Development of TRF Assay for Detection of Actinobacteria

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Abstract

Actinobacteria are prominent taxa in most soil communities and play an important role in bioremediation. To assess community structure, a terminal restriction fragment (TRF) assay was developed for the specific detection of actinobacterial communities in soils. Soil samples were collected from a coastal sand dune that had been contaminated with petroleum hydrocarbons. Samples were screened for cultivatable actinobacteria by cultivation on Czapek yeast agar, starch casein agar and colloidal chitin agar. The specificity of primers F243 and 814R for the amplification of actinobacteria 16S ribosomal RNA genes (rDNA) was evaluated using DNA extracted from various strains of actinobacteria and other non-actinobacteria. The primer pair was shown to be specific for actinobacteria. TRF peaks produced by individual soil isolated actinobacteria were successfully matched to TRF peaks found in the community soil TRF pattern from which the isolates were obtained. Application of TRF analysis with primers F243 and 814R was performed using soil collected from the Natural Attenuation site at the Guadalupe Dunes. A distinct difference in the actinobacterial community structure was detected when samples containing varying physical/chemical parameters (i.e. TPH, CO_2, CH_4, O_2 etc.) were analyzed. Some TRF peaks had strong correlations to physical/chemical parameters commonly associated with anaerobic TPH degradation. Although changes in some TRF peaks correlated strongly with changes in physical/chemical parameters, an insufficient GenBank database made it difficult to specifically identify different genera of the actinobacterial community. Using primers F243 and 814R, 16S rDNA TRF analysis allowed for the specific detection and monitoring of actinobacteria communities associated with TPH degradation.

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Introduction

The study of microbial diversity and community dynamics is growing rapidly. Through the use of culture-independent molecular techniques, new insights into the structure of uncultivated microbial communities have been gained (Head et al., 1998). Molecular tools have a great potential to assist in developing strategies for identifying previously uncultured bacteria using known rDNA sequences. Identification of organisms via molecular tools can also help in determining appropriate culture media, most effective for isolation and purification of specific microorganisms. These tools allow us to further investigate or exploit microorganisms (Kane et al., 1993; Teske et al., 1996A). Identification and/or isolation of microorganisms capable of performing bioremediation could potentially aid in deciphering the mechanism by which organisms work together in a community structure to bioremediate contaminants.

The Guadalupe Oil Field is located on the central coast of California approximately 15 miles south of San Luis Obispo. It is part of the Unocal LeRoy Lease, which covers approximately 3,000 acres within the Nipomo Dunes system. The Nipomo Dunes system is one of the largest dune systems along the California coast. Due to the presence of extensive sand dunes, dune uplands, lakes, and wetlands, the U.S. Secretary of Interior has designated the area as a National Natural Landmark (Little, 1997).

Oil exploration and production began on the site with the Sand Dune Oil Company in October, 1947. Unocal acquired the field in the early 1950s and continued to operate it until March, 1990. At its peak in 1988, there were 215 potential producing wells, and oil production rates for the field were approximately 4,500 barrels per day (bpd). As many as 23 wells remained in operation until April, 1994 (Little, 1997).

Crude oil pumped from the site was extremely viscous and difficult to extract. Unocal employed several methods, including diluent mixing, to enhance recovery of the heavy crude oil. The "diluent" was used to thin the crude oil so as to make pumping it more efficient. The diluent used mostly at the Guadalupe field was a refined hydrocarbon blend that was piped into the field from the Santa Maria Refinery on the Nipomo Mesa (Little, 1997).

During the time hydrocarbon diluent was used at the site, numerous leaks developed in the tanks and pipelines used to distribute it around the field. Over time, these leaks led to serious contamination of the groundwater below the site. Diluent accumulated in plumes at the water table in the dune sand aquifer (about 3 to 40 meters down, depending on the location). As the ground water passed through these areas, some of the diluent dissolved into the water and moved downstream with the groundwater flow, generally from east to west. This resulted in groundwater contamination beneath much of the site, with a flux towards the ocean and the Santa Maria River (Little, 1997).

Petroleum hydrocarbons have been harvested all over the world. For years the refining and transport of petroleum has been the major contributor to vast amounts of polyaromatic hydrocarbons (PAH's) in the environment. There are a variety of methods used to remediate contaminated areas include landfilling and incineration. Most methods are not only difficult to perform, but they can be very costly and have a negative affect on the surrounding environment and habitat. As an alternative to these remediation methods, bioremediation has been established as an efficient, economic, versatile, and environmentally sound treatment (Margesin et al., 2001). Bioremediation uses indigenous microorganisms and works by either transforming or degrading contaminants to less hazardous chemicals.

Natural Attenuation (NA) is the process by which bioremediation of a contaminated site occurs in a natural state. The NA site at the former Guadalupe Dunes has no external interference including physical (tilling the soil etc.) or chemical (addition of nutrients, water, supplements etc.) to help stimulate microbial or any other activity. Bioremediation is simply monitored as it occurs in a natural state. NA monitoring at the former Guadalupe Oil Field was started in 1994 and consists of two general programs: dissolved-phase diluent plume natural attenuation and source zone natural attenuation. The primary objectives for dissolved-phase diluent natural attenuation monitoring at the site are (1) to assure that surface water is not at risk in areas where natural attenuation is the designated remedial action and (2) to provide information on natural attenuation processes at the site. Natural Attenuation of dissolved-phase diluent is being monitored at seven plumes throughout the site. In general, the minimum monitoring requirements for a designated dissolved-phase diluent natural attenuation-monitoring program consists of two years of quarterly sampling. Groundwater samples are sent to a fixed laboratory (Zymax, San Luis Obispo, CA) for analysis of total petroleum hydrocarbon (TPH), Benzene, Toluene, Ethylbenzene, Xylene (BTEX), alkalinity, dissolved iron, nitrate such as nitrogen, sulfate and dissolved gasses (hydrogen, oxygen, methane, carbon dioxide, and nitrous oxide) and ferrous iron (Schroeder, 2003). After two years, the monitoring was reduced to semi-annual sampling for TPH and dissolved oxygen (Schroeder, 2003). Microbial activity is also monitored through the Environmental Biotechnology Institute (EBI) via Terminal Restriction Fragment TRF analysis.

Source zone natural attenuation monitoring consists of two general programs: groundwater sampling at multilevel well clusters and soil-gas (oxygen, methane, carbon dioxide, and nitrous oxide) sampling at multilevel soil-gas probe clusters. For each program, samples

were collected from each discrete well or probe within a cluster on a quarterly basis for the first year following installation, sampling frequency was then reduced to semi-annually. As with the dissolved-phase diluent natural attenuation monitoring, parameters used to monitor the progression of NA are obtained.

Actinobacteria

One group of microorganisms that partake in bioremediation is the actinobacteria (Stackebrandt et al., 1997). These organisms are Gram-positive bacteria characterized by high G-C content in their DNA. They can be found in a variety of habitats and are particularly abundant in soil. Most biodegradative actinobacteria are saprophytes, colonizing or originating from soil where they are usually present at $10^5 - 10^8$ cfu/g. Actinobacterial growth is typically made up of branching filaments, producing a mycelium. This may be of two types, one prostrate, forming a vegetative growth, sometimes referred to as substrate mycelium and the other an erect or aerial mycelium. The spore-bearing hyphae of the aerial mycelium usually have a somewhat greater diameter than the hyphae of the substrate mycelium. Actinobacteria typically produce two types of spores: true conidia, and arthrospores (Waksman, 1959). Actinobacteria are very metabolically diverse and growth of a new colony from a single spore (developed on sporophore), a sporangium, a fragment of hypha or a small part of an old colony, account for their ubiquity (McCarthy and Williams, 1992).

Among gram-positive bacteria, the actinobacteria exhibit the greatest morphological differentiation, which is based on a filamentous degree of organization. It is nearly impossible to summarize all the variations of colonial characters as far as known for the different species. Colonies may be raised or flat, sometimes covered with a leathery layer. Their consistency varies from very soft and pasty to extremely hard. They have a color range, which includes

white, yellow, orange, rose, red, purple, blue, green, brown, and black. Surfaces may be smooth, rigid wrinkled, granular or squamous. The colonies may be completely compact or may demonstrate different zones of growth, in concentric rings with a radial orientation, and frequently a combination of the two. Colony size depends on the species, age and growth conditions, varying from a fraction of a millimeter up to the diameter of centimeters (Miyadoh, 1997).

Actinobacteria help with the recycling of complex biopolymers such as lignocellulose, hemicellulose, pectin, keratin, and chitin (Willimas et al., 1984). Polycyclic aromatic hydrocarbons (PAHs) are hazardous by-products of combustion processes and petroleum refining. It has been shown that PAHs containing three or more rings are recalcitrant to microbial degradation (Cerniglia, 1993). Although higher molecular mass poly aromatic hydrocarbons (PAH's) are difficult to degrade, studies indicate that various strains of *Mycobacterium* spp. may play an important role in the degradation of these compounds (Grosser et al.,1991; Boldrin et al., 1993; Kleespies et al., 1996; Bottger et al., 1997). *Rhodococcus* also exhibit a wide range of metabolic activities. They have been shown to degrade chemical pollutants including simple hydrocarbons (Goodfellow and Minnikin, 1981), chlorinated hydrocarbons, numerous aliphatic and aromatic hydrocarbons, nitroaromatics, and chlorinated polycyclic aromatics such as polychlorinated biphenyls (Warhurst and Fewson, 1994; Iwabuchi et al., 2002). *Gordonia* is another group of actinobacteria with great metabolic diversity. Various species of *Gordonia* have been shown to possess a variety of metabolic pathways that include hydrocarbon-oxidizing and degrading (Kummer et al., 1999; Xue et al., 2003), rubber-degrading (Linos et al., 2002), aromatic-desulphurizing (Kim et al., 1999), and 3-ethylpyridine-degrading pathways (Yoon et al., 2000).

Actinobacteria are also important in the pharmaceutical industry, where they are best known as a source of antibiotics. They produce a variety of metabolites, which are the source of antibiotics, enzymes, and bioactive products in industry (Bull et al., 1992; Goodfellow et al., 1988).

Plating techniques for the isolation of Actinobacteria

Aquatic and terrestrial ecosystems depend heavily upon the activity of bacteria. The cycling of nutrients such as carbon, nitrogen, sulfur etc. is accomplished through everyday interactions with bacteria and their diverse metabolic functions. Despite their vast importance and diversity it is estimated that less than 99% of microorganisms present in nature typically are cultivated by using standard techniques (Amann et al., 1995).

A variety of non-molecular methods, including microscopic observations, plate culture studies and selective culture procedures have been developed for studying actinobacteria from a wide variety of environments including soil, compost, water, plants and animals. Although they vary greatly, each method has certain advantages and disadvantages, and helps to reveal more about the nature and abundance of actinobacteria in a specific environment. The plate methods are one of the oldest methods for determining the nature and abundance of microbial populations.

Selective media, which promote the growth of actinobacteria and suppress the growth of accompanying organisms, have been developed (Waksman, 1959). Many synthetic media containing special nutrients exhibit selective qualities, which can be altered by changing various physiological parameters like pH, salinity, temperature and period of incubation (Hunter-Cevera et al., 1986).

The growth of actinobacteria may be encouraged by the addition of natural substrates and other biopolymers, which are used as energy sources. These energy sources include organic acids, sugars, starches, hemicelluloses, and cellulose, proteins, polypeptides, amino acids, nitrogenous bases, and numerous other organic compounds. Some species utilize fats, hydrocarbons, benzene ring compounds, and even to some extent lignin, tannin, and rubber. The most readily utilizable sources of energy are glucose, mannose, maltose, dextrin, starch glycerol, amino acids, and proteins. Sucrose lactose, xylose, raffinose, rhamnose, inositol, and certain sugar alcohols and sugar acids are generally used less readily. Chitin, sterols, and polyuronides can also be utilized by certain organisms (Waksman, 1967).

In general, if one is interested in obtaining a large variety of actinobacteria for isolation purposes, a relatively simple synthetic media can be prepared with asparagine, glutamic acid, or sodium nitrate as a source of nitrogen, and glycerol, starch, glucose, or a salt of an organic acid, such as malate, as a source of carbon (Waksman, 1959). Such medium, containing the reduced concentration of nutrients, favors the growth of actinobacteria rather than fungi and bacteria. Typically, plates are incubated at 28-30 ^{o}C and examined after 2-7 days with a total incubation period of up to 4 weeks (Waksman, 1959). Lechevalier (1989) recommends agar with traces of nutrients such as: casein hydrolysate, yeast extract, or starch. It was also noted that an extract of the natural habitat (such as soil) could be very effective.

Just prior to pouring plates, the addition of antibiotics and other antimicrobial substances such as cycloheximide (40 μg/ml) and nystatin, will not only repress fungal growth, but also give much greater counts of actinobacteria (Nolan and cross, 1988; Waksman, 1959; Waksman, 1967). As with the fungi, non-actinobacteria counts can also be repressed with the addition of different antibiotics such as polymyxin and penicillin (Williams and Davies, 1965).

Additionally, air-drying soils at specified temperatures can enormously reduce the number of non-actinobacteria, and samples can be preserved in dry conditions for long periods of time (Dietz and Thayer, 1980).

Molecular Techniques

Many studies of bacterial community structure employ conventional phenotypic methods of pure cultures to characterize the bacterial community (Torsvik et al. 1990). Conventional cultivation techniques of microorganisms is laborious, time consuming and most importantly, selective and biased for the growth of specific microorganisms (Eilers et al., 2000). The majority of microscopically visualized cells are viable but do not form visible colonies on plates (Amann and Ludwig, 2000). This is likely a reflection of the artificial conditions in the culture media (e.g. lack of specific nutrients required for growth) and conditions in which the organisms are grown.

The ability to detect a population or even an individual species of bacteria by a method which does not rely on gene expression or cultivation, is ideal for studying bacterial community dynamics. Until recently, there has been no good way to describe microorganisms without growing pure cultures. Recombinant DNA and molecular phylogeny techniques have now allowed us to overcome many of the stumbling blocks of cultivation. It is now possible to perform ecological studies of microbial communities without cultivation. Complex microbial communities can be classified and surveyed in situ by relatively unbiased methods (Hugenholtz and Pace, 1996). Results obtained from molecular techniques thus far have revealed an array of novel organisms, some of which are profoundly different from those known from culture studies. This is particularly relevant to actinobacteria, since they are often likely to be present in the

environment as dormant spores rather than active metabolizing hyphae (McCarthy & Williams, 1992).

