies nearly 2,700 acres located on California's central coast. From 1946 to March 1994 the principal land use at the site was the production of oil and natural gas. In the 1980s the Union Oil Company of California (Unocal) reached their peak oil production of approximately 4,500 barrels per day (3). The crude oil was extremely viscous so it was mixed with a diluent to enhance production and transportation. This diluent, which contained petroleum hydrocarbons, leaked from the pipelines and storage tanks into the surrounding soil and groundwater (3). Unocal constructed a pilot land treatment unit (LTU) with help from the Environmental Biotechnology Institute at Cal Poly, San Luis Obispo. The goal of the LTU was to analyze the

numerous chemical, physical, and biological factors that can affect the rate and extent of hydrocarbon degradation.

The pilot LTU was made up of four cells with each one receiving a standard of nitrogen (ammonium sulfate) and phosphorus (monopotassium phosphate) sources. The soil was tilled to increase the amount of oxygen and moisture levels were held between 6% and 10% (3). Cell 1 was used as a control while the other three cells received an additional carbon source. Parameters that differentiated cells 2-4 included the treatment of corn steep to cell 2, dextrose to cell 3, and cornstarch to cell 4 (3).

One objective of the LTU was to analyze the changes in microbial diversity among the varied cell conditions and parameters. Microbial populations in complex environments, however, are difficult to characterize. Plating techniques, although a relatively easy method, fall short of true community representation because of the large fraction (>85-99.999%) of organisms in nature that can't be cultured (4). Several genetic approaches have been devised to overcome this problem. Currently, the majority of polymerase chain reaction (PCR) methods depend on amplifying, cloning, and sequencing 16S rRNA genes (rDNA) from sample DNA (5). This process is time consuming however, because thousands of clones must be sequenced from a single sample to precisely characterize the microbial community (5). Amplified ribosomal DNA restriction analysis (ARDRA) is another technique employed to study a community of microorganisms. ARDRA produces various patterns by digesting amplified community 16S rDNA with restriction enzymes (5). The fragments that create these patterns are visualized using a polyacrylamide gel. Although this is a faster process than cloning, there are still problems with it. Visual detection, for example, may not resolve small fragments or small amounts of a particular fragment (5). All these problems led to technique that produces fluorescent labeled terminal restriction fragments (TRFs).

Like ARDRA this method also makes use of the divergent sequences in rDNA between species to study community diversity. A selected region of rDNA is fluorescently labeled and amplified by PCR. Most previous experiments employing TRF analysis have focused on an amplified region of the 16S rDNA in bacteria. The samples are then digested with restriction enzymes to produce fluorescent labeled terminal restriction fragments of different lengths. Patterns of these fragments are produced by electrophoretic separation and then detected on an automated gene sequencer.

TRF pattern analysis was used in this study on soil samples obtained from cell 3 of the LTU at three different time intervals. The PCR primers used are homologous to regions of fungal 18S rDNA and were designed to amplify a 550 bp region containing a zone of divergent sequences between different fungal species (6).

The observed TRF patterns (TRFPs) were then compared to predicted patterns to try and identify particular species that were present. Definitive identification of particular fungi wasn't achieved but a few high probabilities were formulated. Changes in the TRFPs were also represented graphically to help visualize the changes in diversity from each of the samples.

II. Materials and Methods

DNA Extraction

Soil samples from cell 3 of the LTU were obtained at three different time intervals that consisted of 14, 49, and 98 days. Microbial DNA was extracted from each of the three soil samples by following the protocol given in the Ultraclean MoBio Soil DNA Kit (MoBio Laboratories Inc., Solano Beach CA.) with one modification: the cells were lysed in a FP-120 Bio 101 Sevant fast prep machine at 5.0m/s for 45 seconds.

Gel Electrophoresis

To ensure a successful extraction from the soil samples the DNA was run on a 1.5% agarose gel of pH 8.5 in TBE buffer (89.2 mM Tris, 88.9 mM boric acid, 2.47 mM disodium EDTA). Each well contained 5µl of extracted DNA and 1µl of 5X loading buffer. The gel ran at 100V for 35 minutes. The gel was then stained with ethidium bromide and the bands of DNA were observed.

DNA Quantification

A SPECTRAmax UV spectrophotometer[â] (Perkin-Elmer, Applied Biosystems Inc., Fremont, CA.) was used to measure the concentration of DNA in each of the three samples. Dilutions of 1:20 were prepared for quantification. The observed concentrations and the volumes used to make 100µl of 10ng/µl samples are recorded in Table 1.

PCR, 18S Labeled

Two primers homologous to conserved regions of fungal 18S rDNA were used to amplify a selected 550 bp region of the extracted DNA samples. The sequences of the primers are:

Fun 18S1 5′CCATGCATGTCTAAGWTAA3′ (forward)

Fun 18S2 5′GCTGGACCAGACTTGCCCTCC3′ (reverse) (7)

Fun 18S1 was fluorescently labeled. Each DNA sample was amplified by PCR in triplicate. The reaction began with a 2 minute denaturation at 95°C followed by a 40 cycle series of 1 minute at 95°C, 1 minute at 55°C, and then 2 minutes at 72°C. The PCR was completed with a 10 minute 72°C cycle to complete elongation. The reaction volumes for each sample totaled 50µl. Reagents and their volumes are shown in Table 2.

