Analysis of Fungal Diversity in a Petroleum Land Treatment Unit Using Terminal Restriction Fragment Patterns (TRFPs)

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Abstract

The genetic composition of the fungal community in a petroleum remediation pilot scale land treatment unit (LTU) was analyzed using terminal restriction fragment patterns (TRFPs). The objective of the LTU was to create a suitable environment in order to promote the biodegradation of petroleum-contaminated sand. The LTU consisted of four separate cells treated with different soil amendments. Cell 2, amended with corn steep, was the cell of interest here. The 18S ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR) using primers that were previously designed and which proved to give the expected fragment sizes for *Aspergillus* genomic DNA. The primers yielded bands of approximately 550 base pairs (bp). One primer was a fluorescent-labeled primer. The tagged rDNA product was digested using either *Msp*I, *Hae*III, or *Dpn*II restriction enzymes. The fragments were separated by capillary electrophoresis on an ABI310 Genetic Analyzer® to observe the terminal restriction fragments (TRFs) generated by restriction digestion. The TRF patterns changed over time for each of the enzymes used. Both predicted and unpredicted peaks were observed for all three enzymes. Accurate predictions of diversity were not accomplished by the TRFs produced. TRF patterns for all enzymes showed a decrease in number of peaks from day 14 to day 49. By day 98, however, the number of TRFs had increased again. The range of TRFs observed was between 6 and 15.

1. Introduction

Bioremediation is a process in which microorganisms play a significant role in degrading organic compounds including petroleum hydrocarbons. The degradation rate and success depends on environmental conditions affecting the microorganisms as well as factors like specific hydrocarbon compound structures. Fungal genera producing abundant small conidia such as *Penicillium* and *Verticillium* spp. have been reported to be efficient in hydrocarbons' degradation (Davies and Westlake, 1979). Biological land treatment was identified as one remedial method for treating diluent-affected soil at the former Guadalupe Oil Field located on the Central Coast in San Luis Obispo and Santa Barbara Counties (4). The Union Oil Company of California (Unocal) provided Environmental Biotechnology Institute (EBI) of Cal Poly, San Luis Obispo with a grant to help establish the operational requirements needed to maximize the rate and extent of hydrocarbon degradation at a pilot Land Treatment Unit (LTU).

The pilot LTU was divided into four test plots designated Cell 1, Cell 2, Cell 3, and Cell 4, each measuring 225-ft by 125-ft by 5-ft high. All cells were treated with standard application of nitrogen (ammonium sulfate) and phosphorous (monopotassium phosphate) sources. Moisture levels (6% and 10%) were maintained throughout the 24-week study. In addition, each cell was tilled to a depth of 18 in. in order to increase the amount of oxygen available. Cells 2, 3, and 4 also received an additional source of carbon to encourage microbial activity. Cell 1, not amended, was used as a control. Cell 2 was treated with 0.5% w/w corn steep liqueur, a by-product in the production of corn syrup. Corn steep is 20-40% protein and high in nitrogen as

well as acid. Cell 3 was treated with 0.5% w/w dextrose. And Cell 4 was treated with 0.5% w/w cornstarch (Pilot Land Treatment Report, 2000).

The total petroleum hydrocarbon (TPH) concentrations over time were determined for all cells; results obtained for Cell 2 are shown in Figure 1. It was observed that petroleum levels dropped significantly in the first few weeks of the treatment period and continued to decline slowly throughout the remaining period with Cell 2 showing the highest TPH degradation when compared to other cells. Fungal plate counts were also obtained (Figure 2). Fungal counts started low for all cells but increased significantly by the sixth week of treatment. Counts in Cells 2 and 4 seemed higher by one order of magnitude than in Cells 1 and 3, especially at later time periods. The fungal community, however, cannot be defined based only on culture-dependent techniques. The identification of uncultured organisms is very important since it is well known that large fractions of the organisms existing in nature appear to be refractory to cultivation (Ward,Weller *et al*, 1990).

Several genetic approaches for assessing microbial diversity in complex environments now provide powerful addition to the culture-dependent techniques. However, these techniques are time consuming and somewhat incomplete. For example, most polymerase chain reaction (PCR) based methods depend on amplifying, cloning, and sequencing the desired genes from sample DNA. This process is time consuming since several thousand clones from a single sample must be cloned to precisely characterize a microbial community (Borneman, Skroch *et al* 1996). Another technique, amplified ribosomal DNA restriction analysis (ARDRA), creates

patterns by restriction digestion of amplified community ribosomal DNA (rDNA). However, ARDRA data relies on the visual resolution of all restriction fragments. Since a significant fraction of the fragments may not be resolved visually, the amount of data available for analysis may be reduced (Clement, Kehl *et al*, 1998). Therefore, it was important to consider other techniques that may prove to be quick and efficient.

In this study, the genetic diversity of the fungal community in Cell 2 was analyzed using a PCR-based method called terminal restriction fragment pattern (TRFP) analysis, which is rapid because it does not require culture or cloning (Clement, Kehl *et a*l, 1998; Liu, Marsh *et al*, 1997). In this technique, the 18S rDNA was amplified and fluorescent-labeled using PCR. The primers used were homologous to conserved regions of fungal 18S ribosomal genes were designed to amplify a region of approximately 550-bp that spanned a region of divergent sequence. It was reported that these primers amplified DNA of the predicted size from *Aspergillus* genomic DNA as well as from DNA extracted from LTU same samples (Shank, P., 1999). Labeled PCR products obtained from LTU samples were cut with 4-cutter restriction enzymes that yield fluorescently labeled terminal restriction fragments (TRFs). Capillary gel electrophoresis separation generated patterns reflecting genetic diversity. The resulting number of TRF peaks reflects the genetic diversity of the community present in the sample. The TRFPs' results obtained for Cell 2 are reported here.