Studies based on molecular techniques tend towards the development of probes from phylogenetically related species or rRNA sequence analysis in population studies (Clegg et al., 1988). Though much less biased than traditional culture techniques, molecular techniques also have their biases. An essential tool for microbial molecular ecology is the isolation of nucleic acids (DNA or RNA) from different habitats, with a purity suitable for molecular techniques like hybridization, cloning, and amplification. Problems which may be encountered during the extraction of nucleic acids are the reliable and reproducible lysis of all bacterial cells, extraction of intact nucleic acid, and the removal of substances, such as humic acids and bacterial exopolysaccharides, which may inhibit DNA digestion with restriction enzymes and polymerase chain reaction (PCR) amplification (e.g., Wheeler and Stahl, 1996). Spores (commonly found with actinobacteria) will be less readily lysed than vegetative cells, and gram-positive cells are more resistant to cell lysis than gram-negative cells (Head et al., 1998). Inadequate cell lysis could potentially lead to a lower amplification rate of certain species during the PCR. Hence the abundance and diversity of certain organisms could ultimately become underrepresented.

Polymerase Chain Reaction (PCR), has rapidly become one of the most widely used techniques for quantitative studies in molecular biology and for good reason: it is rapid, inexpensive, and will allow minute quantities of source DNA to be amplified to about a million fold so that one can determine the number of nucleotides, and nucleotide sequence, etc. The particular stretch of DNA to be amplified, called the target sequence, is identified by a specific pair of DNA primers, oligonucleotides usually about twenty nucleotides in length. By using PCR, a gene fragment can be selectively amplified from mixed DNA. Typically the 16s rRNA

gene is targeted because it is highly conserved in bacteria and has an easily accessible and extensive database for which sequences can be compared. When using mixed template reactions, several factors can also bias gene frequency in PCR products. These factors include the moles percent guanine-plus-cytosine (G+C) content of template DNA (Dutton et al., 1993; Reysenbach et al., 1992) annealing temperature, PCR cycle number, influence of template folding and reannealing (Suzuki and Giovannoni, 1996), gene copy number and genome size (Farrelly et al., 1995), as well as the formation of chimeric molecules (Liesack et al., 1991; Kopczynske et al., 1994).

Cloning and sequencing is one of many methods that can use amplification and analysis of 16S rRNA genes (rDNA) to compare the composition, richness and structure of microbial communities (Dunbar et al., 1999; Suzuki et al., 1997). Cloning and sequencing of rDNA's from environmental samples has also shown that the most common genes recovered belong to undescribed species and in some cases, novel groups of bacteria (Suzuki et al., 1997; Ward et al., 1990).

Cloning is essentially the transfer of a DNA fragment of interest from one organism to a self-replicating genetic element such as a bacterial plasmid. Other cloning vectors include viruses, bacterial artificial chromosomes (BACs), and yeast artificial chromosomes (YACs). The DNA fragments are usually either PCR amplicons or they are isolated from chromosomes by using restriction enzymes. Once the DNA of interest has been successfully joined with its cloning vector in the lab, it is called a "recombinant DNA molecule." The recombinant DNA molecule can then be transformed into a suitable host cell where it will reproduce along with the host cell DNA. Once the plasmids have had sufficient time to replicate (clone themselves) they can be isolated and sequenced. Sequence data can then be compared to a sequence databank,

which may potentially provide large amounts of material for an experimental study, including possible identification of the unknown sequences.

Although the study of microbial diversity is important when trying to understand relationships and interpopulation interactions, it is only one of many aspects relating to microbial ecology. Another important aspect is successional population changes over time. The cloning and sequencing approach is not well suited to study population changes over time, simply because it is too laborious, time consuming and expensive. One alternative method used to analyze the diversity and dynamics of microbial community structures which are subject to change over a period of time (e.g seasonal fluctuations) is PCR followed by denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) (Muyzer, 1999). To perform the DGGE/TGGE analysis, nucleic acids (DNA and RNA) are first extracted, then genes encoding the 16s rRNA are amplified by PCR. Commonly, a sequence of guanines (G) and cytosines (C) are added to the 5′ -end of one of the PCR primers, resulting in amplified DNA fragments with a GC-rich sequence or GC-clamp. The GC-clamp has a high melting domain and helps to prevent the two DNA strands from complete dissociation, which increases separation of varying sequences (Sheffield et al., 1989; Sheffield et al., 1992). The PCR product can then be purified by a variety of methods. The purified PCR product will contain PCR amplicons of the same length (number of base pairs) but different sequences (Muyzer et al., 1993). Theoretically, there should be amplified copies of all 16S rRNA genes that were extracted with the original community DNA. The purified PCR products can then be separated through a DGGE or TGGE. Separation is based on the decreased electrophoretic mobility of polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient. DNA fragments of the same size are separated by their denaturing profile, or how the

double stranded DNA (dsDNS) becomes single stranded DNA (ssDNA). When dsDNA is subjected to an increasingly denaturing physical environment, it partially melts. As the denaturing environment increases, the DNA fragments will eventually completely dissociate. The physical denaturing of the DNA fragment does not proceed in a zipper-like manner but instead separates in discrete so-called melting domains. These melting domains are stretches of base-pairs with an identical melting temperature. The position in a gel where the dsDNA fragment melts and becomes ssDNA is dependent on the nucleotide sequence in the melted region. Different sequences will result in different positions in the gel where the DNA fragment halts (Muyzer and Smalla, 1998). By using DGGE/TGGE, 50% of the sequence variants can be detected in DNA fragments up to 500 bp. This percentage can be increased to nearly 100% by the attachment of a GC rich sequence (GC-clamp) to one side of the DNA fragment (Myers et al., 1985; Sheffield et al., 1989).

Another characteristic that makes DGGE popular is that it can be used as a quantitative tool. Nubel et al. 1999 used DGGE analysis of 16S rDNA fragments to quantify the diversity to oxygenic phototrophs in eight hypersaline microbial mats. The number of bands in the gel was a measure of "richness", whereas the proportional abundance (or "evenness") of the different sequence-defined populations was calculated from the intensity of the bands. In order to obtain more detailed information about specific community members, DGGE profiles can also be used in hybridization experiments with radioactively labeled, group specific oligonucleotide probes for specific detection of bacteria (Brinkhoff and Muyzer, 1997; Muyzer et al., 1993; Teske et al., 1996B). DNA eluted from excised DGGE bands can also be sequenced to further identify community members (Muyzer and deWaal, 1994).

As with most techniques, DGGE/TGGE also has its limitations. The formation of heteroduplex molecules during PCR may contribute to difficulties in interpreting banding patterns (Myers et al., 1989 and Ferris et al., 1997). Also it is not always possible to separate DNA fragments, which have a certain amount of sequence variation (Vallaeys et al., 1997). Another limitation is that DGGE/TGGE can only separate out relatively small fragments, up to 500 bp (Myers et al., 1985). Overall the flexibility, reproducibility, reliability and speed of DGGE/TGGE make them powerful tools for determining community structure and functions of microorganisms in functioning ecosystems.

Fluorescent *in situ* hybridization (FISH) uses rRNA-targeted probes, or "phylogenetic stains" and can provide a unique insight into microbial ecology, enabling both the visualization of whole cells, and the study of microbial ecosystems *in situ* (Amann et al., 1990; Amann et al., 1996). Oligonucleotide probes are designed based on specific sequences in the 16s rRNA and may be used to target either a narrow group of organisms or a broad group of organisms (Christensen et al., 1999). FISH allows for the determination of cell morphology of an uncultured microorganism, its abundance, and spatial distribution *in situ* (Amann et al., 1995). Quantification of the signal conferred by rRNA-tagged oligonucleotides should even allow the estimation of *in situ* growth rates of individual cells (Poulsen et al., 1993). One downfall with FISH is that protocols for bacterial cells extracted from soil are difficult to perform because bacteria associated with soil particles are typically excluded. Excluding bacteria associated with soil particles helps decrease background signal from minute clay and organic soil particles (Christensen et al., 1999). Other problems include target accessibility, low signal intensity, and identification of one organism (possibly in very low numbers) amongst other organisms (in large numbers). Using FISH it is difficult to detect less than 10^3 cells per cm^2. The technique is also

limited in the number of probes which can be applied in a single hybridization experiment (Amann et al., 1995).

DNA microarrays are new technology that allows the whole genome of an organism to be monitored on a single chip so that the interactions of thousands of genes can be observed simultaneously (Brazma et al., 2000). A DNA microarray is composed of pieces of DNA ranging from 20-5000 base pairs concentrated into specific areas on a solid support such as a glass chip (Schena, 1998). Thousands of oligonucleotides are attached to specific locations on the support (glass chip) and gene expression can be observed by quantifying the amount of oligonucleotides that are bound. Therefore, the array works as a capturing device for specific complementary gene products. However, only recently have microarray technologies been extended into the field of environmental microbiology (Rudi et al., 2000; Small et al., 2001; Guschin et al., 1997). A large collection of SSU rRNA-targeted DNA probes has been developed for studies in determinative and environmental microbiology. With the use of such probes the sequence diversity of SSU rRNAs recovered from different microbial populations of various abundances could be analyzed by a single hybridization to the microchip (Guschin et al., 1997). Microarray technology can be a valuable tool for the study of microbial ecology but it too has pitfalls. Microarray printing and imaging equipment is very expensive and costly to maintain, and the manual handling and nucleic acid purification are very time consuming. There are also multiple steps involved which can have negative effects including inefficient purification or concentration of nucleic acids at low target concentrations, especially in environmental samples, and the coextraction of inhibitory compounds that interfere with subsequent molecular manipulations, such as PCR (Small et al., 2001).

Terminal restriction fragment (TRF) fingerprinting is another powerful tool commonly used in microbial ecology. It can be used to monitor the diversity, structure, and dynamics of microbial populations in a statistically adequate manner (Kitts, 2001). TRF fingerprinting is based on the PCR amplification of a mixture of genes (representing different microorganisms), using a fluorescently labeled primer. After amplification, the amplicons are digested with a restriction enzyme usually with 4 bp recognition sequence (Kitts, 2001). Depending on the DNA sequence of the bacteria analyzed, and the restriction enzyme used in the digestion, labeled fragments of varying lengths will be generated. Only the terminal restriction fragments are visualized after electrophoretic separation on a DNA sequencing machine. Ideally, one fragment or TRF per organism is observed. However, 16S rDNA sequences do not always produce only one data point for each species. Different species, regardless of their taxonomic classification, may produce the identical TRFs with one restriction enzyme but may produce unique TRFs when digested with a second restriction enzyme (Brunk et al., 1996; Dunbar et al., 2001; Marsh et al., 1999). Thus, a more accurate picture of the complete bacterial community can be obtained by analyzing TRFs obtained from multiple enzyme digests.

Advantages to using TRF length polymorphism over other techniques include high speed and high sample throughput (hundreds of samples in under a week), highly precise fragment length determination, and rapid production of data, which allows for quick analysis of large amounts of information. Data collected from TRF patterns can also be compared with data predicted from rapidly expanding sequence databases in order to infer the potential composition of a sample (Dunbar et al., 2001; Kitts, 2001; Marsh et al., 1999). Osborn et al. 2000 showed TRF length polymorphism to be a highly repeatable method. This is important when comparing different microbial communities or changes in community structure over time. A highly

repeatable method helps ensure that variability between samples is due to actual differences in community structure and not reflections of the technique being used. Because of its sensitivity, high throughput and supporting sequence databases (Marsh et al., 2000), TRFLP is an ideal technique for comparative microbial community analysis (Clement et al., 1998; Dunbar et al., 2001; Kaplan et al., 2001; Liu et al., 1997; Ludemann et al., 2000; Markus et al., 1999). As with all other molecular techniques, TRF length polymorphism also has negative aspects. Since TRF length polymorphism is a PCR based technique, the inherent biases found in any PCR based technique also apply to TRFLP (Tillmann and Friedrich, 2003). One disadvantage of TRFLP includes the fact that the DNA used in creating the TRF patterns is destroyed and cannot be used in any further analysis. Depending on the gene being targeted and available databases used to compare TRF patterns against species identification can also be difficult. In some cases identification down to the level of genus or even species may be possible while in other cases identification may be limited to a group of closely related organisms or no identification at all may be made.

Regardless of which molecular technique is used, they all seem to excel in some areas while having certain drawbacks in other areas. Even with their drawbacks, today's molecular techniques show a much more detailed picture of what the microbial communities are doing and how they may be interacting than earlier methods (e.g. culture techniques). If the speed and progress with which the current molecular techniques have improved continues, it may not be long before we are able to precisely determine exactly what organisms are in a given environment, and what role they play in the community.

Enzyme Selection

To optimize the effectiveness of TRFLP as a tool for bacterial community analysis, it is necessary to have some idea of the distribution of restriction sites on the 16S ribosomal DNA as well as the relationship of terminal fragment size to phylogeny. When choosing an enzyme, one should ask which enzyme(s) will provide the most distinguishing estimate of population density, which enzyme(s) will provide the best resolution for the phylogenetic group(s) of interest and what primer-enzyme combination will be optimal for the community under investigation. One way to answer these questions is by the use of an in silico digestion of the 16s rRNA database (Marsh et al., 2000; Brunk et al. 1996; Liu et al., 1997). Existing computer analysis and web based programs can be used to perform in silico digestions of the 16s rRNA databases. Marsh et al. 2000 describes one such web-based tool located at the Ribosomal Database Project web site http://rdp.cme.msu.edu (Cole et al., 2003). This site provides the user with a rapid way to determine optimal primer and restriction enzyme combinations for community analysis. It also tentatively allows the user to experimentally determine phylotypes from similar phylotypes in the database. Due to the fact that many actinobacteria have highly conserved 16S rRNA gene regions, it can at times be difficult when trying to identify specific organisms using TRF analysis. To more accurately identify different species with highly conserved regions, incorporating the use of two different restriction enzymes (*Taq*I and *Hha*I for this study) can help to increase the chances of distinguishing one organism from another.

Primer Selection

When studying microbial ecology, the identification of genes or families of genes in a single or multiple species is commonly achieved with the use of PCR. PCR is performed by designing primers, which target conserved regions of homologous genes (Polz and Cavanaugh,

1998). Primer selection is of the utmost importance because the primers should be sensitive enough to allow the detection of bacteria at low concentrations but also have the ability to discriminate among a wide range of bacterial taxa, which are often found in a complex environmental sample. The primers must be able to hybridize to all small subunit rRNA (SSU rRNA) genes present in the sample so that all of the different SSU rRNA's are represented in the final PCR product. Simultaneously, the region between the primer sites needs to have enough sequence variability to differentiate between the bacteria present in the sample (Brunk et al., 1996).

The purpose of this study was to develop a PCR assay to analyze actinobacterial community dynamics using TRF analysis. Initial stages of the project included the development of a PCR assay using well-characterized laboratory strains to validate primer specificity and determining whether or not the application of TRF would work when applied to mixed communities of actinobacterial and non-actinobacterial rDNA. The methods used throughout this study included amplification of rDNA (via PCR) from known strains of actinobacterial and non-actinobacterial rDNA, cloning and sequencing, and comparison of soil isolate TRF patterns to soil community TRF pattern from which the isolates were obtained. A practical application of the actinobacterial specific TRF assay was then performed using samples collected from the Natural Attenuation site at the Guadalupe Dunes. With the actinobacterial specific TRF assay it was possible to identify specific TRF peaks and show that they had strong correlations with physical/chemical parameters involved in the anaerobic degradation of TPH.