Clean-up of PCR Products

The PCR products were run on a 1.5% agarose gel, as described earlier, to confirm the success of the PCR. All successful reactions for each of the three samples were combined together and purified according to the protocol for the Ultra Clean-Up DNA Purification Kit (MoBio Laboratories Inc., Solano Beach CA.). This kit was used to remove the primers and other reactants in order to concentrate the amplicons.

Quantification of Labeled PCR Products and Enzyme Digestion

The labeled PCR products were quantified using the SPECTRAmax UV spectrophotometer[â] as described earlier. The concentrations of the amplified samples of DNA are displayed in Table 3. This table also shows the proper volume calculated and transferred to digest tubes such that each digest reaction contained 100ng of amplified DNA. Since the least concentrated sample required the largest volume of the PCR product to obtain 100ng of DNA, PCR water was added to the other two samples in order to equalize the volume of all three samples.

The enzymes used in the digest were *Msp*I, *Dpn*II, and *Hae*III (New England Biolabs, Beverly, MA.). The *Dpn*II and *Hae*III reactions contained 0.2µl of 10U/µl of each endonuclease respectively. For the *Msp*I reaction 0.1µl of the 20U/µl enzyme was used. In addition to the enzymes and DNA, each digest sample contained the reaction buffer recommended by the

manufacture and enough PCR water to bring the total reaction volume to 20µl (Tables 4a-4c). The first and last samples also received 1µl of the digest standard.

The samples were digested for 5 hours at 37°C and then heated to 65°C for 20 minutes to deactivate the enzymes.

Ethanol Precipitation of Digest

Each digest reaction was mixed with 50µl of cold 95% ethanol and 1µl 3M sodium acetate (pH 4.6) and incubated at 4°C for 30 minutes. After the incubation period the DNA was centrifuged for 15 minutes at 15 x kg to pellet the DNA. The ethanol was removed and the pellet was washed in 50µl of cold 70% ethanol and then centrifuged again for 15 minutes at 15 x kg. The ethanol was remove completely and the pellet was dried by vacuum centrifugation.

Capillary Electrophoresis

The DNA pellets were dissolved in 20µl of formamide (BioRad, Benecia CA.), 1µl of load buffer, and 1µl each of Genescan 500 ROX^{â} (Perkin-Elmer, Applied Biosystems Inc., Fremont CA.) and ROX^{â} 550-700 (BioVenture, Murfreesboro, TN.) size standards. The samples were denatured at 95°C for 5 minutes and snap-cooled in an ice bath for 10 minutes. They were then run on an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems Inc., Fremont CA.) at 15 kV and 60°C. Analysis of the output was performed on Genescan 3.1 software (Applied Biosystems Inc., Fremont, CA.) using Local Southern method with heavy smoothing. The size standard peaks were used to resolve the electropherogram peaks to one base pair.

Data Analysis

Sample data consisted of peak areas for a given TRF pattern. The amount of DNA loaded onto the ABI Prism™ 310, however, can't be accurately controlled so the total sample area varied between TRFPs. In order to adjust for these differences and make it possible to

validly compare the three samples to each other, the peaks were truncated and the peak areas were converted to parts per million (ppm).

Truncation was done by calculating the ratio of each peak area to the area of the smallest peak and then multiplying that ratio by the smallest peak area the ABI Prism™ can detect (580 area units). The peaks that had areas lower than this new threshold level were removed from the data. Table 5 shows an example of how the data was truncated.

Changing their areas to ppm normalized the peaks remaining after truncation. This was achieved by calculating the ratio of each peak area to the total area and multiplying by $1x10^6$.

Table 1. Extracted DNA Concentrations and Volumes used for PCR Amplification

Sample	[DNA]	DNA Volume (µl)[1]	H_2O (µl)[2]
C3:14	41.2	24.3	75.7
C3:49	10.9	91.7	8.3
C3:98	14.6	68.5	31.5

[1]$C_1V_1=C_2V_2$ where C_1= Extracted [DNA] V_1= Volume needed to make dilution
C_2= Desired [DNA] or 10ng/µl V_2= Final desired volume or 100µl
[2]The required amount of DI H_2O added to each sample so that the total volume was 100µl

Table 2. PCR Reagents and Volumes used for each Reaction

Template DNA	10.0µl[1]
10X Buffer 10mM	5.0µl
DNTPs 10µg/ml	3.0µl
BSA 20µg/ml	2.0µl
$MgCl_2$ 25mM	7.0µl
FUN 18S 1	1.1µl
FUN 18S 2	1.1µl
H_2O	20.5µl
Taq 5U/µl	0.3µl
Total Volume/Reaction	50.0µl

[1]This is 10µl of the 10ng/µl dilution calculated for each sample in Table 1.