2. Materials and methods

2.6.*DNA extraction*

Microbial community DNA was extracted from soil samples obtained from Cell 2 at days 14, 49, and 98. The extraction was carried out using a modified Ultraclean MoBio Soil DNA Kit (MoBio Laboratories, Inc. Solano Beach, CA). The protocol provided was follow.

2.2. *Gel electrophoresis*

In order to confirm the presence of DNA from the extraction, a 1.5% agarose gel was electrophoresed in pH 8.5 TBE buffer (89.2 mM Tris base, 88.9 mM boric acid, 2.47 mM disodium EDTA), and stained with ethidium bromide. The bands were checked for consistency.

2.6.*DNA quantification by A_{260} UV spectrophotometer*

The DNA concentration in each sample was quantified using SPECTRAmax UV spectrophotometer® (Perkin-Elmer, Applied Biosystems Inc., Fremont, CA, USA). Dilutions of 1:20 were used for quantification. The DNA concentrations (Appendix A) were diluted with DI water to a final concentration of 10 ng/µl (Table 2, Appendix C) for running PCR.

2.7.*PCR (18s labeled)*

Primers homologous to conserved regions of fungal 18S ribosomal genes, previously designed, were used to amplify a region of approximately 550 bp. The sequences of the labeled primers were FUN18s1 5′CCATGCATGTCTAAGTWTAA′3, forward, and FUN18s2 5′GCTGGCACCAGACTTGCCCTCC ′3, reverse. Reactions were carried out with the

following reagents in triplicate, resulting in a 54 µl (Table 3, Appendix C) reaction per sample: Template DNA, 10 ng; 10X buffer (Promega); dNTPs, $3X10^{-5}$ mmols; bovine serum albumin, $4X10^{-2}$ µg; $MgCl_2$, $1.75X10^{-4}$ mmols; FUN18s1; FUN18s2; Taq DNA polymerase (Promega), 2U; and PCR water. Reaction temperatures and cycling were: 95°C for 2 minutes, 40 cycles of 95°C for 1 minute, 49°C for 1 minute, 72°C for 2 minutes, followed by 72°C for 10 minutes.

2.5. *Combination and clean-up of labeled PCR reactions*

The amplified DNA was visualized by agarose gel electrophoresis (section 2.2). All successful PCR reactions were combined together. In order to remove the primers and concentrate the amplicons, the Ultra Clean PCR Clean-Up DNA Purification Kit (MoBio Laboratories, Inc. Solano Beach, CA) was utilized according to the protocol included with kit.

2.6.*Combined amplicons quantitation by A_{260} UV spectrophotometer*

The labeled PCR products were quantified using SPECTRAmax UV Spectrophotometer® as described in section 2.3. The amplicon concentrations (Appendix B) were diluted with DI water to a final concentration of 10 ng/µl (Table 4, Appendix C) for performing digestions.

2.7. *Enzyme Digest*

The labeled PCR product was digested with MspI, DpnI, and HaeIII enzymes. The digestion reactions contained 3.9 µg of labeled DNA and were incubated for 5 hours at 37˚C. The 20 µl reactions contained 0.4 U/L of either MspI or DpnII or 0.2 U/L of HaeIII restriction

endonucleases (New England Biolabs, Beverly, MA, USA) in the manufacture's recommended reaction buffers. Once the digestion was finished, the tubes were placed in a 65˚C water bath for 20 min to deactivate the enzymes.

2.8. *Ethanol Precipitation of Digest*

The digest reactions were precipitated with 95% ethanol. Each digest was added with 50 µl, 2.5 x digest volume, of cold 95% ethanol and 1 µl 3M Sodium Acetate (pH4.6), 5% digest volume. After incubating the tubes for at 4˚C for 30 min, the DNA was spelled by centrifugation for 15 min at 15 x kg, washed in 50 µl cold 70% ethanol, and centrifuged again for 15 min at 15 x kg. The ethanol was removed completely, and the pellet dried.

2.9. *Capillary Electrophoresis and fragment size determination*

The precipitated pellet was dissolved in 18 µl of formamide (BioRad, Benecia, CA, USA), 1µl load buffer, and 1 µl each of Genescan 500 ROX® (Perkin-Elmer, Applied Biosystems Inc., Fremont, CA, USA) and ROX 550-700® (BioVenture, Murfreesboro, TN, USA) size standards. The samples were denatured at 95˚C for 5 minutes and snap-cooled for 10 minutes, and run on an ABI Prism™ 310 Genetic Analyzer at 15 kV and 60˚C. TRF sizing was performed on electropherogram output from Genscan 3.1 software using Local Southern method with heavy smoothing. Electropherogram peaks were resolved to one base pair with size standard peaks.

2.10. *Data analysis*

Truncation and normalization were used to analyze the data. Sample data consisted of peak areas (Appendix D) for a given TRF in a pattern. The total sample area varied between TRFPs due to the uncontrolled amount of DNA loaded on the capillary, so it was necessary to truncate peaks, and convert peak areas to parts per million to normalize the data. Creating new detection thresholds for each sample normalized peak detection threshold. Truncation was done by multiplying the smallest detectable peak area by the multiple of its total area to that of the sample with the smallest totals area. Peaks under the threshold value for a sample were removed from the data set (Table 1).

Table 1. Truncation example.

SAMPLE	TOTAL AREA	RATIO OF TOTAL PEAK AREA	SMALLEST PEAK AREA ANALYZED
Smallest	200000	1:1	580*
Big	400000	2:1	1160
Bigger	2000000	10:1	5800

*Minimum detectable peak area with ABI Prism™ 310

Converting each peak remaining after truncation to ppm normalized samples. This was done by finding the ratio of each peak to the total area, and multiplying by 10^6.

3. Results and discussion

The TRFPs in Cell 2 changed significantly over time. The structure of the fungal community fluctuated for all three enzymes used at days 14, 49, and 98.