Materials and Methods

Plating and Isolation of Actinobacteria

Four soil samples (HAC8CS-6-6.0-060701, HAC8HDW-5-1.0-060701, HAC8MDC-1-1.0-061101, HAC8MDW-5-6.5-061201) obtained from the C8 site at the Guadalupe dunes were used to isolate pure cultures of actinobacteria. Samples were collected by Unocal employees and sent to the Environmental Biotechnology Institute for analysis. The C8 site contained areas that had previously been contaminated by TPH. Some of these areas were excavated and backfilled with clean non-contaminated soil. After backfilling with the non-contaminated soil, willow and cottonwood trees were planted over selected areas of the C8 site. The purpose of this was to study the affects phytoremediation has on contaminated groundwater directly below the C8 site. The control area of the C8 site has remained unaffected by TPH and hence was not excavated as previously mentioned. The CS or control site, located within the C8 site, contains only natural coastal scrub and is not planted in willow or cottonwood trees. Drilling down various depths into the soil using a hand agar, four soil cores were obtained from the C8 site. Sample HAC8CS-6-6.0-060701 was collected from the control site at a depth of 1.8 meters, HAC8HDW-5-1.0-060701 was collected from a high-density willow site at a depth of 0.3 meters, HAC8MDC-1-1.0-061101 was collected from a medium density cottonwood site at a depth of 0.3 meters and HAC8MDW-5-6.5-061201 was collected from a medium density willow site at a depth of 2.0 meters. From each of the four cores, 1 gram of soil was placed into 9 ml of sterile H_2O and 10 fold serial dilutions were carried out to 10^{-7}. 100µl of each dilution (ranging from 10^{-1} to 10^{-7}) were plated onto czapek yeast agar, starch casein agar and colloidal chitin agar. Czapek yeast agar, starch casein agar and colloidal chitin agar were prepared as outlined in the Handbook of

Microbiological Media (ref. Ronald M. Atlas, 1993). The inoculated plates were then placed into a 35 ^{o}C incubator and over a 4 week period 135 actinobacteria colonies were isolated and purified onto ISP-2 media (Becton Dickinson, Sparks MD). Actinobacteria isolates were identified by morphological characteristics as described in Actinomycete Taxonomy (Dietz and Thayer, 1980).

Isolate DNA Extractions

DNA was extracted from the actinobacteria isolates by transferring 10-15 loops full of pure culture from the agar into MoBio® bead lysis tubes. The protocol given in the MoBio UltraClean® soil DNA extraction kit (MoBio Laboratories Inc., Solano Beach CA.) was followed for the extraction process with the following exception: cells were lysed in the Bio 101 FP-120 FastPrep machine running at 4.5 m/s for 30 seconds.

Collection of Natural Attenuation Samples and Physical/Chemical Data

Natural Attenuation (NA) groundwater samples (H2-2A,B,C,D,E, H2-3A,B, G3-3B,C, and J1-3A,B,C,D,E) used for the application portion of this study were collected in the field by Unocal employees and then sent to Zymax laboratories (San Luis Obispo, CA) as well as to the Environmental Biotechnology Institute (EBI). Zymax was responsible for collecting all physical/chemical data including Total Petroleum Hydrocarbon (TPH), Benzene, Toluene, Ethylbenzene, Zylene (BTEX), Carondioxide (CO_2), conductivity (cond), alkalinity (alk), methane (CH_4), Nitrate (NO_3), depth to water (d2w), sulfate (SO_4), dissolved iron (dFe), pH and oxygen (O_2). After collecting the physical/chemical data for each sample, data was sent to the EBI for analysis and comparison against TRF data. 1-gallon of each NA groundwater sample (sent to the EBI) was filtered through a 2-micron filter. The filters were subjected to a vacuum pump, which pulled the gallon of water through the 2-micron filter over a period of time (up to 8

hours in some cases). After filtering the 1-gallon of water, each filter was individually mixed with liquid nitrogen inside of a mortar and crushed to a fine powder using a pestle. DNA was then extracted using exactly the same method as for the soil samples (see soil DNA extractions) with the exception that one crushed filter was added to each MoBio® bead lysis tube instead of 1 gram of soil.

Soil DNA Extractions

Community DNA was extracted from the same soil samples in which the isolates were obtained (HAC8CS-6-6.0-060701, HAC8HDW-5-1.0-060701, HAC8MDC-1-1.0-061101, HAC8MDW-5-6.5-061201). Community DNA was also extracted from the Natural Attenuation (NA) samples (H2-2A,B,C,D,E, H2-3A,B, G3-3B,C, and J1-3A,B,C,D,E) used in the application of the F243 and 814R primer pair (Appendix A, Figure 1). Community DNA from each soil sample was isolated in triplicate (three separate extractions for each sample). To perform the DNA extraction, one gram of soil (or one crushed filter if referring to the NA groundwater samples) was placed directly into the MoBio® bead lysis tube. The protocol given in the MoBio® soil DNA extraction kit was followed for the extraction process with the following exception: cells were lysed in the Bio 101 FP-120 FastPrep machine running at 5.0 m/s for 45 seconds. The isolated DNA was then visualized by agarose gel electrophoresis. Success of each extraction was determined by measuring DNA concentration. 1:20 dilutions of each sample were made and the DNA was quantified using a SPECTRAmax UV spectrophotometer® (Perkin-Elmer, Applied Biosystems Inc., Fremont, CA.) Triplicate soil extractions were combined prior to performing the PCR.

Primer Optimization

DNA from 325 known actinobacteria (including the 135 soil isolates previously mentioned) and 49 non-actinobacteria were extracted using a MoBio UltraClean® soil DNA extraction kit and used as template DNA for the optimization of primers F243 and 814R. The primer F243 (F243-D4 (5'-D4Pa-GGA TGA GCC CGC GGC CTA-3') was taken from a published report on primers selective for actinobacteria (Heuer et al. 1997). The F243 primer was selected for this study because of the various publications on amplification of actinobacterial rDNA the F243 primer appeared to be most applicable to this study in that it is capable of selectively amplifying the greatest variety of actinobacterial 16S rDNA fragments during PCR. A reverse primer labeled 814R (ACTN814R (5'-ACC AGG GTA TCT AAT CCT GTT-3') was developed by aligning actinobacterial 16S rRNA sequences and observing areas of good sequence homology (personal communication, R. J. Cano). By altering various parameters (e.g. $MgCl_2$ and annealing temperature) the reactions were optimized so that predominantly actinobacterial DNA was amplified. A single prominent band of approximately 570 bp was repeatedly produced for most of the actinobacterial cultures.

Polymerase Chain Reaction (PCR) of Isolates & Community Soil rDNA

PCR was performed using 16s rDNA labeled primers F243-D4 (5'-D4Pa-GGA TGA GCC CGC GGC CTA-3') (forward primer) and ACTN814R (5'-ACC AGG GTA TCT AAT CCT GTT-3') (reverse primer). PCR reactions for all community soil DNA extractions were carried out in triplicate. The 135 actinobacterial isolates and 190 other known strains of actinobacterial DNA (obtained from EBI stock) were used to test the specificity of F243 and 814R primers. The following reagents were added to carry out the PCR in 50µl reactions. 4µL of undiluted extraction product (isolates DNA) or 5µL of a 1/10 dilution (community DNA),

5µL of 10x Ampli*Taq* Gold Buffer (Applied Biosystems, Fremont, California, USA), 3µL of 10 mM DNTP, 2µL of 20 µg/mL BSA, 4µL of 25 mM $MgCl_2$, 1µL of 10 µm ACTN814R, 1µL of 10 µm F243-D4, 0.3mL of 5 U/mL Ampli*Taq* Gold DNA polymerase (Applied Biosystems) and enough water to total 50µl. Reaction temperatures and times were 94 °C for 10 min; 35 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 2 min; and 72 °C for 10 min. PCR products (of approximately 570 bp) were visualized by agarose gel electrophoresis. Positive and Negative controls used the same master mix that was made for each PCR throughout the study. For the negative controls no template DNA was added to the master mix and for each positive control 3 µL of Streptomyces griseus (ATCC# 23345) genomic DNA at a concentration of 1 ng/µL was added. Reactions that were inconsistent or unsuccessful were discarded. Following confirmation of PCR product, successful reactions (if in triplicate the PCR reactions were first combined) were cleaned using the MoBio UltraClean® PCR Cleanup Kit as was specified by the protocol sent with the kit. The amplicons for each sample were again quantified using the Spetramax spectrophotometer.

Amplicon Digestion

An enzyme digest was performed on each sample using the New England Biolabs restriction endonuclease *Hha*I. Each 20µL digestion used 50ng of DNA, 2U of enzyme, 2µL of buffer, and 0.2µL of BSA. Samples were digested for 3h at 37 °C and inactivated for 20 min at 65 °C. Samples were additionally digested with New England BioLabs *Taq*I using the same volumes and concentrations as with *Hha*I. The samples were digested for 3h at 65 °C and inactivated for 20 min at 80 °C. The digestion products were then purified by ethanol precipitation.

Capillary Gel Electrophoresis

TRF patterns were generated for the 135 isolates as well as for the soil samples from which they were obtained. Additionally, TRF peaks were generated for the Natural Attenuation samples (H2-2A,B,C,D,E, H2-3A,B, G3-3B,C, and J1-3A,B,C,D,E) used in the application of the TRF assay. To obtain TRF patterns, the precipitated DNA was resuspended in 20µl of Sample Loading Solution (formamide) (Beckman Coulter®) and 0.25µl of CEQ DNA size standard 600 (Beckman Coulter®). One drop of mineral oil was added to each sample to prevent evaporation. Samples were then run in the Beckman Coulter CEQ 8000 Genetic Analysis System. CEQ 8000 run parameters were set at a slope threshold of 2, include peaks at 2%, inject voltage of 5 kilovolts (kv) with an inject duration of 5 seconds (sec) and a separation voltage of 5 kv with total time separation of 65 minutes (min).

Cloning & Sequencing

Three soil samples O13 Soil ID compA18-9.0-111302 and O13 Soil ID# compA82-10.0-111102 which were isolated from capillary fringe soil at the O-13 site and Nereus soil sample ID #13-3-2 were used to construct the 16s rDNA clone library for sequencing. Cloning was performed using the TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA). Bacterial community rDNA was isolated and amplified as previously described (Soil DNA extraction and Polymerase Chain Reaction (PCR) of Isolates & Community Soil rDNA) with the exception that unlabeled forward primer (at a concentration of 10µM) was used in the PCR. The ligation reaction was set up as outlined in the protocol for eventual transformation into chemically competent TOP10 *E. coli.* The protocol was followed as outlined with the following exception: 3 µl of fresh PCR product were used when performing the TOPO® cloning reaction. The ligated vector was then transformed into the One Shot® TOPO10 Chemically Competent *E.*

coli cells as outlined in the protocol. Cells were then plated onto prewarmed LB plates containing 150 μg/ml of ampicillin (amp) and incubated overnight at 37 ^{o}C. Colonies which grew were picked and inoculated into LB/amp broth. The cultures were shaken at 200 rpm while incubating overnight at 37 ^{o}C. The cells were then pelleted and plasmids extracted using the Wizard® SV 96 Plasmid DNA Purification System (Promega) as directed by the manufacturer.

Isolated plasmids were sequenced in both forward and reverse directions. The forward M13 (5′-GTAAAACGACGGCCAG -3′) or reverse M13 (5′-CAGGAAACAGCTATGAC-3′) primer was used in the sequencing reactions. The 10 μl sequencing reactions consisted of 4 μl Big Dye terminator (Applied Biosystems, Foster City, CA), 2 μl of 0.8 μm primer (either forward or reverse), and 4 μl of isolated plasmid. Reaction temperatures and times were 96 ^{o}C for 30 sec; 40 cycles of (96 ^{o}C for 30 sec, 50 ^{o}C for 10 sec, 60 ^{o}C for 4 min). The samples were then ethanol precipitated. Precipitated DNA was resuspended in formamide and analyzed on an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA). Resulting sequences were assembled and analyzed using SeqMan^{TM} II software program (DNAStar, Madison, WI USA). Clone sequences were also analyzed with Chimera Check on the Ribosomal Database Project (RDP) website (Maidak et al., 2000).

Statistical Analysis of Physical/Chemical and TRF Data

The application of the F243 and 814R primer pair was performed using samples H2-2A,B,C,D,E, H2-3A,B, G3-3B,C, and J1-3A,B,C,D,E which were collected from the H2-2, H2-3, G3-3, and J wells respectively (Appendix A, Figure 1). Analysis of physical/chemical parameters including Total Petroleum Hydrocarbon (TPH), Benzene, Toluene, Ethylbenzene, Zylene (BTEX), Carbon dioxide (CO_2), conductivity (cond), alkalinity (alk), methane (CH_4), Nitrate (NO_3), depth to water (d2w), sulfate (SO_4), dissolved iron (dFe), pH and oxygen (O_2) was

performed on all of the samples. The physical parameters along with TRF data collected from the same wells were used as indicators for the identification of actinobacteria potentially involved in the degradation of TPH and/or other possible contaminants. To best understand the actinobacterial community dynamics, analysis of samples from three different subsets of wells were performed. The first analysis included samples from wells H2-2, H2-3, G3-3, and J, the second analysis included samples from H2-2, H2-3, and G3-3 wells and the third analysis included samples from H2-2, and G3-3 wells. The rationale for removing samples from well J for the analysis (second analysis), was that the J samples had a much higher O_2 concentration and much lower TPH concentration. The J samples were also low in CH_4 and CO_2, which could potentially indicate anaerobic degradation of TPH. Analysis of H2-2, and G3-3 wells (third analysis) was performed because the wells were very close in proximity to each other and many of the physical/chemical parameters were very similar. Also in order to take a closer look at potential anaerobic actinobacteria, wells with the lowest O_2 concentrations (H2-2 and G3-3) were closely scrutinized.

Physical/chemical and TRF data were analyzed using the statistics programs S+ (Insightful, Seattle, WA), Minitab (Minitab Incorporated, State College, PA), and Excel (Microsoft Corporation, Seattle, WA). Excel macros and correlation formulas were used to correlate the physical components with TRF data and also for the comparison of *Taq*I TRF peaks to *Hha*I TRF peaks. Principal Component Analyses (PCA) were performed using S+ and Minitab. PCA was used because it provides a rapid way to assess variation with multidimensional data such as those provided by TRF patterns. PCA is a method of data reduction that transforms the data into new variables (the principal component scores), which are constructed to represent the greatest variation in the data set. The first principal component

(PC1) accounts for as much of the variability in the data as possible, and each succeeding PC accounts for as much of the remaining variability as possible. Assigning a loading factor to each variable and summing the total of all variables multiplied by their loadings generate PC scores. Plotting the first PC scores on the x-axis and the second PC scores on the y-axis creates a score plot. A good result would be obvious grouping of samples within the plot. Plotting the first PC loadings on the x-axis and the second PC loadings on the y-axis creates a loading plot. A loading plot indicates which variables are important to each PC score. Score plots and loading plots can be overlaid to increase the information content.