Table 3. Final DNA Concentrations after PCR Amplification and the Volumes Needed to Obtain 100ng for Digestion

Sample	[DNA]	Volume (µl) needed for 100ng of DNA	H_2O (µl)[1]
C3:14	38.6	2.6	0.3
C3:49	34.3	2.9	0.0
C3:98	37.8	2.6	0.3

[1]PCR water was added to samples C3:14 and C3:98 to bring their final volumes equal to that of C3:49

Table 4a. Reagents and Volumes used for Digest Reactions of C3:14

	*Dpn*II	*Hae*III	*Msp*I
Enzyme	0.2µl	0.2µl	0.1µl
10X Buffer	2.0µl	2.0µl	2.0µl
H_2O	14.9µl	14.9µl	15.0µl
DNA[1]	2.6µl (+ 0.3µl H_2O)	2.6µl (+ 0.3µl H_2O)	2.6µl (+ 0.3µl H_2O)
Total Volume/Reaction	20.0µl	20.0µl	20.0µl

[1]The volumes shown here contain 100ng of amplified DNA. Additional PCR water was added to bring the volume equal to that of C3:49; 2.9µl, which represents 100ng of DNA for that sample.

Table 4b. Reagents and Volumes used for Digest Reactions of C3:49

	*Dpn*II	*Hae*III	*Msp*I
Enzyme	0.2µl	0.2µl	0.1µl
10X Buffer	2.0µl	2.0µl	2.0µl
H_2O	14.9µl	14.9µl	15.0µl
DNA[1]	2.9µl	2.9µl	2.9µl
Total Volume/Reaction	20.0µl	20.0µl	20.0µl

[1]The volumes shown here contain 100ng of amplified DNA. Note that no extra water was added to these reactions because C3: 49 was the most dilute sample

Table 4c. Reagents and Volumes used for Digest Reactions of C3:98

	*Dpn*II	*Hae*III	*Msp*I
Enzyme	0.2µl	0.2µl	0.1µl
10X Buffer	2.0µl	2.0µl	2.0µl
H_2O	14.9µl	14.9µl	15.0µl
DNA[1]	2.6µl (+ 0.3µl H_2O)	2.6µl (+ 0.3µl H_2O)	2.6µl (+ 0.3µl H_2O)
Total Volume/Reaction	20.0µl	20.0µl	20.0µl

[1]The volumes shown here contain 100ng of amplified DNA. Additional PCR water was added to bring the volume equal to that of C3:49; 2.9µl, which represents 100ng of DNA for that sample.

Table 5. Example of Truncation for TRFP Data Analysis

Sample	Total Area	Ratio of Total Peak Area[1]	Peak Area Analyzed[2]
Smallest Peak	200,000	1:1	580[3]
Medium Peak	400,000	2:1	1160
Biggest Peak	2,000,000	10:1	5800

[1]Ratio= Peak area/Area of smallest peak
[2]Peak area analyzed= ratio x 580
[3]Minimumdetectable peak area with ABI Prism™

III. Results and Discussion

The TRFPs generated from each of the three restriction enzymes were compared to each other in order to illustrate the changes in the microbial community over the 98 day period. Peaks appearing in the range of 500 bp or greater were not incorporated in the analysis or characterization of the TRFPs because these peaks were believed to be uncut or the result of incomplete or partial digestion of the amplicons. The TRFPs produced from the three enzymes were first observed individually and then looked at as a group to identify general trends.

Figure 1 displays the TRFPs for all three samples produced by *Hae*III, which created a total of 12 unique peaks. Day 14 had the greatest number and day 49 the least at 8 peaks and 1 peak respectively. By day 98 that number had risen back up to 4 peaks. The two largest peaks were at 213 bp and 217 bp on day 14. These two peaks represent the most dominant organisms in the soil at this point in time. The TRFP for day 14 also shows six smaller peaks that range from 160 bp to 489 bp. Except for the peak at 489 bp all the peaks that were produced on day 14 were absent from the other two samples. Day 49 had only one peak and it was unique to all three samples having a length of 467 bp. The TRFP for day 98 reveals a small return of the peak at 489 bp that was originally present in the day 14 sample. There were also three relatively small peaks in the day 98 sample that are missing from days 14 and 49.

The TRFPs for all three samples created by *Msp*I are shown in Figure 2. Out of all three enzymes, *Msp*I produced the least number of peaks for all three samples. Similar to *Hae*III, the greatest number of peaks generated by *Msp*I occurred on day 14. The other two samples had the same number of observable peaks. The 184 bp peak was the most dominant in the first and last samples, and although a minor peak, it was still present in the day 49 sample. Two additional peaks at 324 bp and 335 bp were also present in all three samples. A 425 bp peak that was

initially present on day 14 disappeared on day 49 but was present again in a small amount on day 98. The peak at 435 bp, however, is visible on both the day 14 and 49 TRFPs but is missing from the day 98 TRFP. The 331 bp peak was first produced on day 49 and by day 98 it was greatly diminished. *Msp*I created at least one original peak for each sample.