The enzyme *Msp*I gave different number of TRFs for days 14, 49, and 98 (Figure 3A, 3B, and 3C, respectively). The least number of peaks was observed at day 49. The greatest number of peaks was observed at day 98 (Figures 3C and 6). Peaks 1, 2, and 4 disappeared at day 49 and reappeared by day 98; peaks 3, 6, 7, and 8 disappeared completely; peaks 5 and 9 were larger at day 49 compared to days 14 and 98. New peaks were observed at days 49 (peak) 10, and 98 (peaks 11-14).

The enzyme *Dpn*II gave different number of TRFs for days 14, 49, and 98 (Figure 4A, 4B, and 4C, respectively). The greater number of peaks was observed at day 14. Peaks 1 and 2 were larger at day 14 but persisted through day 98. Peak 6 was present throughout but larger at day 98. Peak 8 was also present throughout but larger at day 49. Peak 3 disappeared by day 49 while peaks 4 and 5 disappeared by day 98. Peak 7 disappeared at day 49 but reappeared by day 98.

The enzyme *Hae*III gave different number of TRFs for days 14, 49, and 98 (Figure 5A, 5B, and 5C, respectively). Peaks 1, 3, 4, and 6 disappeared at day 49, then reappeared by day 98; peaks 2 and 5 disappeared entirely by day 49. Peaks 7, 8, and 9 appeared at day 49 and remained until day 98; peaks 10, 11, and 12 appeared only at day 98.

A database was created by downloading fungal ribosomal sequences from Genbank[â] and submitting them to a program that mimics TRF analysis. This program predicted some of the observed TRFs, but not all (Tables 1 and 2). For example, observed TRF peak number 1 (Table 1) was predicted to consist of several genera and TRF peak number 2 (Table 1) was not predicted from the databases. Unpredicted TRFs may be due to the incomplete databases available. Although not shown, similar results were apparent for digestion with *Hae*III.

A summary of the TRF patterns observed in Cell 2 for all three enzymes for days 14, 49, and 98 is shown in Figure 6. An overall trend toward a decrease at day 49 and an increase at day 98 in TRF peaks was observed. The number of TRF peaks for all three enzymes ranged between 6 and 15. The enzyme *Hae*III yielded the greatest number of peaks and the enzyme *Dpn*II yielded the least number of peaks. Additionally, digestions with *Msp*I and *Hae*III gave new peaks at days 49 and 98, but no new peaks were observed for *Dpn*II after day 14.

Figure 7 shows the observed TRF lengths for the fungal community after digestion with *Msp*I, *Dpn*II, and *Hae*III. TRF peaks were consistently sized from approximately 120 bp to 530 bp. Digestion with *Hae*II appeared to give slightly more abundant short TRFs. TRFs bigger than approximately 500 bp were believed to be undigested amplicons. Digestions with enzyme *Dpn*II resulted in the greater number of larger fragments. Over 50 genera were predicted to give an uncut fragment, which agreed with the observed results. Digestion with *Msp*I also resulted in a slightly higher number of large fragments and two organisms were predicted to give peaks greater than 500 bp for *Msp*I (Table 2).

4. Conclusion

In this study, changes over time in the fungal community of the pilot LTU Cell 2 were observed using TRFPs (Figures 3-5). Distinctive fungal community patterns were rapidly generated using fluorescently labeled, PCR- amplified 18s rDNA terminal restriction fragments. Two enzymes , *Msp*I and *Hae*III, showed similiar community dynamics overtime. TRF peaks that were predicted and some that were not predicted were observed (Tables 1 and 2). Because several TRF peaks were predicted from a large number of genera, use of 18s for community studies may not reflect real diversity. It was not possible to predict, based on the peaks observed, which organisms might be present over time. However, an overall trend of a decrease and increase in peak number was observed for all three enzymes from day 49 to day 98 (Figure 6). This may be due to the addition of corn steep liqueur at day 48. Database analysis predicted a larger number of TRFs greater than 500 bp for *Dpn*II, but not ther other two enzymes, fitting with the data (Table 1 and Figure 4). Overall, this analysis was useful in observing changes in fungal community structure.

Table 1. *Dpn*II observed and predicted TRFs.

	***Dpn*II**
TRF peak observed (bp)-peak 1*	**212-216**
Lophiostoma crenatum	212
Alternaria (2 species)	213
Cochliobolus sativus	213
Cucurbidothis (3 species)	213
Herpotrichia (2 species)	213
Leptosphaeria (3 species)	213
Mycosphaerella mycopappi	213
Pleospora herbarum	213
Pyrenophora tritici-repentis	213
Rhytidhysteron rufulum	213
Setosphaeria (2 species)	213
Sporomia lignicola	213
Westerdykella dispersa	213
Curvularia brachyspora	214
Pneumocystis carinii	214
Scolecobasidium sp.	215
TRF peak observed (bp)-peak 2*	**217-218**
No predicted peaks from database	
TRF peak observed (bp)-peak 3*	**268-271**
No predicted peaks from database	
TRF peak observed (bp)-peak 4*	**>500**
Over 50 genera predict an uncut fragment	

*See Figure 4.

Table 2. *Msp*I TRF peaks observed and predicted

	***Msp*I**
TRF peak observed (bp)-peak 11*	**131**
No predicted peaks from database	
TRF peak observed (bp)-peak 12*	**139**
Aciculosporium take	140
Claviceps paspali	140
Halosphaeriopsis mediosetigera	140
TRF peak observed (bp)-peak 1*	**177-179**
Thanatephorus praticola	178
Candida maltosa	179
Lecanora dispersa	179
Ramichloridium anceps	179
Neocosmospora vasinfecta	180
Podospora anserina	180
Talaromyces bacillisporus	180
TRF peak observed (bp)-peak 2*	**~224**
Bensingtonia musae	224
Bensingtonia (2 species)	225
Candida krusei	225
Kondoa malvinella	225
TRF peak observed (bp)-peak 10*	**~229**
Exophiala mansonii	229
Nadsoniella nigra	229
Phaeoannellomyces	229
Phaeococcomyces exophialae	229
74 genera	230-232
TRF peak observed (bp)-peak 4*	**313**
No predicted peaks from database	
TRF peak observed (bp)-peak 5*	**334**
30 genera	332-335
TRF peak observed (bp)-peak 8*	**>500**
Hansenula polymorpha	521
Saccharomycodes ludwigii	522

* See Figure 3.