RESULTS

Actinobacteria Isolates

Over a 4-week period, 135 actinobacteria colonies were isolated from czapek yeast agar, starch casein agar or colloidal chitin agar and purified onto ISP-2 media. With respect to the actinobacteria isolated from the three media types, there was no preferential selectivity (of actinobacteria) by one agar over the others (Appendix A, Table 3). Typically, a particular strain of actinobacteria growing on one medium was usually present on one or both of the other plates. This observation was based on visual appearance as well as on results of TRF patterns obtained from the isolates (Dietz and Thayer, 1980).

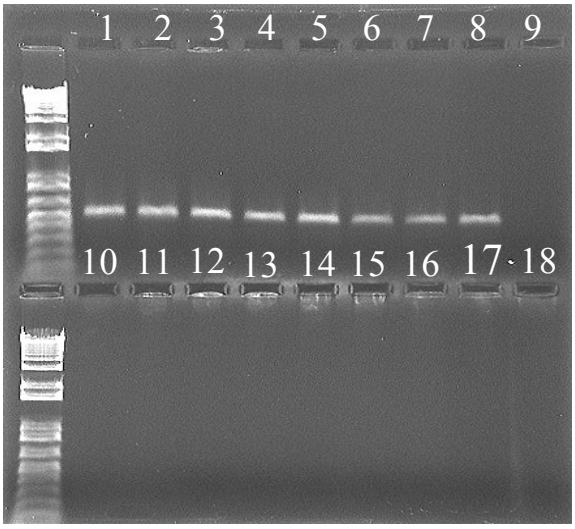
Testing the Primers for Specificity on Known Bacteria

The F243 and 814R primer pair used for PCR successfully amplified rRNA genes from 16 out of 19 known actinobacterial strains gathered from the American Type Culture Collection (ATCC). The ATCC samples were used as controls to compare other samples. The resulting amplicon consisted of a clean, single band of the predicted size (570 to 580 bp) with little or no primer dimer formation (Figure 1). Thirteen additional ATCC non-actino strains were also tested; only one (*Citrobacter freundii*) gave a weak positive result (Table 1, Figure 1). A private library (maintained by the Environmental Biotechnology Institute) of bacterial strains was also tested and 274 out of 306 actinobacterial strains were successfully amplified while amplification failed for 16 out of 16 non-actinobacteria (Appendix A, Table 1 and Table 2). In total, 290 out of 325 (89%) actinobacteria strains were successfully amplified by the primer pair and only 1 out of 49 (2%) non-actinobacterial strains were amplified. Based on these results, these primers were considered sufficiently specific and were used for the rest of the study.

Table 1. Known strains from the ATCC tested for amplification with primers F243 and 814R.

ATCC #	Genus and species	Actino?	PCR?
13181	*Pseudonocardia autotrophica*	Yes	Yes
13685	*Amycolatopsis mediterranei*	Yes	Yes
19425	*Actinomadura madurae*	Yes	No
21411	*Amycolatopsis mediterranei*	Yes	Yes
23057	*Microtetraspora glauca*	Yes	Yes
23345	*Streptomyces griseus*	Yes	Yes
23491	*Dactylosporangium*	Yes	Yes
23867	*Planobispora longispora*	Yes	Yes
27643	*Amycolatopsis mediterranei*	Yes	Yes
27980	*Nocardioides albus*	Yes	No
31121	*Actinoplanes teichomyceticus*	Yes	No
31497	*Saccharothrix australiensis*	Yes	Yes
33774	*Streptomyces setae*	Yes	Yes
39323	*Kibdelosporangium aridum*	Yes	Yes
43688	*Streptomyces megasporus*	Yes	Yes
49499A	*Actinokineospora riparia*	Yes	Yes
51143	*Crossiella cryophila*	Yes	Yes
51423	*Planotetraspora mira*	Yes	Yes
53649	*Actinoplanes teichomyceticus*	Yes	Yes
31006	*Bacillus sp.*	No	No
7966	*Aeromonas hydrophilia*	No	No
6633	*Bacillus subtilis*	No	No
8090	*Citrobacter freundii*	No	Poor
13048	*Enterobacter aerogenes*	No	No
19433	*Enterococcus fecalis*	No	No
55151	*Escherichia coli*	No	No
29605	*Haloferax vulcanii*	No	No
13315	*Proteus vulgaris*	No	No
10145	*Pseudomonas aeruginosa*	No	No
13525	*Pseudomonas fluorescens*	No	No
13880	*Serratia marcescens*	No	No
155	*Staphylococcus epidermidis*	No	No

Figure 1. Gel electrophoresis of PCR product using F243 and 814R primers. DNA from ATCC strains of actinobacteria was used as template DNA for lanes 1-8. DNA from non-actinobacteria was used as template DNA for lanes 10-17. Negative controls are in lanes 9&18.



Sequencing of Amplified DNA to Confirm Actinobacterial Origin

Under most circumstances, soil rDNA extract will contain a mixed slurry of actinobacterial and non-actinobacterial rDNA. If the F243 and 814R primer pair could not selectively amplify actinobacterial rDNA under these conditions, then they would not be suitable for the analysis of actinobacterial community dynamics. To test this, PCR amplified DNA from two soil samples obtained at the phyto (O-13) site plus one other sample from a private collection (Nereus Pharmaceuticals) were cloned into *E. coli* and 176 separate clones representing individual amplified fragments of rRNA genes were sequenced. A comparison of these sequences to the GenBank database confirmed that 158 (90%) of the amplified sequences came from actinobacteria. The remaining 18 sequences were from either *Verrucomicrobium* spp. or delta-proteobacteria origins (Appendix A, Table 4A, 4B, and 4C). Through the use of cloning and sequencing it was shown that the primer pair was also selective for the amplification of actinobacterial rDNA from environmental samples.

Comparison of Soil TRF Patterns with TRF Peaks from Actinobacteria isolated from the Corresponding Soils

TRF peaks produced by individual isolated actinobacteria were successfully matched to TRF peaks found in the soil TRF pattern from which the isolates were obtained. This was the case for both *Taq*I TRF patterns (Figure 2) and *Hha*I TRF patterns (Figure 3). With only one exception, the TRF peaks for all of the soil-isolated actinobacterial strains were clearly observed in the community TRF patterns from these soils (Appendix A, Table 3). Conversely, the actinobacterial TRF patterns from the tested soils showed many TRF peaks that did not match any of the isolated actinobacteria.

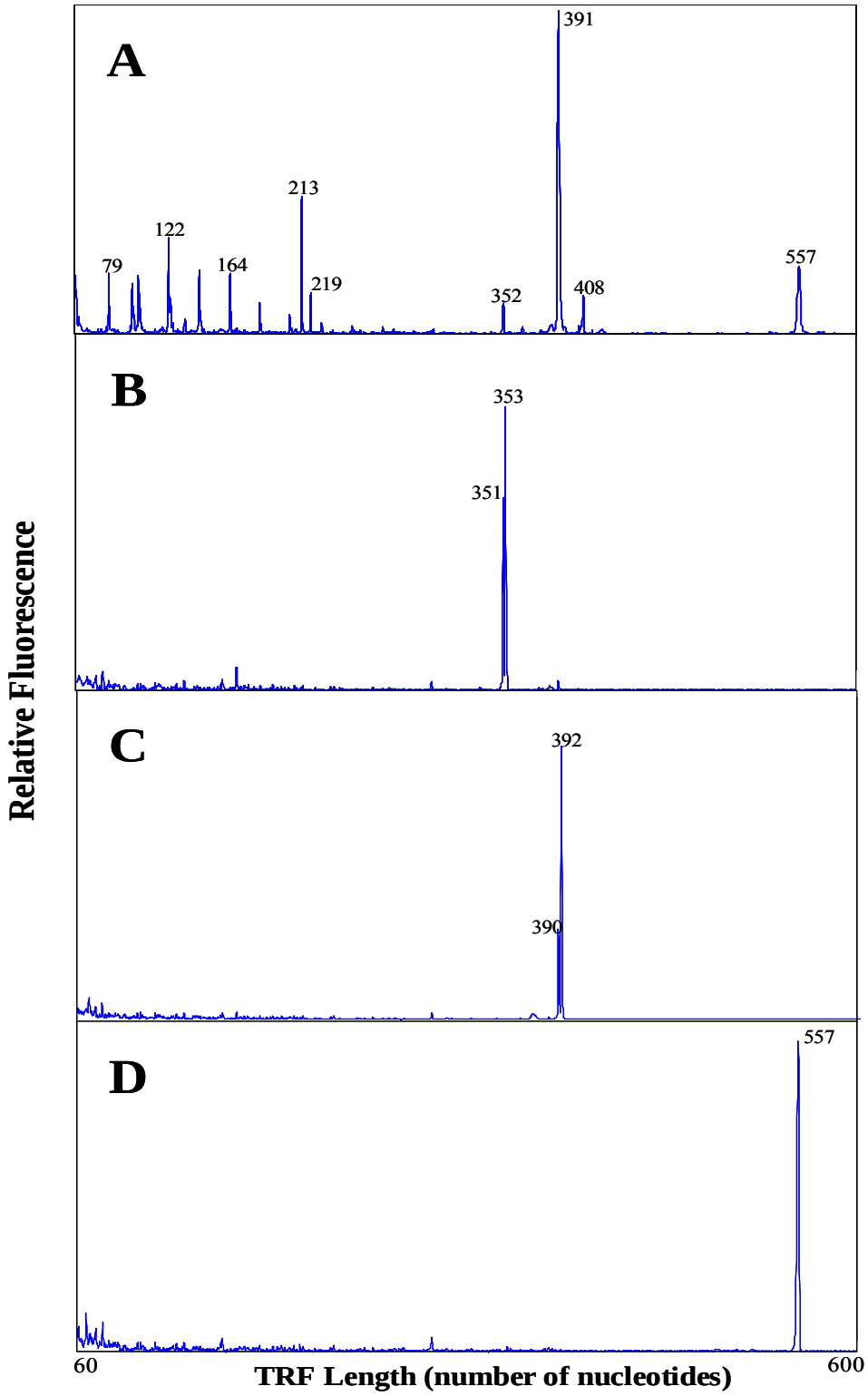


Figure 2. Caption (A) is a representative soil TRF pattern obtained from the C8 site. Captions (B), (C), and (D) are TRF patterns from representative actinobacteria isolates obtained from the C8 phytoremediation soils. TRF fragments were generated using the restriction endonuclease *Taq*I.

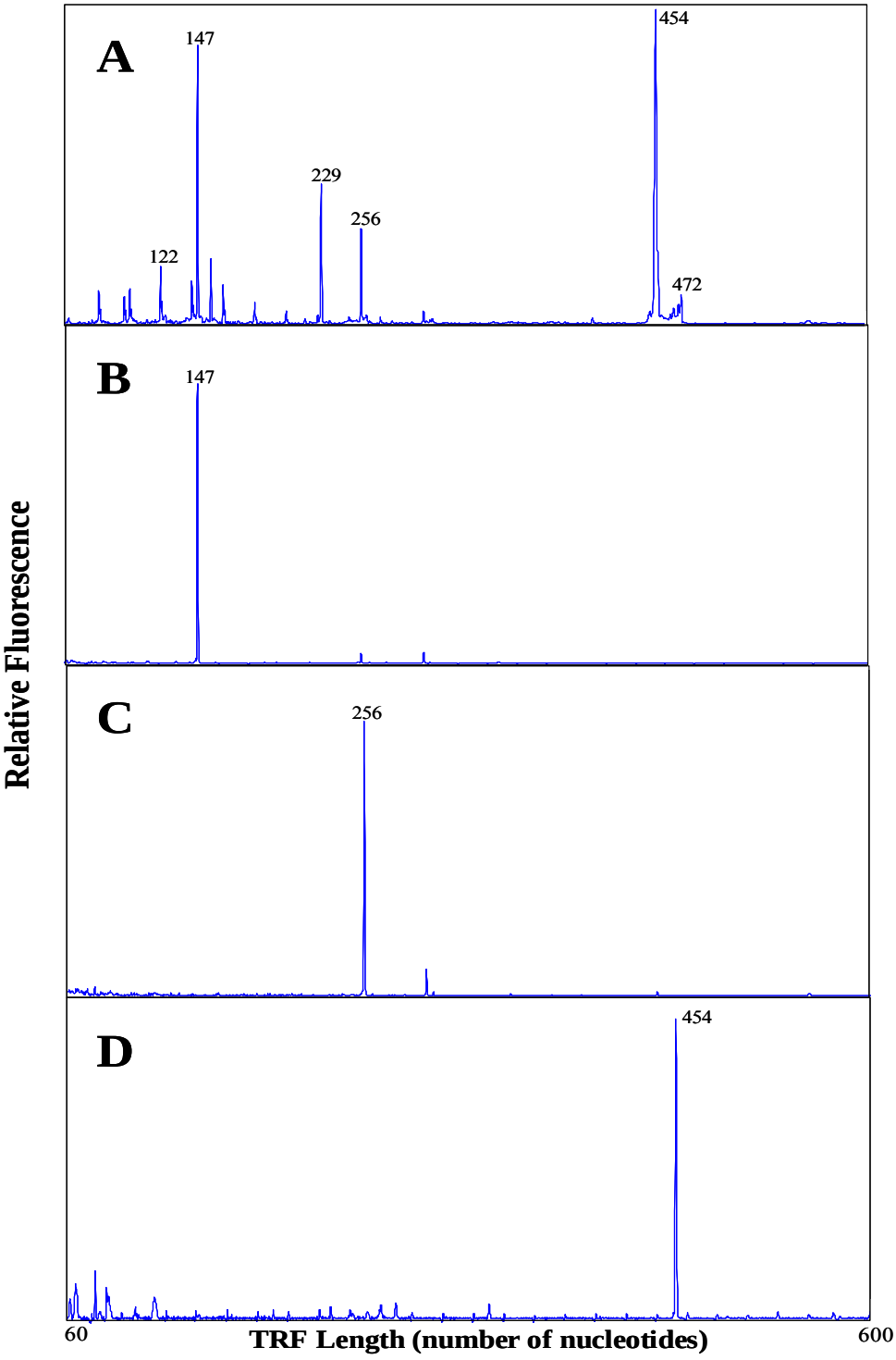


Figure 3. Caption (A) is a representative soil TRF pattern obtained from the C8 site. Captions (B), (C), and (D) are TRF patterns from representative actinobacteria isolates obtained from the C8 phytoremediation soils. TRF fragments were generated using the restriction endonuclease *Hha*I.