Out of the three enzymes *Dpn*II created the greatest number of total peaks (Figure 3) and like the other two it produced more peaks in the beginning on day 14 than on day 98. There are three very prominent peaks on day 14 occurring at 213 bp, 218 bp, and 475 bp. The 213 bp peak was greatly reduced by day 49 and then had an enormous increase again on day 98. The 218 bp peak was just the opposite in that it had an increase from day 14 to day 49, but then declined a little in the 98 day sample. Although the 475 bp was the most dominant peak on day 14 it was completely absent from the other two samples. There were several other minor peaks on day 14 that followed this same pattern. The peak at 479 bp, however, was present in all three samples but was least abundant on day 49. Five new peaks were created on day 49, two of which persisted through day 98. These two peaks had lengths of 160 bp and 488 bp. Day 98 also had three minor peaks that were unique to this sample.

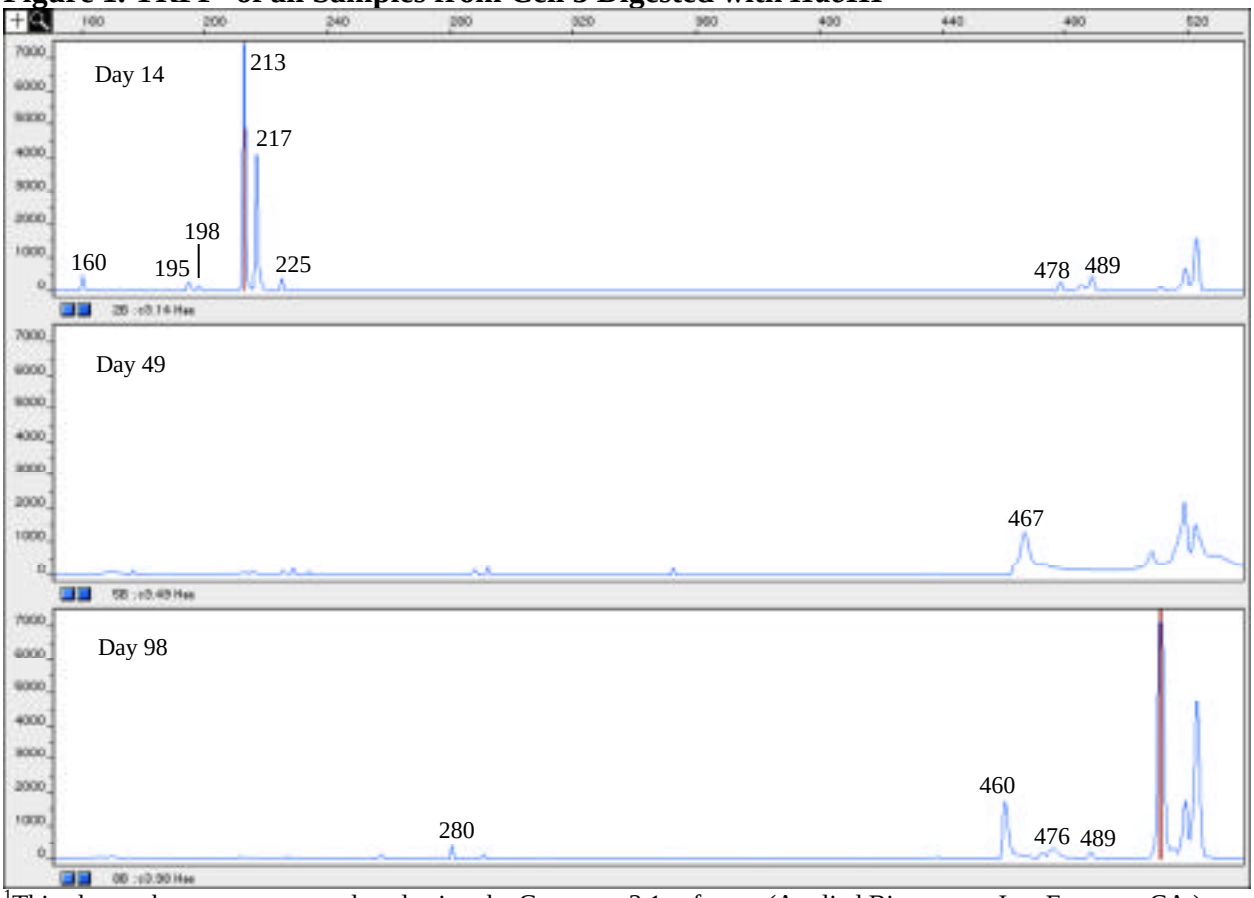
By looking at each TRFP produced by the three enzymes together a few general trends are evident. For example, each enzyme generated at least one new peak in every sample and the presence of most peaks diminished in the sample following its initial appearance. A second trend universal to all three enzymes was that their greatest number of peaks were generated in the beginning on day 14 (Figure 4). This figure also shows that *Dpn*II created the most peaks in all three of the samples.