Figure 1. LTU total TPH concentration over time observed for Cell 2, which was amended with corn steep.

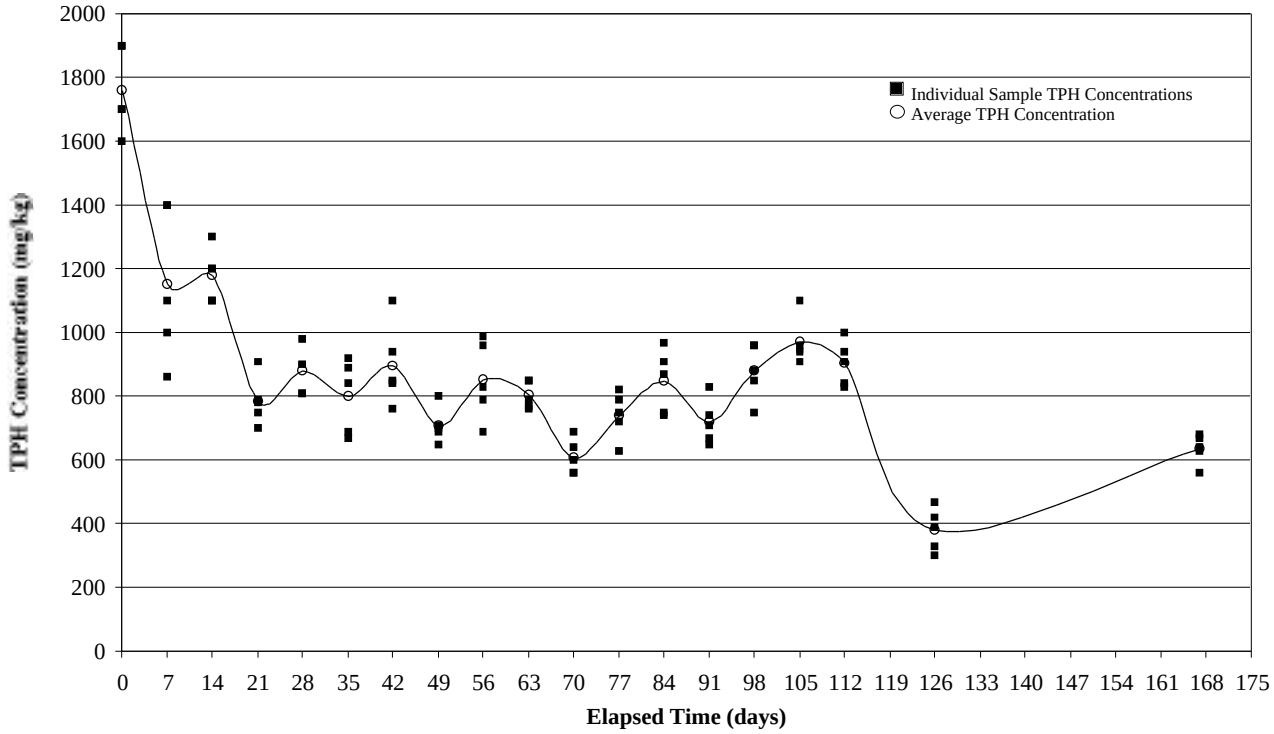


Figure 2. Fungal Plate Count for Cells 1 through 4.