Analysis of Physical/Chemical and TRF Data Collected from the H2-2, H2-3, G3-3, and J Natural Attenuation Wells

Principal Components Analysis (PCA) of the physical parameters from the H2-2, H2-3, G3-3, and J wells showed most of the variance (65%) to be in PC1 (Figure 4). PC loadings for each physical parameter indicated the importance of that parameter in separating the samples on the PCA plot. TPH, BTEX, CO_2, cond, alk, and CH_4 all had a negative loading whereas NO_3, d2w, SO_4, and O_2 all had a positive loading. The samples from J wells were low in TPH and had no BTEX where as the remaining NA samples were high in TPH and BTEX. Also note that the J samples are much higher in NO_3, d2w, SO_4, and O_2 than the other NA samples. The large variation of physical/chemical parameters resulted in the wide separation of the J's from the other NA samples.

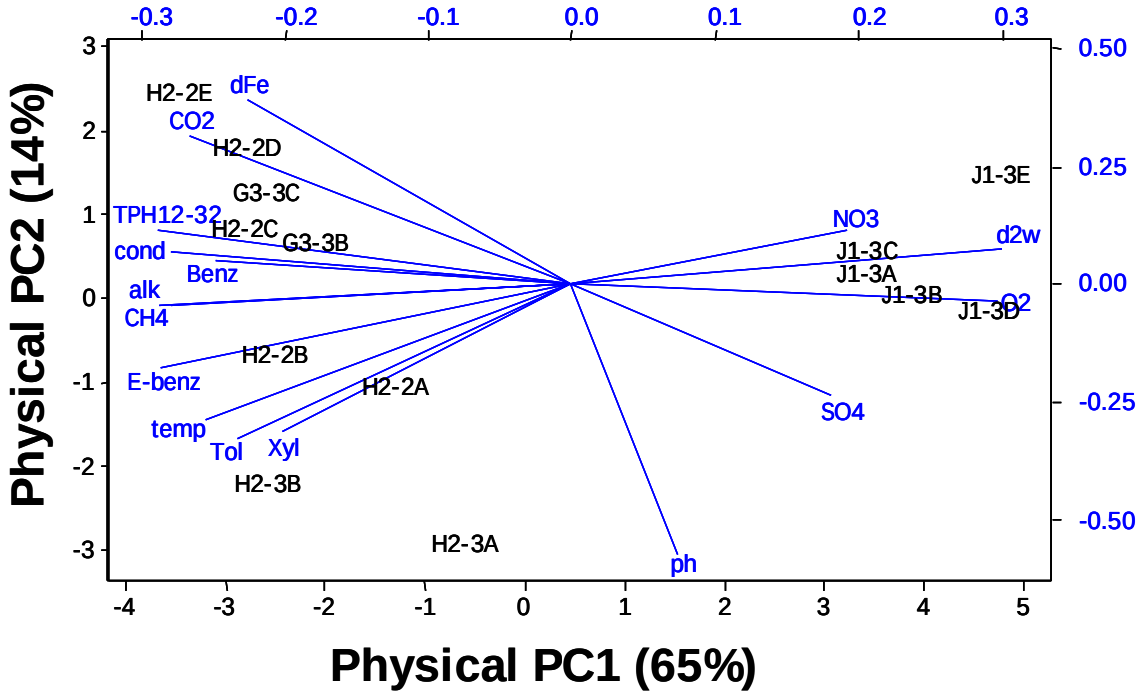


Figure 4. PCA of the physical/chemical parameters from the H2-2, H2-3, G3-3, and J natural attenuation (NA) samples. Note how the J's are clustered together and separated from the remaining samples along PC1. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

PCA of TRF peaks obtained using *Taq*I shows that the sample distribution follows a similar pattern to that of the physical PCA (Figure 5). Note that peaks 557 and 558 had the greatest affect on J well sample distribution due to their large negative PC1 loadings. Again, the J samples tend to spread out across PC1 more so than PC2 and the remaining NA samples are better dispersed along PC2. A fitted line plot (regression) of TRF PC1 against Physical PC1 showed good correlation, indicating that 67.4% of the variation in TRF PC1 was predicted by physical PC1 (Figure 6). This shows that 33% of the variation in Actinobacterial community structure (67.4% of 49%) could potentially be attributed to the TPH degradation parameter physical PC1.

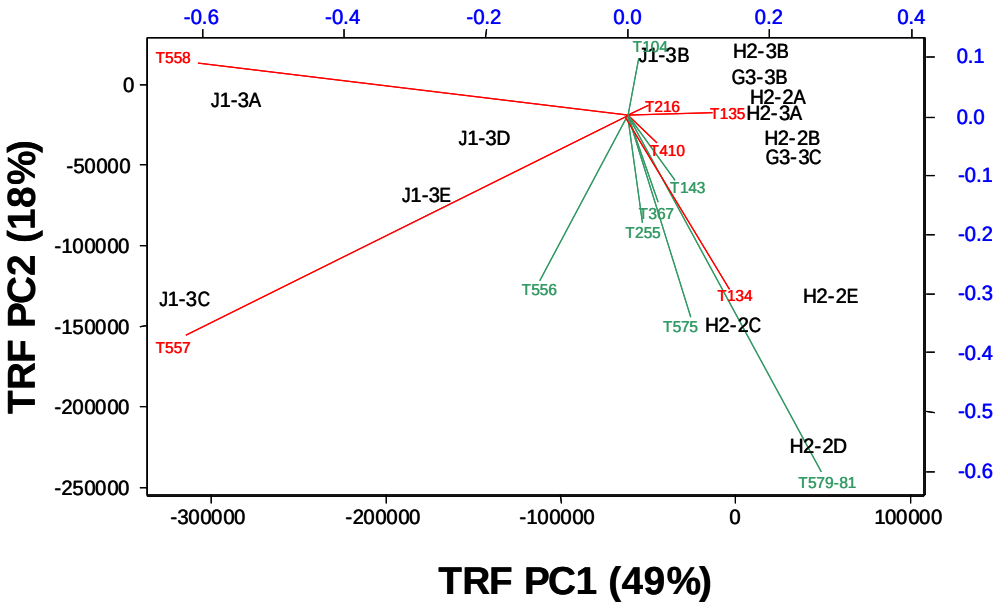


Figure 5. PCA of *Taq*I actinobacterial TRF data from nested wells at H2-2, H2-3, G3-3, and J. Red lines indicate TRF peaks which had a similar standardized abundance to TRF peaks obtained using *Hha*I. Green indicates TRF peaks, which had high loadings but could not be matched to *Hha*I TRF peaks. Only TRF peaks with loading > 0.1 or peaks which had a similar standardized abundance to *Hha*I peaks are shown. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

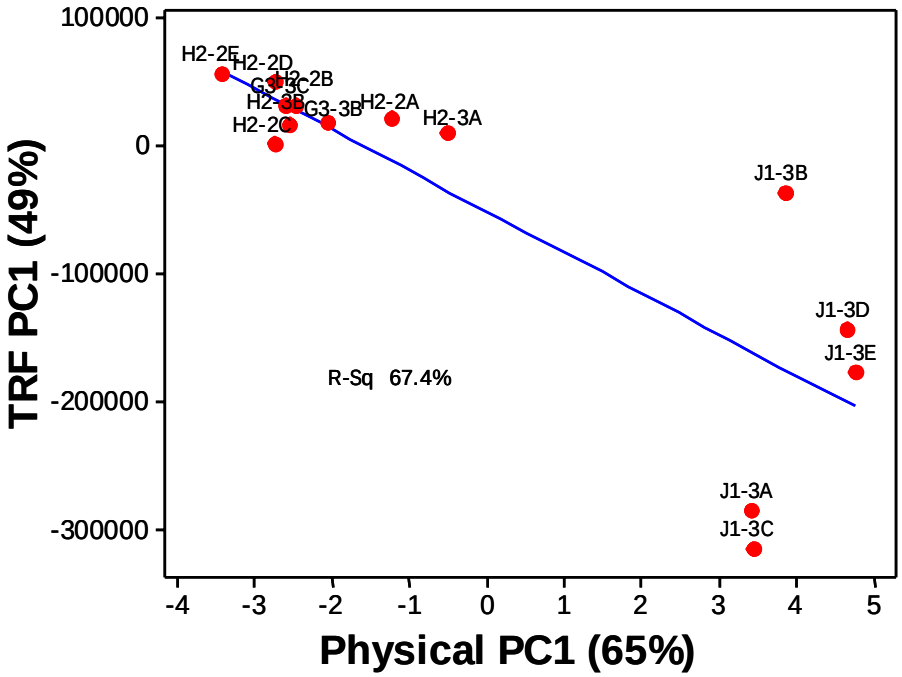


Figure 6. Correlation of the physical/chemical data (represented as physical PC1) against *Taq*I actinobacterial TRF PC1 data from the H2-2, H2-3, G3-3, and J nested wells.

PCA of TRF peaks obtained using *Hha*I shows that 58% of the variance is accounted for in PC1. Important peaks, which have positive PC1 loadings, include 579-81 and 576-80 whereas important peaks, which have negative PC1 loadings, include 156, 454, 557 and 558 (Figure 7). A fitted line plot of TRF PC1 against Physical PC1 showed good correlation, indicating that 74.2% of the variation in TRF PC1 was predicted by physical PC1 (Figure 8). This shows that 43% of the variation in actinobacterial community structure (74.2% of 58%) could potentially be attributed to the TPH degradation parameter physical PC1. Again, the J samples separate from the remaining NA samples.

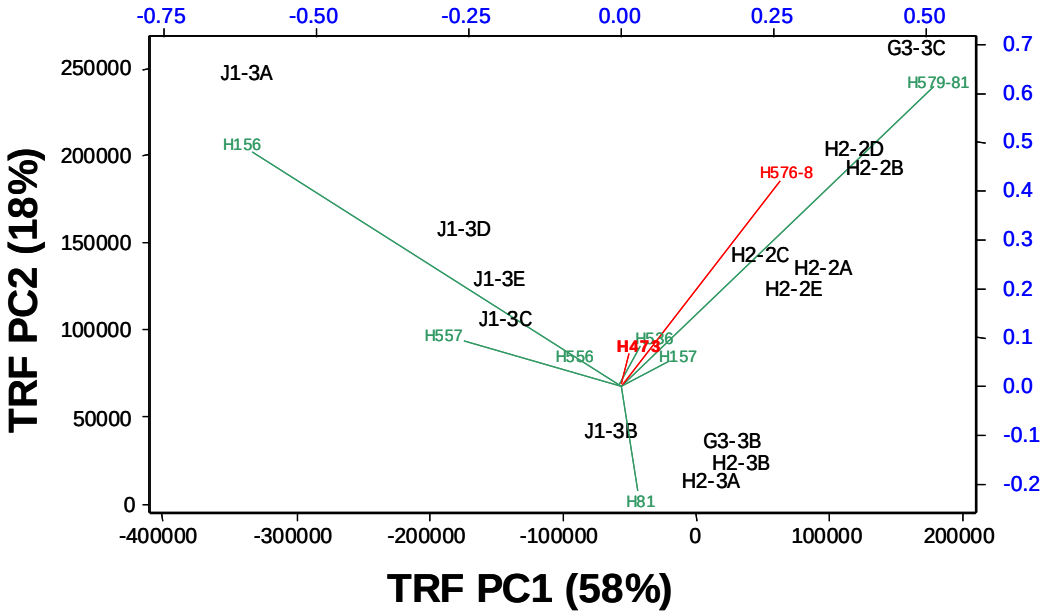


Figure 7. PCA of *Hha*I actinobacterial TRF data from nested wells at H2-2, H2-3, G3-3, and J. Red lines indicate TRF peaks which had a similar standardized abundance to TRF peaks obtained using *Taq*I. Green indicates TRF peaks, which had high loadings but could not be matched to *Taq*I TRF peaks. Only TRF peaks with loading > 0.1 or peaks which had a similar standardized abundance to *Taq*I peaks are shown. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

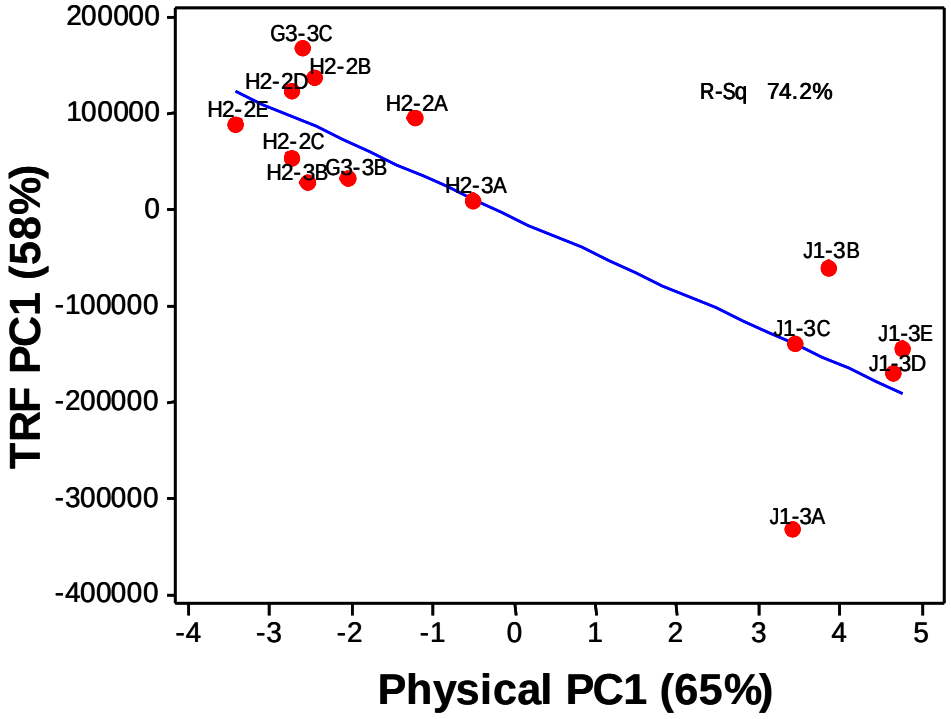


Figure 8. Correlation of the physical/chemical data (represented as physical PC1) against *Hha*I actinobacterial TRF PC1 data from the H2-2, H2-3, G3-3, and J nested wells.

Analysis of Physical/Chemical and TRF Data Collected from the H2-2, H2-3, and G3-3 Natural Attenuation Wells

Both *Hha*I and *Taq*I analyses showed the J samples distinctly separating from the remaining NA samples. This indicates that removal the J samples and re-analysis of the remaining NA samples may help to gain a better understanding of the important peaks in the H2-2, H2-3, and G3-3 wells. PCA of the H2-2, H2-3, and G3-3 nested well physical data begins to show a clearer picture of what physical parameters are affecting sample distribution in wells with higher TPH levels. The PCA shows that O_2, Toluene, Ethylbenzene and Xylene (TEX), SO_4 and pH all have positive PC1 loadings while TPH, Fe, CO_2, CH_4 and cond all have negative PC1 loadings. Sample distribution is most significant across PC1, which accounts for 40% of the variation between samples (Figure 9).