A database containing fungal ribosomal sequences was used in conjunction with a program that mimics TRF analysis. Actual TRFPs were then compared to those predicted by the program in an attempt to identify specific organisms. An organism that has predicted peaks for all three enzymes that match peaks present in the TRFPs for a particular sample is a strong

indication of the presence of that organism in the sample being studied. Tables 6-8 show fungi with their predicted TRF peaks and some observed peaks that closely resemble them. The organisms listed in Table 7 have predicted peaks that are similar to those appearing in the TRFPs for day 49 within a 20%- 30% margin. The rest of the TRFs in day 49 and the other two samples (Tables 6 & 8) fall outside of this margin. These tables aren't all inclusive in that several peaks weren't predicted by the database. This is due to the limitations of the database and perhaps indicates that some of the organisms present in the sample have never been cultured. The tables also show that in some instances 10 or more genera are expected to produce the same TRFPs when using these three specific restriction enzymes.

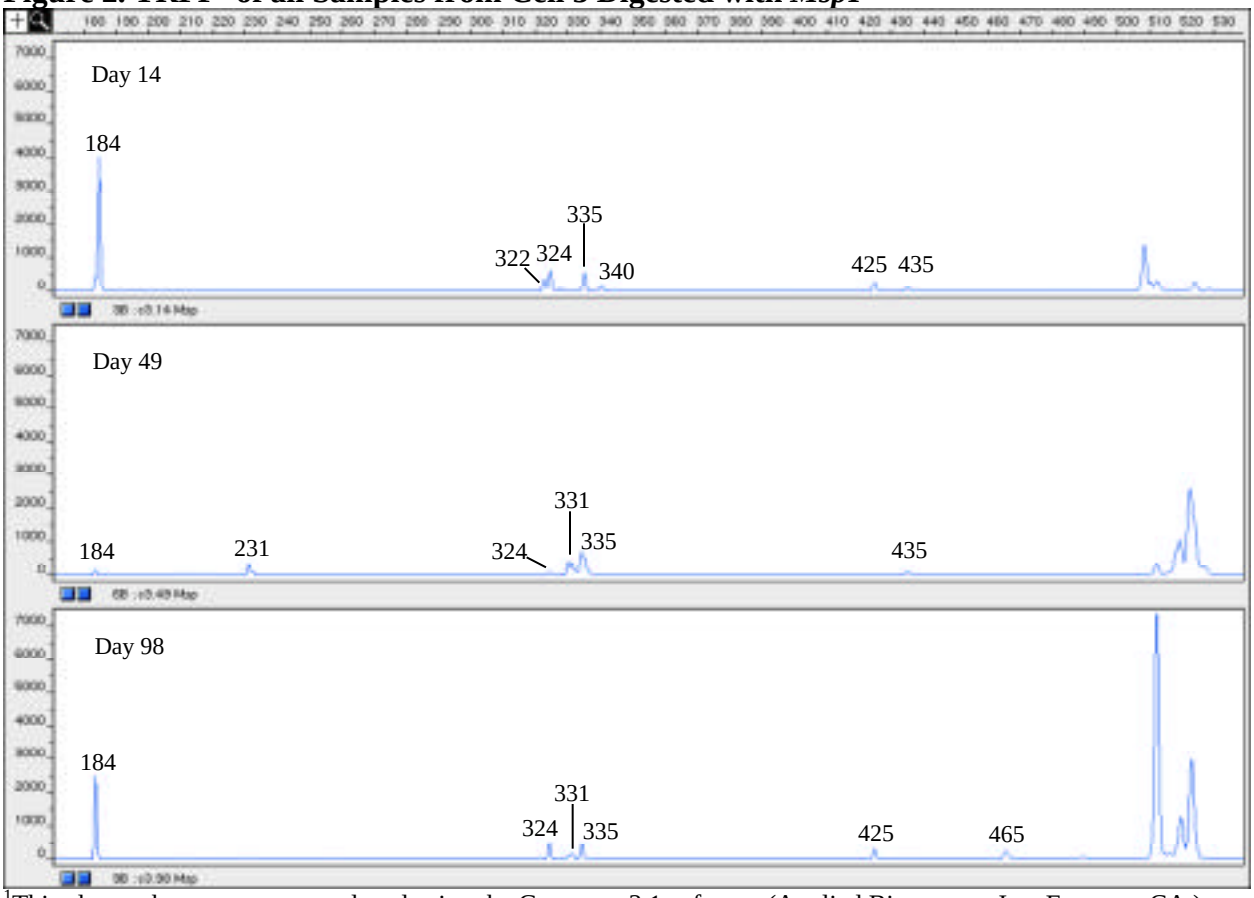
Of the fungi potentially identified, those of the genus *Hansenula* (Table 6.) are known to be common hydrocarbon degraders (1).