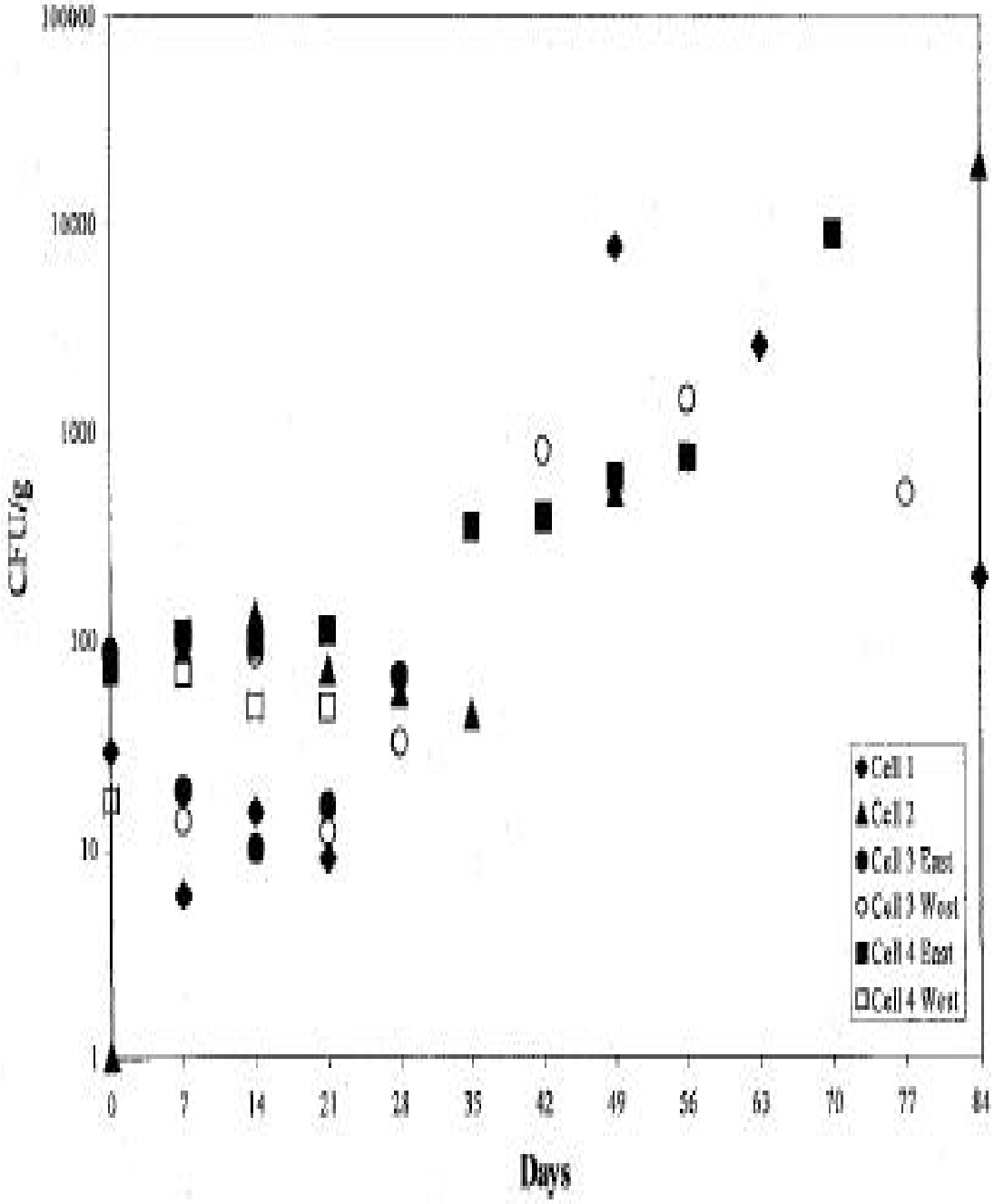


Figure 3. Electropherograms of the TRFPs derived from *Msp*I digestions of fungal community DNA obtained from LTU Cell 2 petroleum contaminated soil samples at days 14 (2A), 49 (2B), and 98 (2C). Major peaks are numbered.

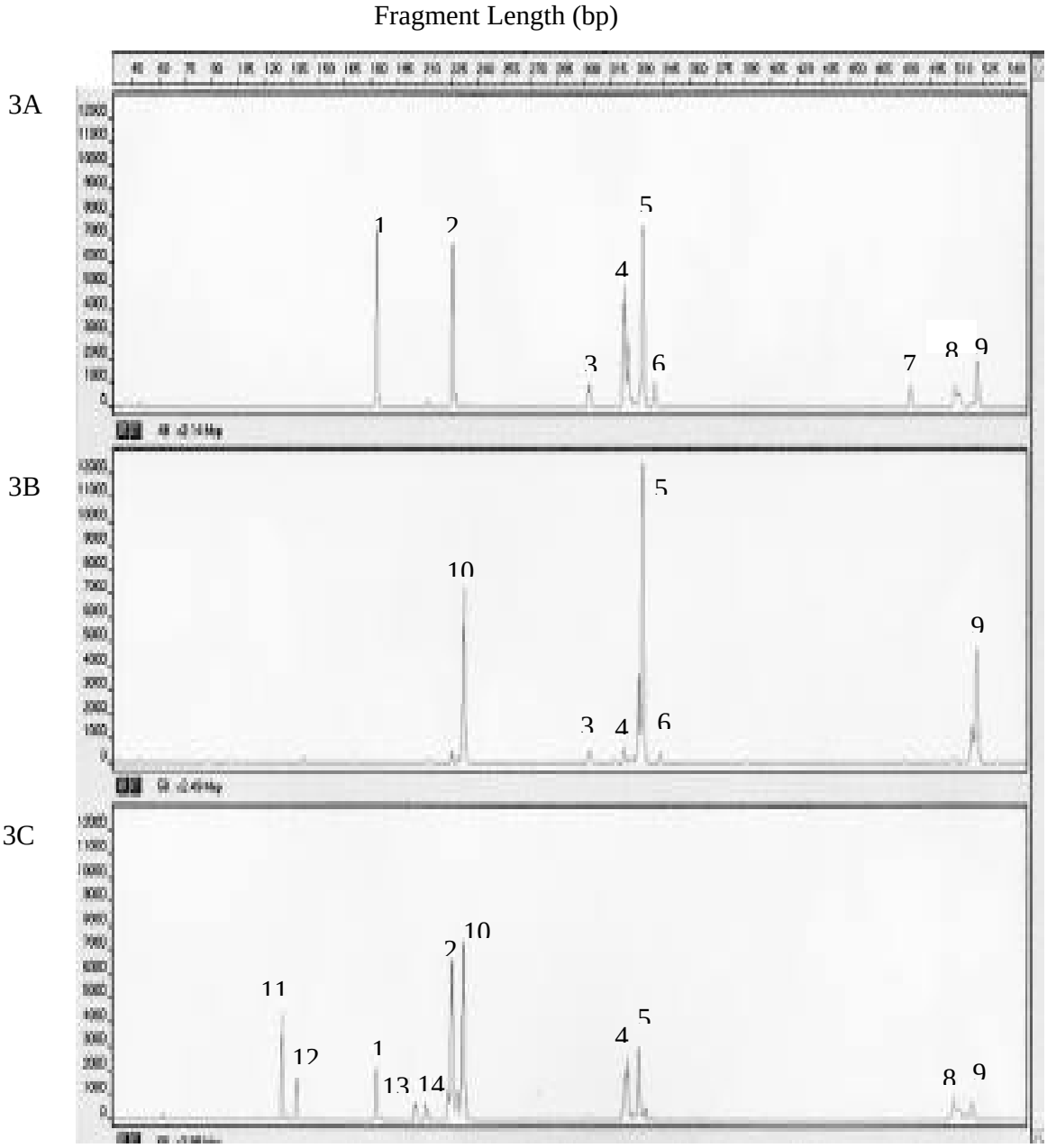


Figure 4. Electropherograms of the TRFPs derived from *Dpn*II digestions of fungal community DNA obtained from LTU Cell 2 petroleum contaminated soil samples at days 14 (3A), 49 (3B), and 98 (3C). Major peaks are numbered.