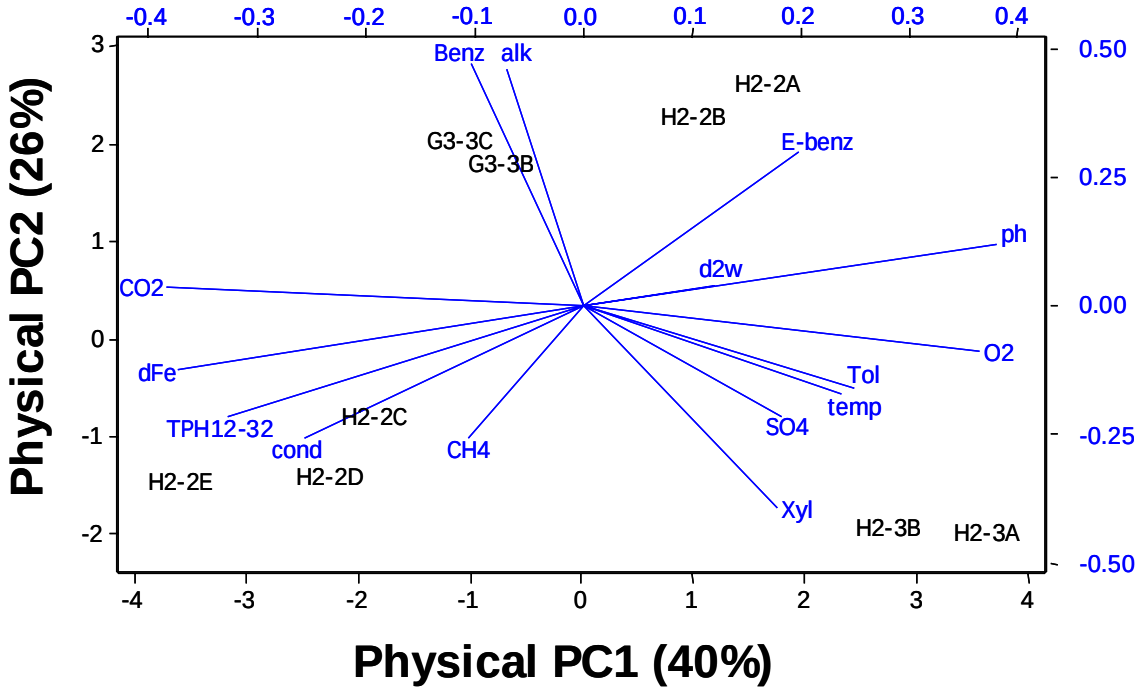


Figure 9. PCA of the physical/chemical parameters from the H2-2, H2-3, and G3-3, natural attenuation (NA) samples. Physical parameters involved in TPH degradation are most notably spread across PC1. Also note that without data from J wells the H and G well samples tend to disperse more along PC1 rather than PC2. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

PCA of the H2-2, H2-3, and G3-3 nested well TRF peaks obtained using *Taq*I show a wide distribution across PC1, which makes up 45% of the variation. Important TRF peaks affecting the percent variation with positive PC1 loadings are 579-81, 575, and 134. TRF peaks with negative PC1 loadings are 104 and 199 (Figure 10). A fitted line plot of TRF PC1 against physical PC1 showed good correlation, indicating that 60.2% of the variation in TRF PC1 was predicted by physical PC1 (Figure 11). This shows that 27% of the variation in actinobacterial community structure (60.2% of 45%) could potentially be attributed to the TPH degradation parameter physical PC1.

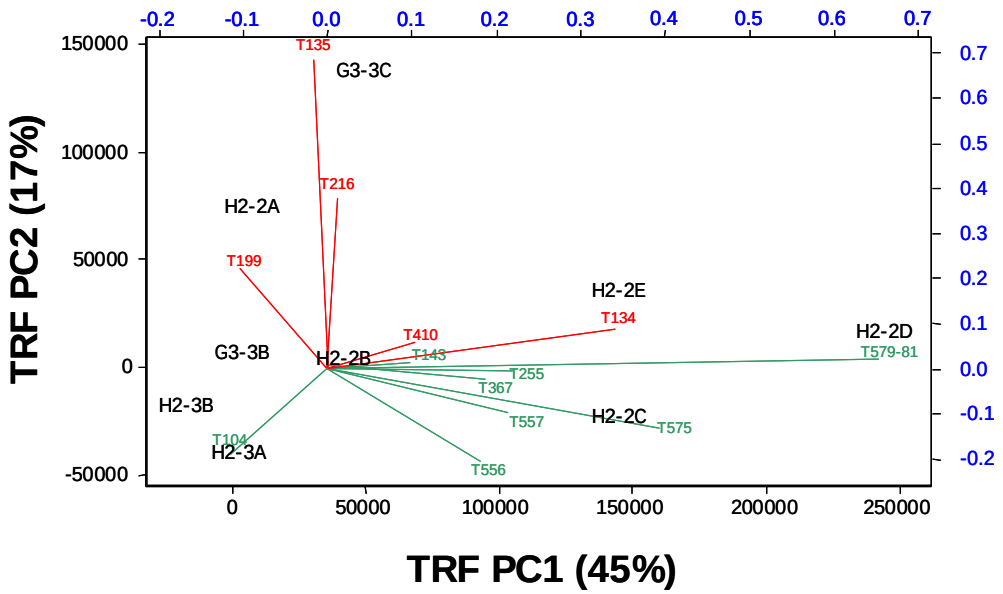


Figure 10. PCA of *Taq*I actinobacterial TRF data from wells at H2-2, H2-3, and G3-3. Without the J wells the sample distribution is along PC1 more so than PC2. Red lines indicate TRF peaks which had a similar standardized abundance to TRF peaks obtained using *Hha*I. Green indicates TRF peaks which had high loadings but could not be matched to *Hha*I TRF peaks. Only TRF peaks with loading > 0.1 or peaks which had a similar standardized abundance to *Hha*I peaks are shown. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

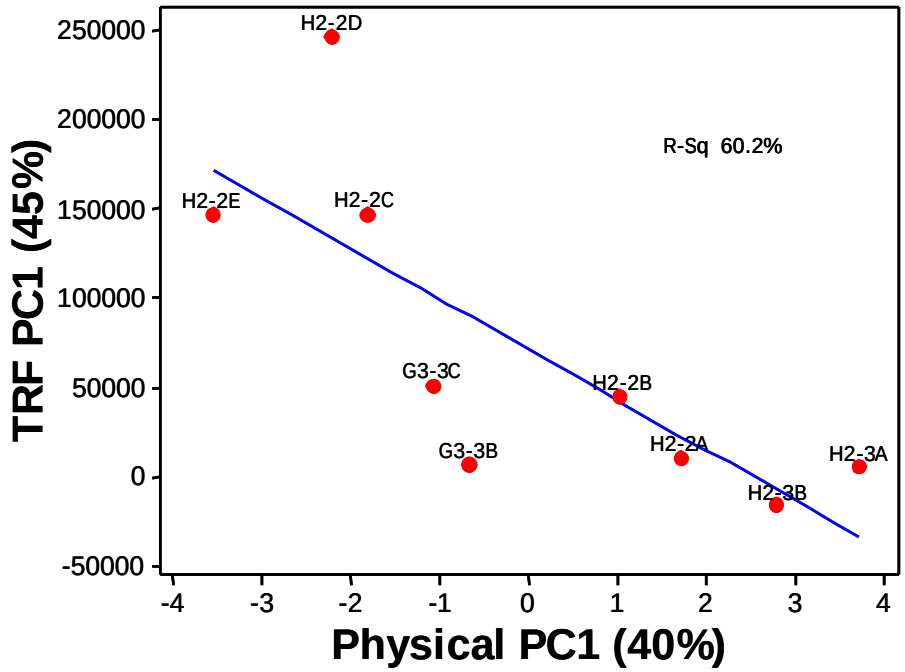


Figure 11. Correlation of the physical/chemical data (represented as physical PC1) against *Taq*I actinobacterial TRF PC1 data from the H2-2, H2-3, and G3-3 nested wells.

PCA of the TRF peaks obtained from *Hha*I show that 64% of the variation is found in PC1 and only 12% is in to PC2 (Figure 12). The large percent variation observed in TRF PC1 is mainly due to peaks 576-8 and 579-81, which has very large negative PC1 loadings. Peaks 576-8 and 579-81 have such large PC1 loadings that they significantly increase the PC1 percent coverage. In reality PC2 gives a better picture of the variation in the bacterial community due to ground water physical parameters. A fitted line plot of TRF PC2 against physical PC1 showed a good correlation, indicating that 45.1% of the variation in TRF PC2 was predicted by physical PC1 (Figure 13). This shows that 5.4% of the variation in actinobacterial community structure (45.1% of 12%) could potentially be attributed to the TPH degradation parameter physical PC1. It is important to keep in mind that the physical PC1 data was plotted against *Hha*I TRF PC2, which only accounted for 12% of the overall variation between samples. If peaks 576-8 and 579-81 had smaller PC1 loadings there may have been a better correlation of TRF PC1 and physical PC1. Consequently, a larger percent variation in the actinobacterial community structure may have been attributed to the TPH degradation parameter physical PC1.

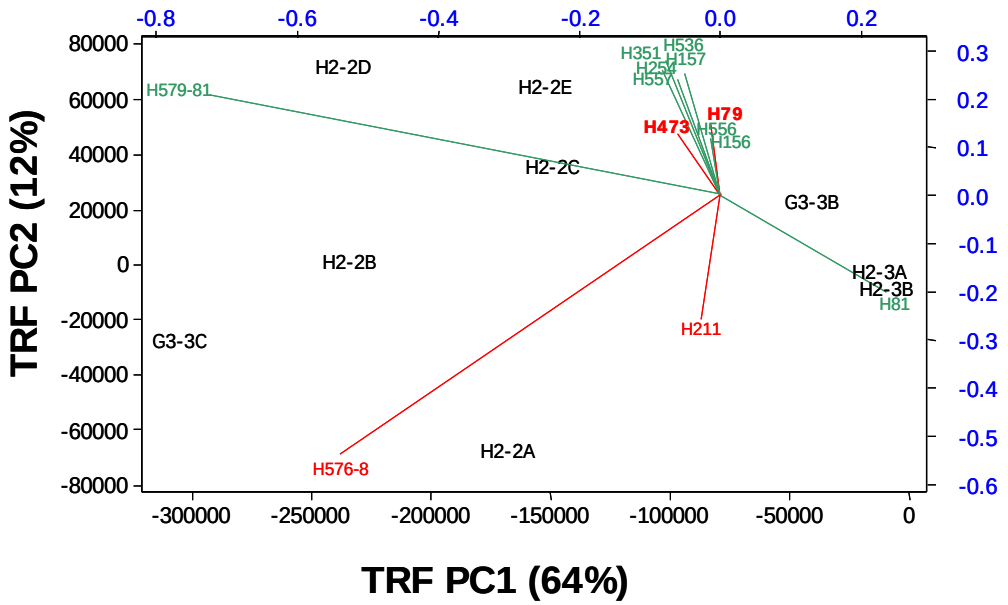


Figure 12. PCA of *Hha*I actinobacterial TRF data from nested wells at H2-2, H2-3, G3-3. Red lines indicate TRF peaks which had a similar standardized abundance to TRF peaks obtained using *Taq*I. Green indicates TRF peaks which had high loadings but could not be matched to *Taq*I TRF peaks. Only TRF peaks with loading > 0.1 or peaks which had a similar standardized abundance to *Taq*I peaks are shown. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

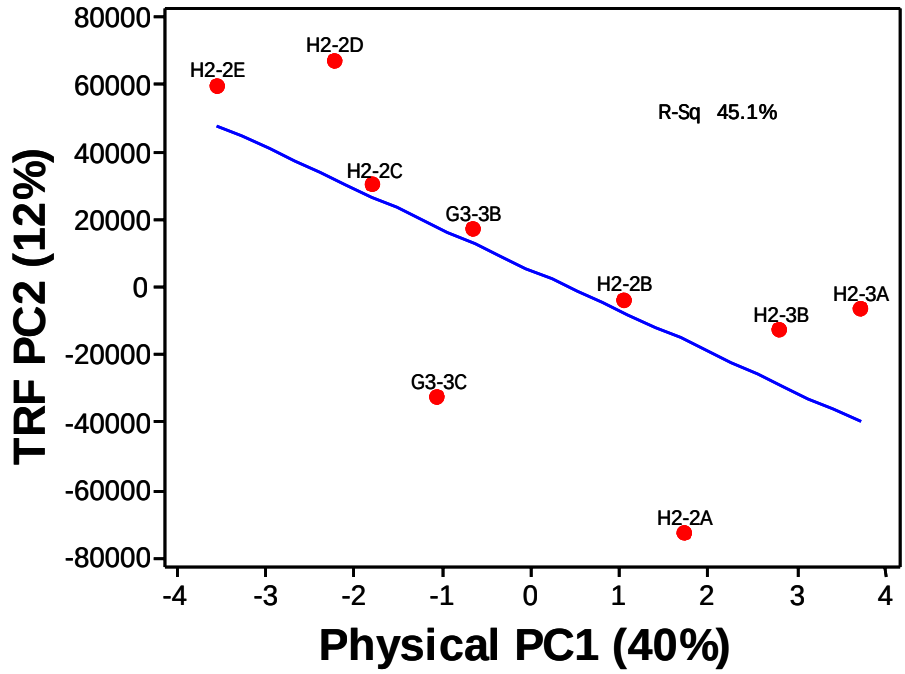


Figure 13. Correlation of the physical/chemical data (represented as physical PC1) against *Hha*I actinobacterial TRF PC1 data from the H2-2, H2-3, and G3-3 wells.

Analysis of Physical/Chemical and TRF Data Collected from the H2-2 and G3-3 Natural Attenuation Wells

When the H2-2, H2-3, and G3-3 wells were sampled together, it was apparent that peak #81 had a significant affect on PC1 variation. The PCA showed TRF peaks 576-8 and 579-81 to have heavily weighted negative PC1 loadings while peak #81 had a heavily weighted positive PC1 loading. PCA showed the H2-3 samples to be responsible for peak #81 (Figure 12). It was decided that the removal of H2-3 samples and reanalysis of the remaining NA samples might help further identify the important peaks in the H2-2 and G3-3 wells. It was also noted that at the Guadalupe Dunes O_2 concentration is generally low in areas with TPH. Samples obtained from wells H2-2 and G3-3 were significantly lower in O_2 concentration than the other NA well samples (J and H2-3). In order to identify organisms potentially involved with anaerobic TPH degradation it was decided to focus on the H2-2 and G3-3 set.