Figure 1. TRFP[1] of all Samples from Cell 3 Digested with *Hae*III



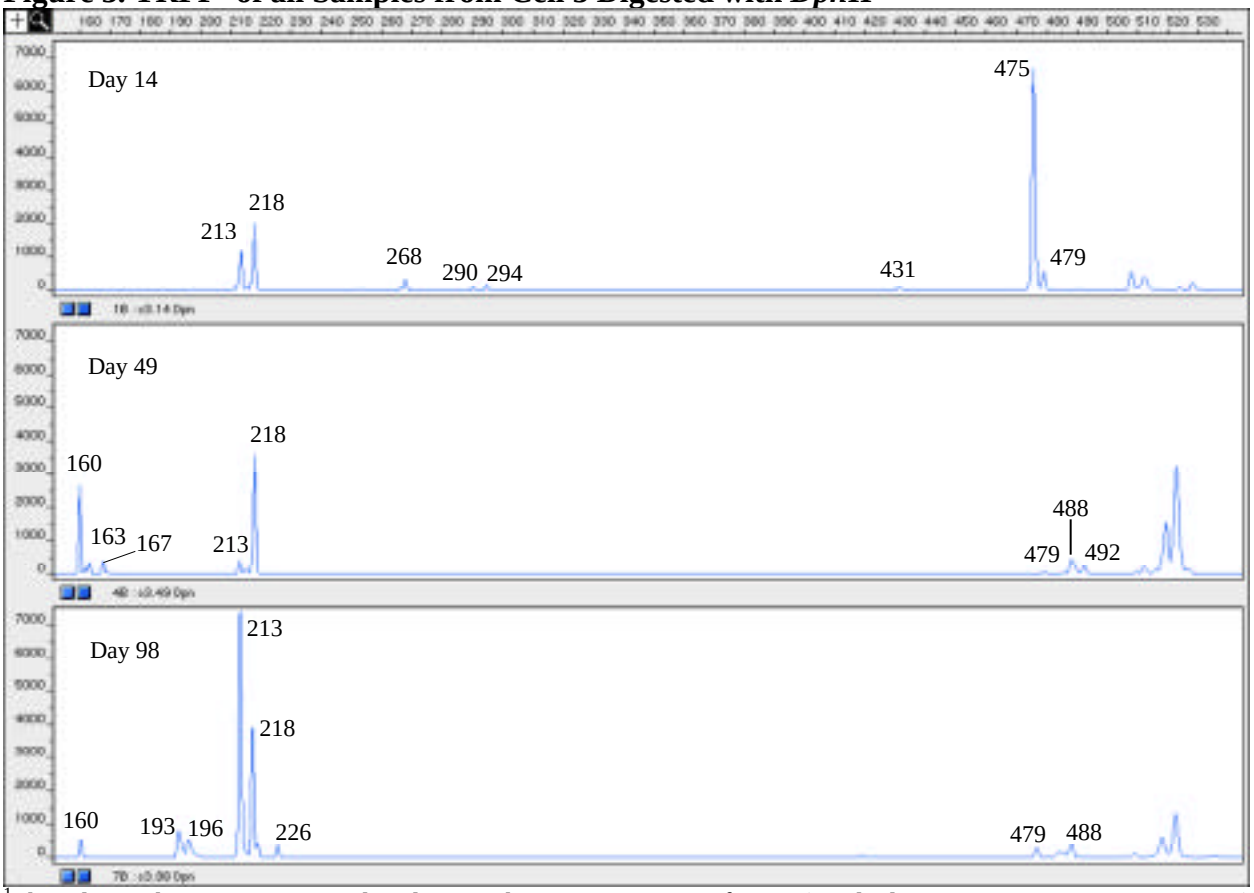
[1]This electropherogram was produced using the Genescan 3.1 software (Applied Biosystems Inc. Fremont, CA.) with the data generated by the ABI Prism™ 310 Genetic Analyzer. Each peak represents a TRF of specific length created the *Hae*III endonuclease. The height of each peak correlates to the amount of each TRF.

Figure 2. TRFP[1] of all Samples from Cell 3 Digested with *Msp*I



[1]This electropherogram was produced using the Genescan 3.1 software (Applied Biosystems Inc. Fremont, CA.) with the data generated by the ABI Prism™ 310 Genetic Analyzer. Each peak represents a TRF of specific length created the *Msp*I endonuclease. The height of each peak correlates to the amount of each TRF.

Figure 3. TRFP[1] of all Samples from Cell 3 Digested with *Dpn*II



[1]This electropherogram was produced using the Genescan 3.1 software (Applied Biosystems Inc. Fremont, CA.) with the data generated by the ABI Prism™ 310 Genetic Analyzer. Each peak represents a TRF of specific length created the *Dpn*II endonuclease. The height of each peak correlates to the amount of each TRF.

Figure 4. Summary of Observed TRFs for all Samples from Cell 3 Created by Each Enzyme