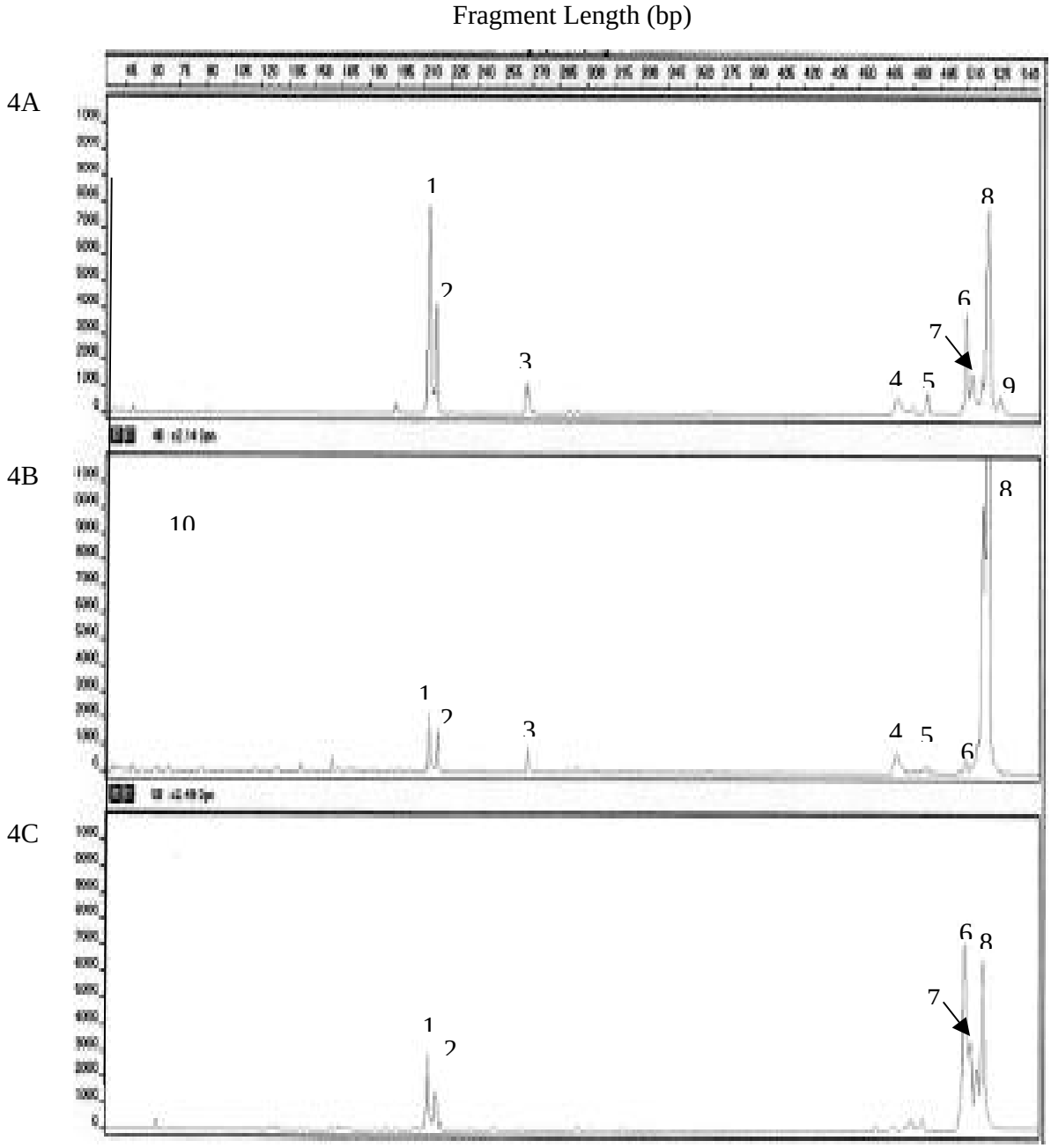


Figure 5. Electropherograms of the TRFPs derived from *Hae*III digestions of fungal community DNA obtained from LTU Cell 2 petroleum contaminated soil samples at days 14 (4A), 49 (4B), and 98 (4C). Major peaks are numbered.