PCA of the groundwater parameters from nested wells at H2-2 and G3-3 showed a good spread of data along PC1. The PC1 actually accounts for 55% of the overall variation between samples (Figure 14). As shown in figure 14 many of the components associated with TPH degradation are dispersed along PC1. Physical components with negative PC1 loadings consist of TPH, cond., dFe, Xyl, CO_2 and CH_4 while physical components with positive PC1 loadings consist of Tol, Benz, alk, E-benz, pH and O_2 (Figure 14).

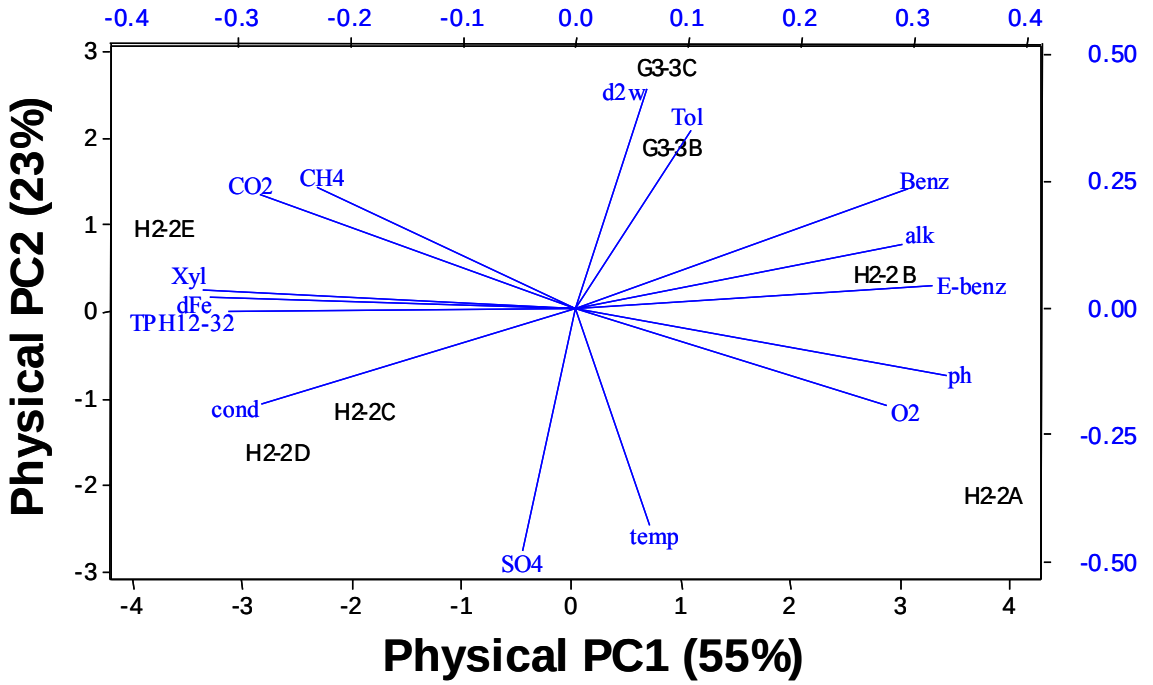


Figure 14. PCA of the physical/chemical groundwater parameters from nested wells H2-2, and G3-3. Physical/chemical parameters involved in TPH degradation are most notably spread across PC1. By removing specific samples from the analysis it is obvious which physical parameters are having the greatest affect on sample distribution. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

PCA of the actinobacterial TRF data obtained using *Taq*I also showed a good spread along PC1, which covered 47% of the variation in TRF data (Figure 15). The order of samples along the TRF PC1 axis appeared quite similar to what was seen along the physical PC1 axis for the same samples. In fact, a plot of TRF PC1 against physical PC1 showed good correlation, indicating that 69% of the variation in TRF PC1 was predicted by physical PC1 (Figure 16). This shows that 32% of the variation in actinobacterial community structure (69% of 47%) could potentially be attributed to the TPH degradation parameter physical PC1.

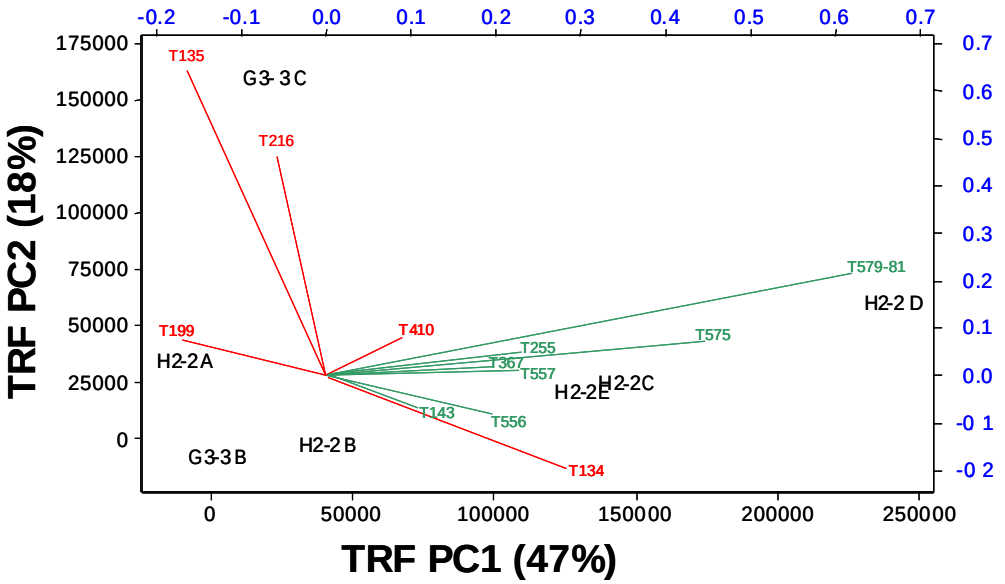


Figure 15. PCA of *Taq*I actinobacterial TRF data from wells at H2-2, and G3-3. Red lines indicate TRF peaks which had a similar standardized abundance to TRF peaks obtained using *Hha*I. Green indicates TRF peaks which had high loadings but could not be matched to *Hha*I TRF peaks. Only TRF peaks with loading > 0.1 or peaks which had a similar standardized abundance to *Hha*I peaks are shown. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

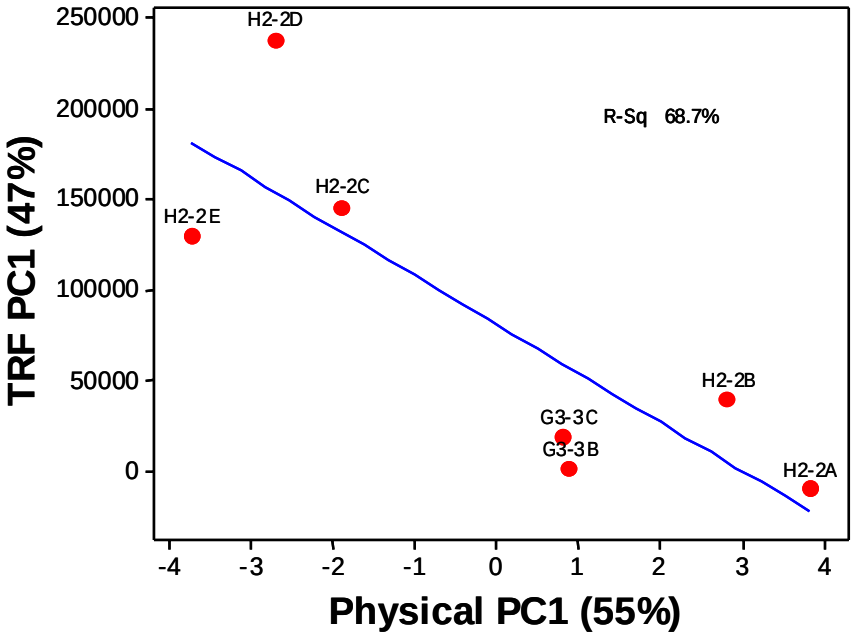


Figure 16. Correlation of the physical/chemical data (represented as physical PC1) against *Taq*I actinobacterial TRF PC1 data from the H2-2, and G3-3 nested wells.

PCA of the actinobacterial TRF data obtained using *Hha*I showed that PC2 accounts for only 19% of the TRF data while PC1 accounts for 58% (Figure 17). The PCA also shows peaks 576-8 and 579-81 to have large negative PC1 loadings. A fitted line plot of TRF PC2 against physical PC1 showed a good correlation, indicating that 80% of the variation in TRF PC2 was predicted by physical PC1 (Figure 18). This means that 15.2% of the variation in actinobacterial community structure (80 % of 19%) could potentially be attributed to the TPH degradation parameter physical PC1. When samples from the H2-2, H2-3 and G3-3 wells were analyzed together, they showed a large percent variation in TRF PC1. This was again the case for the H2-2 and G3-3 analysis. In both cases it appears as if the excessive negative PC1 loadings from peaks 576-8 and 579-81 are mainly responsible for the large percent variation found in PC1. Due to the excessive percent variation in PC1 (caused by peaks 576-8 and 579-81) PC2 resulted in a much lower percent variation. Even though the percent variation accounted for in TRF PC1 was much larger than that of TRF PC2 a fitted line plot shows that physical PC1 correlates much better with TRF PC2 than with TRF PC1 (Figure 18).

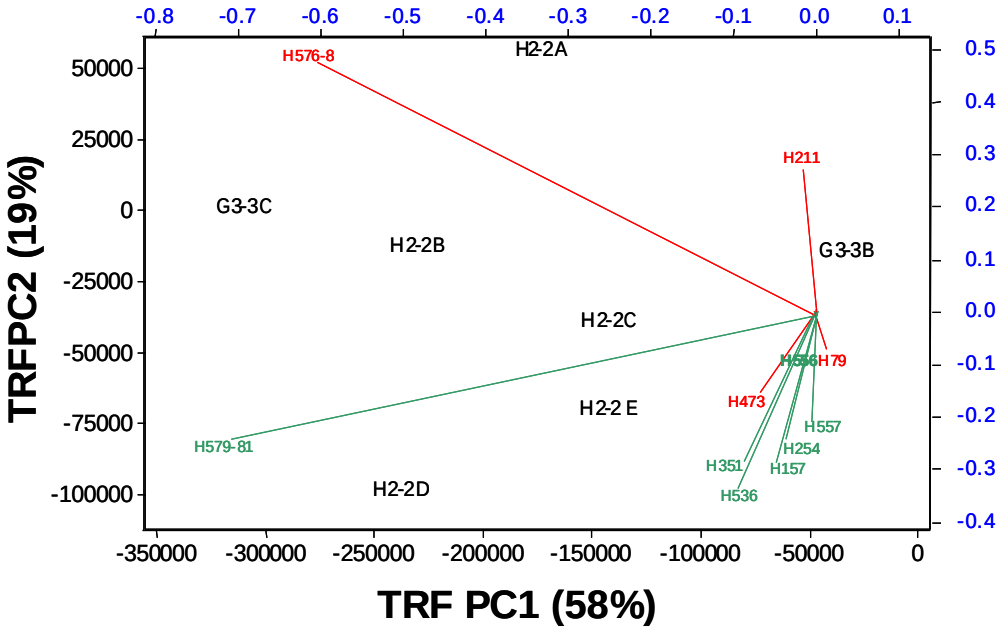


Figure 17. PCA of *Hha*I actinobacterial TRF data from H2-2 and G3-3 nested wells. Red lines indicate TRF peaks which had a similar standardized abundance to TRF peaks obtained using *Taq*I. Green indicates TRF peaks which had high loadings but could not be matched to *Taq*I TRF peaks. Only TRF peaks with loading > 0.1 or peaks which had a similar standardized abundance to *Taq*I peaks are shown. Percent variation covered by each principal component is indicated in parentheses in the axis titles below and to the left, along with the principal component scores. The right and top axes show values for principal component loadings.

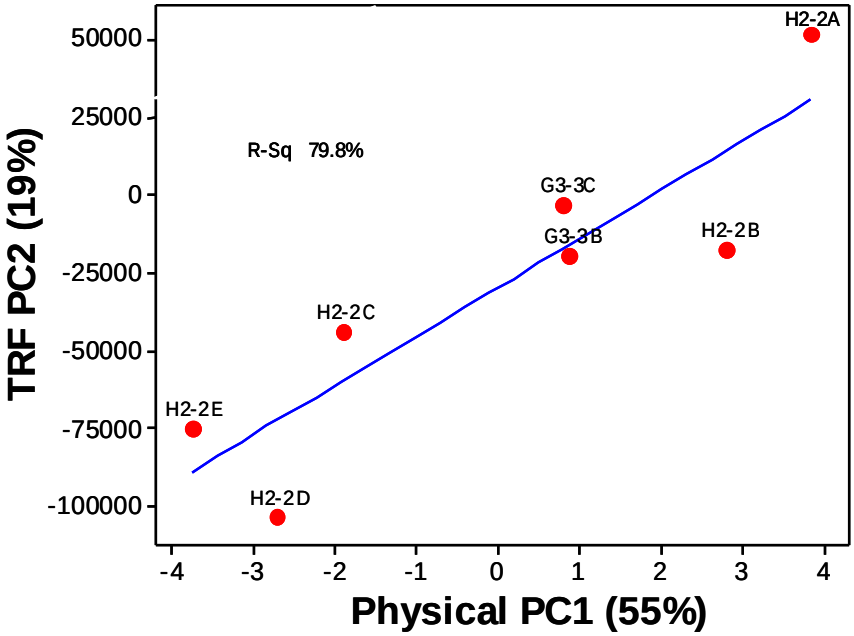


Figure 18. Correlation of the physical/chemical data (represented as physical PC1) against *Hha*I actinobacterial TRF PC1 data from the H2-2, and G3-3 wells.

Correlations Between *Taq*I and *Hha*I TRF Peaks and Physical/Chemical Properties Associated with TPH Degradation

*Taq*I and *Hha*I TRF peaks with a similar standardized abundance, along with TRF peaks that had high PC1 loadings were correlated with TPH, dFe, CH_4, and CO_2. Results of the correlations, which were recorded as R^2 values, showed some peaks to be strongly correlated with physical/chemical properties associated with anaerobic TPH degradation including TPH, dFe, CH_4, and CO_2 (Table 2, e.g. Figure 19). TPH degradation by microorganism's results in a decrease in TPH chain length and an increased production of CO_2, CH_4 and dissolved iron (Fe^{2+}). Strong correlations between these physical/chemical properties and specific TRF peaks can be used as indicators of potential metabolic processes that may be occurring during TPH degradation (Figure 19).

Table 2. This table shows how TRF peaks with high PC1 loadings correlate to specific physical parameters. Results (shown as R^2 values) were calculated using data from the H2-2, H2-3, G3-3 and J wells.