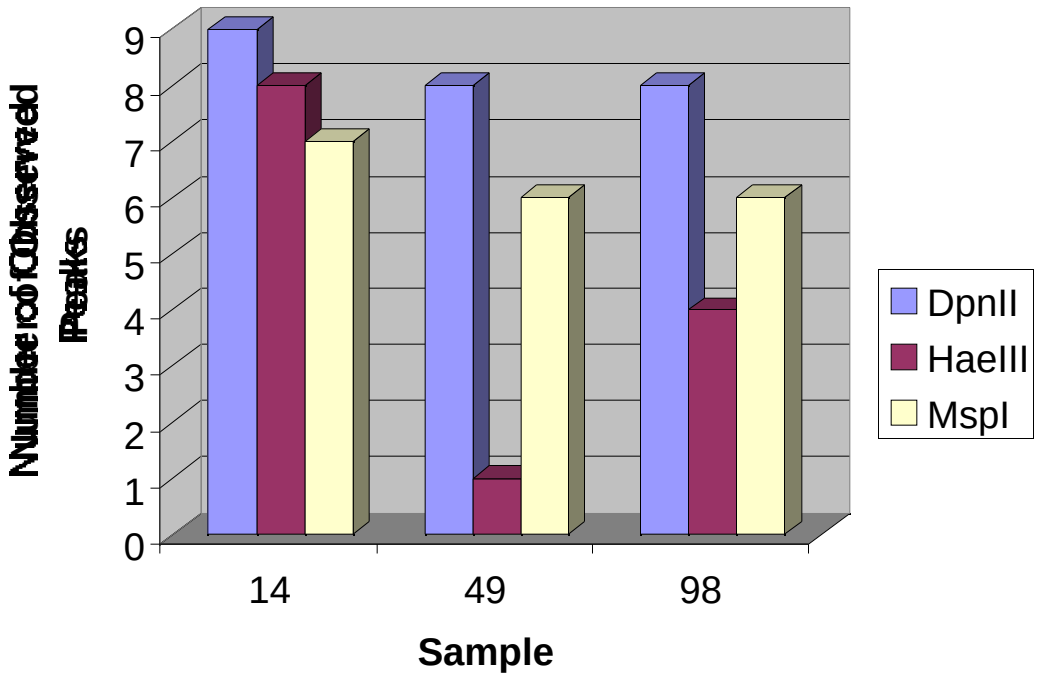


Table 6. Organisms with Predicted TRFs Similar to Those Created on Day 14[1]

Organism	*Hae*III Obs/Pred	*Msp*I Obs/Pred	*Dpn*II Obs/Pred
Porphyra umbilicalis (2 strains)	225/225	324/325	213/215
Hansenula polymorpha	225/225	Uncut[3]	Uncut
Represents ≥ 10 Genera	225/225	335/332	Uncut

[1]This table was generated using a macro (Environmental Biotechnology Institute) that compared the data from TRFPs on day 14 to a database of predicted fragment sizes for particular organisms digested by the three enzymes.
[2]Obs= Observed fragment length from TRF; Pred= Predicted TRF from database
[3] Database predicted that the enzyme wouldn't produce a terminal restriction fragment from this organism

Table 7. Organisms with Predicted TRFs Similar to TRFs Created on Day 49[1]

Organism	*Hae*III	*Msp*I	*Dpn*II
Rhizosolenia setigera (20%)	518	231	213
Climacostomum virens (20%)	483	168	483
Salvinia natans (20%)	172	238	174
Chlamydomonas radiata (30%)	170	175	487
	Obs/Pred[2]	Obs/Pred	Obs/Pred
Alexandrium ostenfeldii	Uncut[3]	335/335	Uncut
Represents ≥ 10 Genera	Uncut	331/330	213/213

[1]This table was generated using a macro (Environmental Biotechnology Institute) that compared the data from TRFPs on day 49 to a database of predicted fragment sizes for particular organisms digested by the three enzymes. The percentages indicate the amount of deviation between the predicted and observed TRFPs.
[2]Obs= Observed fragment length from TRF; Pred= Predicted TRF from database
[3]Database predicted that the enzyme wouldn't produce a terminal restriction fragment from this organism

Table 8. Organisms with Predicted TRFs Similar to TRFs Created on Day 98[1]

Organism	*Hae*III Obs/Pred	*Msp*I Obs/Pred	*Dpn*II Obs/Pred
Mallomonas striata	280/280	184/184	213/215
Synura sphagnicola	280/279	184/184	218/217
Represents ≥ 10 Genera	Uncut[3]	331/330	213/213

[1]This table was generated using a macro (Environmental Biotechnology Institute) that compared the data from TRFPs on day 98 to a database of predicted fragment sizes for particular organisms digested by the three enzymes.
[2]Obs= Observed fragment length from TRF; Pred= Predicted TRF from database
[3]Database predicted that the enzyme wouldn't produce a terminal restriction fragment from this organism