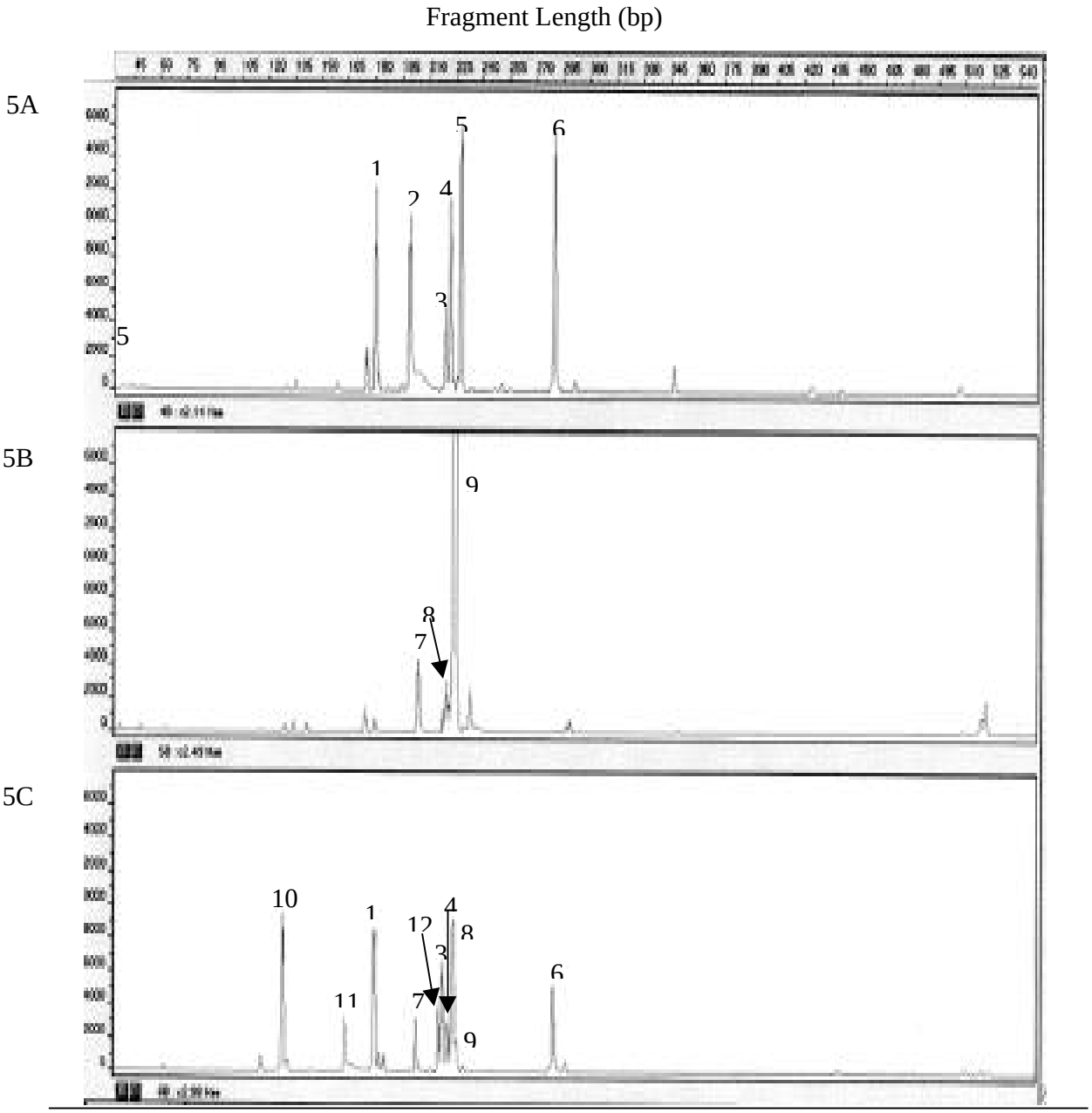


Figure 6. Summary of TRF patterns observed in Cell 2 for enzymes *Msp*I, *Hae*III, and *Dpn*II for days 14, 49, and 98. All peaks were included in this analysis.

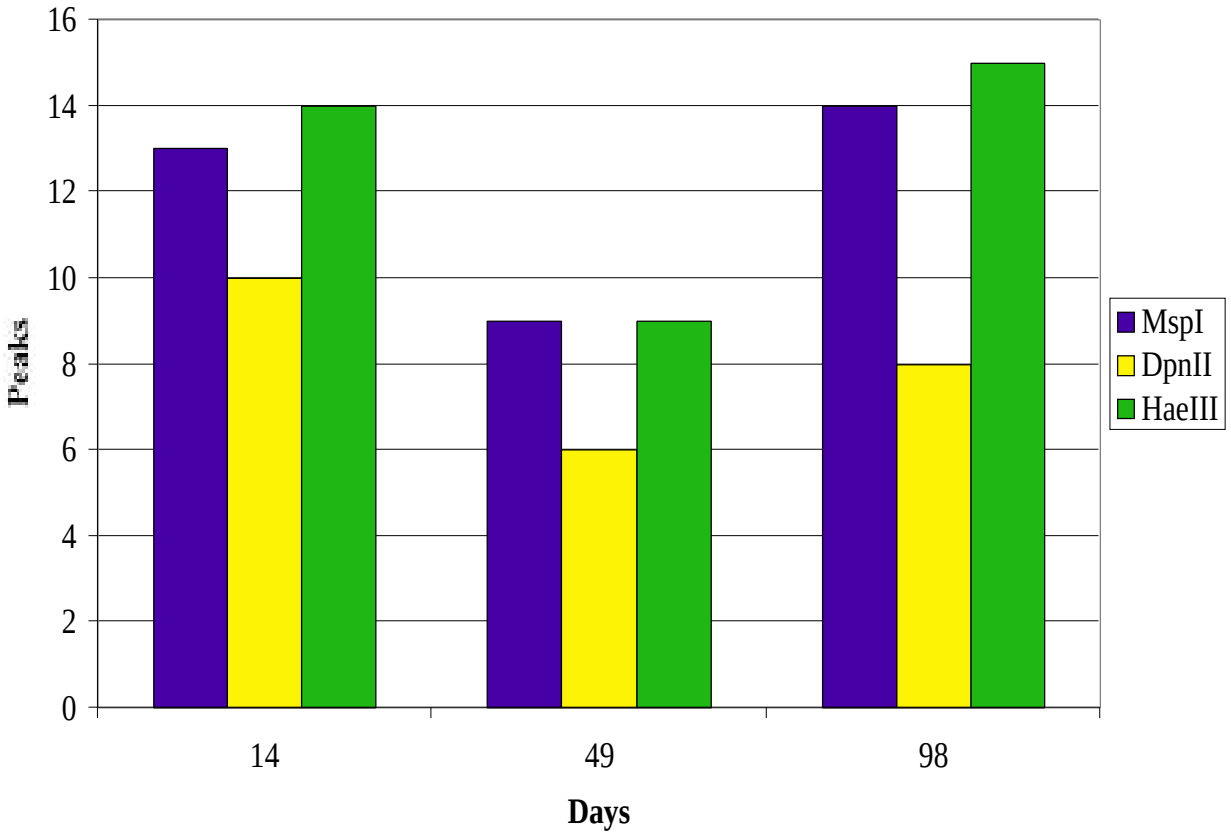
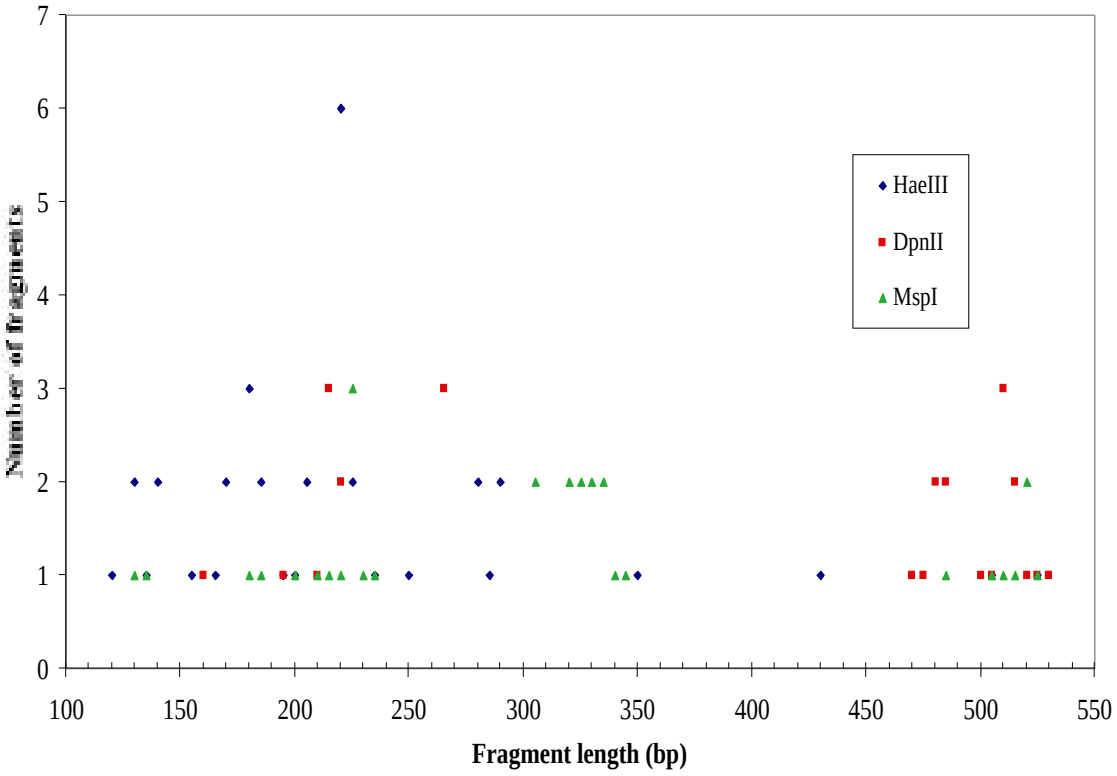