IV. Conclusion

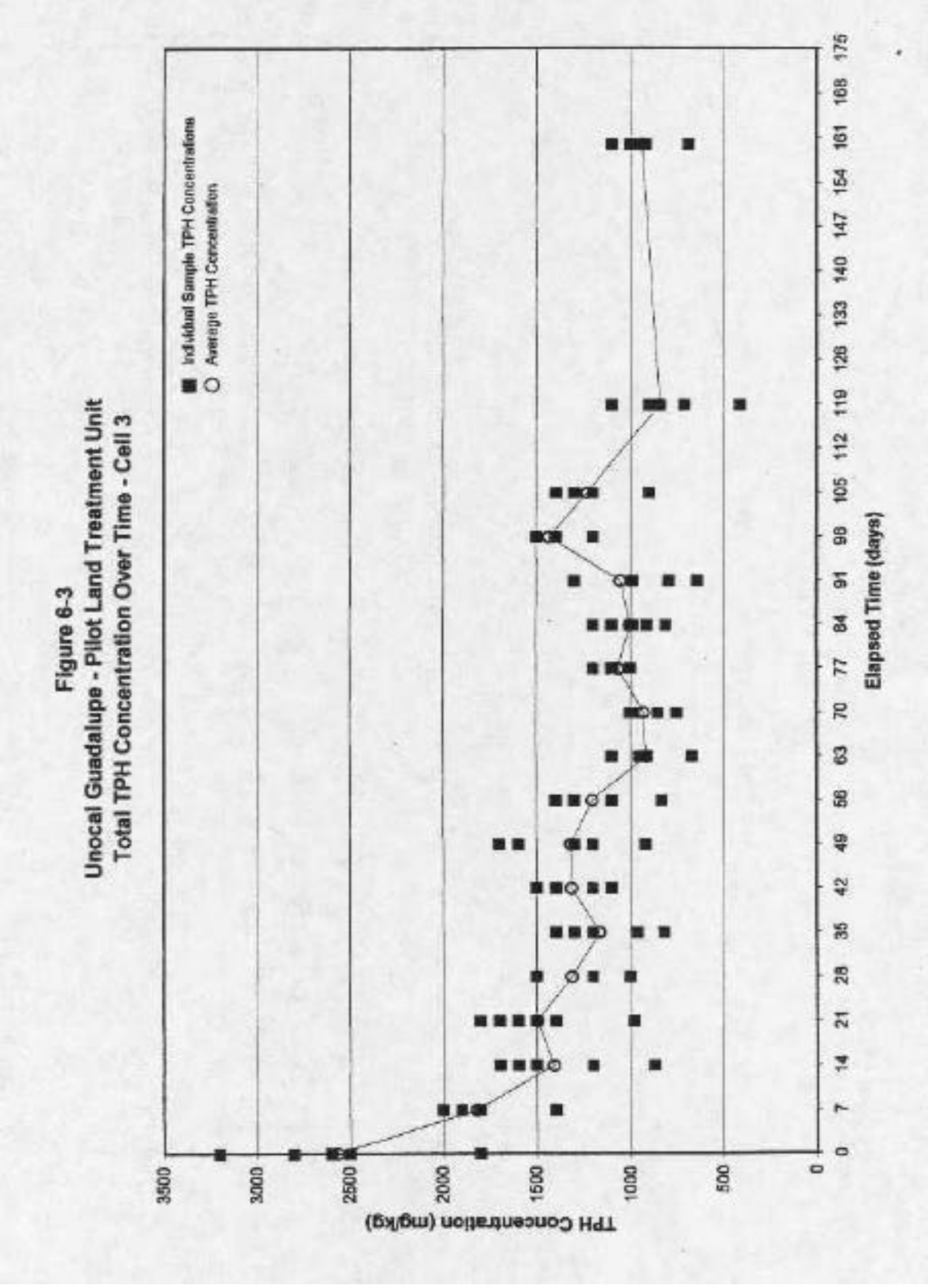
This study demonstrated the perpetual fluctuation of a complex fungal community over a 98 day period. Distinctive patterns of the fungi present were rapidly generated using fluorescently labeled, PCR amplified 18S rDNA terminal restriction fragments. The TRFPs produced depend on the restriction enzyme employed (Figures 1-3). Figure 4 illustrates the total number of peaks observed for each TRFP. This graph shows that the total number of peaks created by each enzyme decreased between days 14 and 49 but seems to be relatively constant between days 49 and 98 for those peaks created by *Dpn*II and *Msp*I. In contrast to this *Hae*III appears to have produced a greater number of peaks on day 98 than on day 49. Although the total number of observed peaks between these latter two periods is the same for both *Dpn*II and *Msp*I this doesn't mean they are the same peaks. In fact the day 49 TRFP and the day 98 TRFP created by *Dpn*II have three unique peaks between them. It isn't certain exactly how many different organisms these six peaks represent but it does indicate a change in the fungal community over that time period. Some ideas can now be formulated to help explain the population dynamics observed.

In the beginning there was an abundance of nutrients for a wide range of fungi to use and metabolize. This is represented by the high number of peaks visible on each TRFP for day 14. As time went on and these substrates were used up, conditions became more demanding, and those fungi that were less efficient at degrading hydrocarbons began to die off. This left only those organisms that could utilize the more complex hydrocarbons for survival. During this time (day 49) these fungi broke down the complex hydrocarbons into simpler ones and the total petroleum hydrocarbon (TPH) levels dropped (Figure 5). This may have provided a less demanding environment in which different organisms could flourish. Evidence for this is seen in day 98 when new peaks began to arise and the TRFP created by *Hae*III had a higher number of

peaks than in the TRFP it created for day 49. The accumulation of bacterial biomass could have also aided in the growth of certain fungi on day 98.

TRFP analysis is good for an overall description of the dynamics of a population and has a great advantage of being able to study all the members of a community but is not yet specific enough to identify particular organisms (at least not in a complex environment). Perhaps using more or different restriction enzymes, or using a less conserved region of DNA might help solve this dilemma. Identification of specific organisms, however, is not mandatory for substantive studies of microbial communities (5).

Figure 5. Total TPH Concentration Over Time for Cell 3 (3)



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