Figure 7. Observed TRF lengths for 18S rDNA for fungal community after digestion with *Msp*I, *Dpn*II, or *Hae*III for days 14, 49, and 98.



Appendix C

The following volumes and concentrations were calculated to obtain a final DNA concentration of 10 ng/µL from initial concentrations obtained using SPECTRAmax UV spectrophotometer®.

Table 2. Final DNA concentrations for running PCR.

SAMPLE	[DNA]	DNA VOLUME (µL) [1]	H_2O (µL) [2]
C2:14	52.0	19.2	80.8
C2:49	13.2	75.8	24.2
C2:98	13.8	72.5	27.5

[1] $C_1V_1=C_2V_2$, where C_1=52.0, C_2= 10ng/µl (the wanted final [DNA]), V_1 = wanted volume, and V_2=100µl: V_1=(10ng//µl*100µl)/(52.0ng/µl)=19.2µl

[2]A total volume of 100 µl was required. 19.2- H_2O=100µl=80.2µl

The following table lists the reagents and volumes used for running PCR with reactions of 54 µL.

Table 3. List of reagents for PCR reactions (per sample)

MASTER MIX	VOLUME (µL)
10X Buffer	5
$MgCl_2$	7
dNTPs	3
FUN18s1	1.1
FUN18s2	1.1
H_2O	26.5
Taq DNA Polymerase	0.3

The following volumes and concentrations were calculated to obtain a final DNA concentration of 10 ng/µL from initial concentrations obtained using SPECTRAmax UV spectrophotometer®.

Table 4. Final DNA concentrations for enzyme digestion of labeled PCR product.

SAMPLE	[DNA]	DNA VOLUME (µL) [1]	H_2O (µL) [2]
C2:14	59.06	1.7	2.2
C2:49	50.1	2.0	1.9
C2:98	25.9	3.9	0.0

[1] $C_1V_1=C_2V_2$, where C_1=59.6, C_2= 10ng/µl (the wanted final [DNA]), V_1 = wanted volume, and V_2=10µl: V_1=(10ng//µl*10µl)/(59.6ng/µl)=1.7µl

[2]A total volume of 3.9µl was required because that was the lowest [DNA]: 1.7- H_2O=3.9µl=2.2µl

References

1. Borneman, J., P.W. Skroch, K.M. O'Sillivan, J.A. Palus, N.G. Rumjanek, J.L Jansen, J. Nienhuis, E.P. Triplett, Molecular microbial diversity of an agricutural soil in Wisconsin, Appl. Environ. Microbiol. 62 (1996) 1935-1943.
2. Clement, Brian G., Lucia E. Kehl, Kristin L. DeBord, Christopher L. Kitts, Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities, Journal of Microbiological Methods 31 (1998) 135-142.
3. Davies, J.S., D.W. Westlake, Crude oil utilization by fungi, Can J. Microbiol. 25 (1979) 146-156.
4. Liu, Wen-Tso, Terence L. Marsh, Hans Cheng, Larry J. Forney, Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphism of Genes Encoding 16S rRNA, Applied and Environmental Microbiology. 63 (1997) 4516-4522.
5. Pilot Land Treatment Unit Report Guadalupe Oil Field, San Luis Obispo, CA. February 25, 2000. Environmental Biotechnology Institute, California Polytechnic State University, San Luis Obispo.
6. Shank, P., Senior Project, California Polytechnic State University, San Luis Obispo, Biological Sciences Dept. (1999)
7. Ward, D. M., R. Weller, and M. M. Bateson. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community, Nature 345 (1990) 63-65.