***Taq*I TRF Peaks**	**TPH12-32**	**dFe**	**CH_4**	**CO_2**
T557	0.31	0.14	0.47	0.26
T558	0.42	0.22	0.46	0.25
T410	0.35	0.82	0.21	0.63
T134	0.21	0.40	0.08	0.23
T579-81	0.71	0.88	0.39	0.69
T143	0.62	0.70	0.39	0.57
T255	0.16	0.59	0.06	0.27
T367	0.19	0.61	0.07	0.29
***Hha*I TRF Peaks**	**TPH12-32**	**dFe**	**CH_4**	**CO_2**
H454	0.58	0.29	0.65	0.36
H473	0.29	0.74	0.13	0.52
H79	0.34	0.53	0.29	0.32
H81	0.02	0.02	0.22	0.00
H156	0.41	0.18	0.39	0.23
H157	0.55	0.80	0.53	0.67
H254	0.48	0.74	0.47	0.66
H351	0.22	0.59	0.09	0.32
H536	0.21	0.57	0.13	0.31
H557	0.50	0.20	0.66	0.40
H579-81	0.54	0.57	0.34	0.72

*Taq*I and *Hha*I TRF peaks colored in blue followed a similar standardized abundance to a corresponding TRF peak from the other enzyme (*Taq*I or *Hha*I) (see table 3 for peak #'s) *Taq*I and *Hha*I TRF peaks colored in brown did not have a corresponding (*Taq*I or *Hha*I) TRF peak but they did have high PC1 loadings. Numbers colored in orange had correlations with R^2 values ≥ 0.40

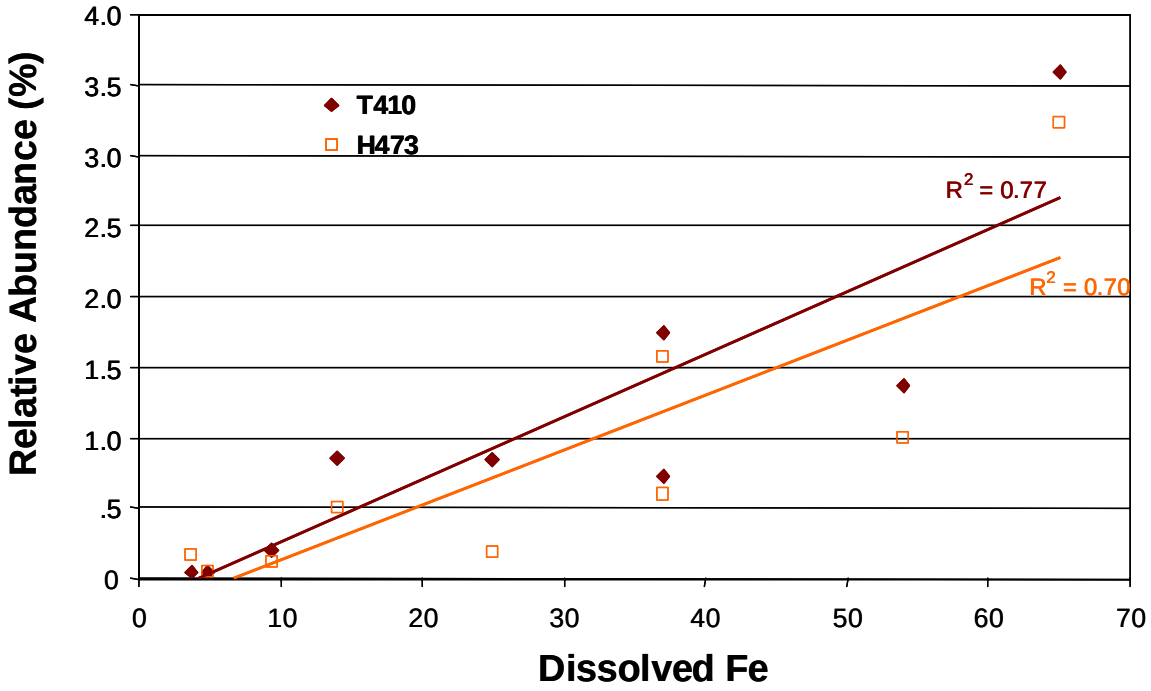


Figure 19. This figure shows TRF peak relative abundance vs dissolved iron for samples collected from the H2-2, H2-3 and G3-3 wells. Note how the *Taq*I peak 410 and *Hha*I TRF peak 473 follow a similar pattern and also have similar R^2 values.

Identification of Actinobacteria Potentially Involved in TPH Degradation

For better identification of actinobacteria, *Taq*I and *Hha*I TRF peaks were matched to one another by comparing the standardized abundance of each peak (Figure 20). *Taq*I and *Hha*I TRF peaks, which had a similar standardized abundance, were likely the result of one organism or group of organisms. Some of the matching *Taq*I and *Hha*I TRF peaks also correlated well with the physical/chemical properties sampled at each well (Table 2). A search of the EBI and Genbank actinobacterial TRF database resulted in a variety of matches to a single enzyme. However, of the four *Taq*I and *Hha*I TRF peaks that followed similar patterns of standard abundance, only one potential identification was made. The

database indicated that organisms resulting in *Taq*I 557 and *Hha*I 454 TRF's are mostly *Corynebacterium* (Table 3). It is clear that the actinobacteria represented by these TRF peaks are not well studied and their physiology cannot be determined at this time. However, their association with physical/chemical indicators of anaerobic TPH degradation suggests that these organisms are key players in the natural attenuation of TPH at the Guadalupe Dunes natural attenuation site.

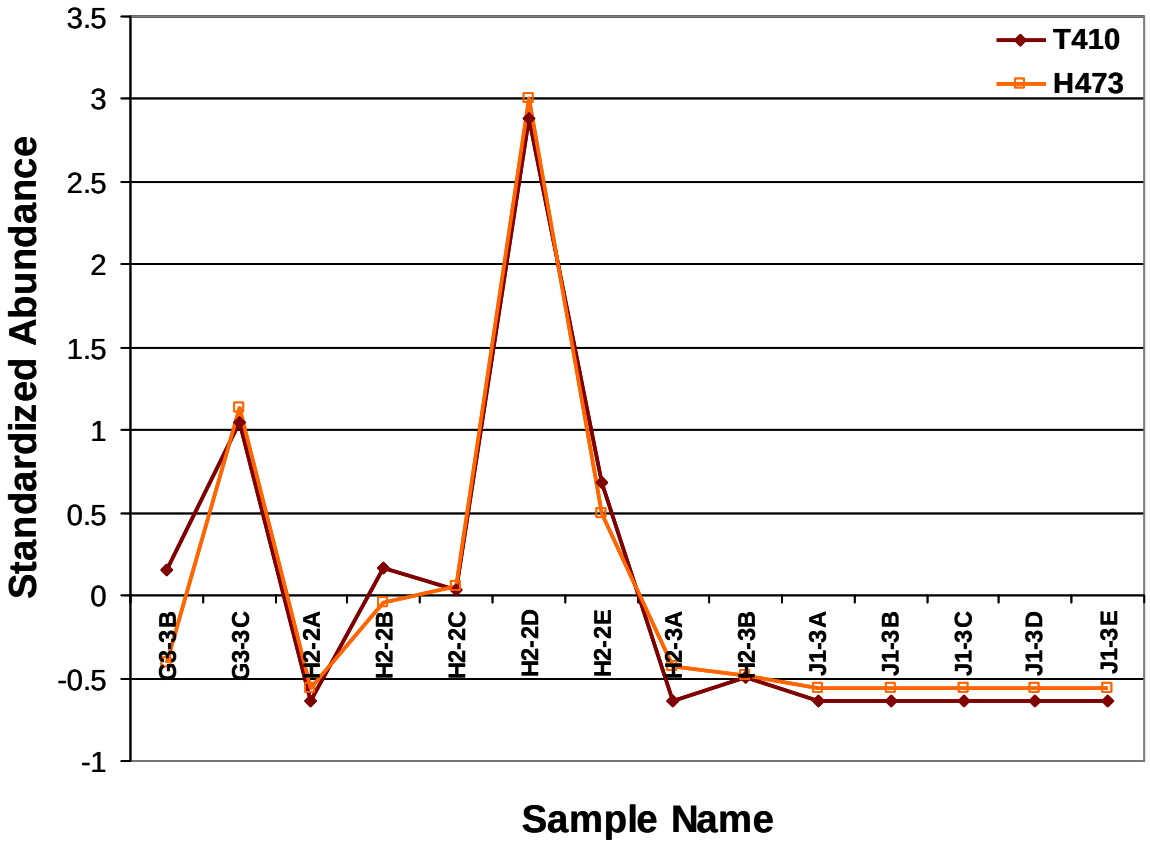


Figure 20. This figure shows a comparison of *Taq*I TRF peak 410 against *Hha*I TRF peak 473 for the H2-2, H2-3, G3-3 and J samples. Note the similar standardized abundance from well to well. This indicates that the same organism or group of organisms is responsible for both peaks.

Table 3. *Taq*I and *Hha*I TRF peaks, which had a similar standardized abundance, are shown in the observed column. Predicted sequences, which were obtained using the Genbank database and sequences obtained throughout this study gave only one match to a potential group of organisms responsible for the different TRF peaks. Other *Taq*I and *Hha*I TRF peaks, which followed a similar standardized abundance, had no database matches.

***Taq*I peak vs *Hha*I peak**	**phylotype (Genbank DB)**	**Enzyme**	**TRF (bp) Predicted**	**TRF (bp) Observed**
1	Mostly *Corynebacterium*	HhaI	454	453,454
		TaqI	557	557,558
2	No match	HhaI	na	473
		TaqI	na	410
3	No match	HhaI	na	282,354
		TaqI	na	84
4	No match	HhaI	na	79
		TaqI	na	134

Discussion

The purpose of this study was to develop a TRF assay to analyze actinobacterial community dynamics via TRF analysis. Through the use of known actinobacteria and non-actinobacteria it was clearly shown that the primer pair F243 and 814R is specific for the amplification of actinobacterial rDNA and is also capable of amplifying rDNA from a wide variety of actinobacteria.

The F243 and 814R primer pair's ability to selectively amplify actinobacterial rDNA in both pure cultures and environmental samples makes them suitable for use in generating TRF patterns for the analysis of actinobacterial community dynamics. By obtaining TRF peaks for individual soil isolates and comparing them to TRF patterns generated from their corresponding soil samples, it was shown that the primer pair is not only capable of generating actinobacterial community TRF patterns but they are also capable of distinguishing between different groups of actinobacteria. It is important to note that as expected, soil TRF patterns showed a large percentage of actinobacteria which were not cultured. This indicates a large number of actinobacteria in the soil were undetected using conventional culture techniques.

The fact that TRF analysis was capable of detecting and differentiating these uncultured bacteria further shows the value of TRF. Liu et al., 1997 showed TRF to be a powerful tool when assessing the diversity of complex bacterial communities and also for rapidly comparing the community structure and diversity of different ecosystems. There have been numerous publications focused on the monitoring of microbial populations via

TRF analysis. However, as with this study, some of the recently published articles report using PCA as one means of analyzing TRF data (Jernberg et al., 2005; Kaplan and Kitts, 2004; Wang et al., 2004).

Application of Actinobacterial TRF to Natural Attenuation at the Guadalupe Dunes

In this study three separate analyses were performed on the physical/chemical and actinobacterial TRF data collected from the natural attenuation site at the Guadalupe Dunes. Data analysis showed strong correlations between TRF and physical/chemical data collected from each well (Table 5). Through the understanding of microbial metabolic cycles, one can speculate that as TPH degradation progresses, certain physical/chemical properties will change as well. Expected physical/chemical changes due to the microbial degradation of TPH would include an increase in CO_2, CH_4, and dissolved iron (Fe^{+2}). A variety of microorganisms can reduce ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron when performing anaerobic respiration. Both chemoorganotrophic and chemolithotrophic bacteria can use Fe^{3+} as an electron acceptor for energy metabolism. Because the reduction potential of the Fe^{3+}/Fe^{2+} couple is very electropositive, Fe^{3+} reduction can be coupled to the oxidation of a variety of both organic and inorganic electron donors. The result of this is that organic compounds (including TPH and other petroleum products) coupled to Fe^{3+} reduction can be converted to CO_2. The CO_2 can then be converted to CH_4. Studies have shown that dissimilatory iron reduction accounts for a significant fraction of anaerobic metabolism in certain freshwater and marine ecosystems (Aller et al., 1986; Lovley et al., 1987). A separate study by Lovley, 1995 also showed that the extent to which iron reduction contributes to mineralization depends in part on the supply rates of organic matter and Fe^{3+}.

Correlations between physical/chemical and TRF peak data can potentially be used to assign certain TRF peaks as indicators of TPH degradation. It is probable that organisms linked to these peaks play a role in the process of TPH degradation. This study successfully correlated multiple TRF peaks to physical/chemical properties commonly associated with anaerobic TPH degradation (Table 2). Unfortunately, it was difficult to assign specific organisms to the different TRF peaks.

Many actinobacteria have highly conserved 16S rRNA gene regions, which can make it difficult to identify specific organisms using TRF analysis. As shown by Clement et al., 1998 a more accurate characterization of bacterial communities can be achieved by combining TRF data derived from multiple enzymes. For this reason two different restriction enzymes (*Taq*I and *Hha*I) were used in this study. Having data from multiple enzymes also allowed for a comparison of the standardized abundance of TRF peaks obtained from both *Taq*I and *Hha*I. If the standardized abundance of an individual *Taq*I TRF peak follows the same standardized abundance as an individual *Hha*I TRF peak, it is very likely that one organism or group of organisms is responsible for producing these peaks. Four different *Taq*I and *Hha*I TRF peak alignments were identified (Table 3). Of the four possible *Taq*I and *Hha*I TRF peaks that matched, only one had a database match for possible organisms. The matches for *Taq*I 557 and 558 with *Hha*I 453 and 454 turned out to be mainly *Corynebacterium*. Identifying *Corynebacterium* as a potential organism was promising because many studies have shown *Corynebacterium* to actively participate in the degradation of a variety of petroleum products (Pineda-Flores et al., 2004; Mun et al., 2004; Rahman et al., 2002A; Rahman et al., 2002B; Williams et al., 1998). Although three other *Taq*I and *Hha*I TRF peak had similar standardized abundances among the 14

wells, no database matches were found. In numerous cases there were multiple organisms matching a single TRF peak either from *Taq*I or *Hha*I, but due to the large number of possibilities, identification of a specific organism could not be established. One possible explanation for the lack of database matches may be that the ground water samples were mostly anaerobic. The problem with identifying anaerobic actinobacteria by TRF is that they have not been extensively studied. Culturing anaerobic actinobacteria can be very difficult and time consuming. The consequence of this is that there are very few anaerobic actinobacterial sequences to compare with TRF peaks.

Each procedure required to obtain a good TRF pattern is open to error and bias. Everything from cell lysis (Head et al., 1998), DNA extraction and purification (Wheeler and Stahl, 1996), PCR conditions and formation of chimeric PCR products (Liesack et al., 1991; Kopczynske et al., 1994) can impact the overall abundance of a community analysis. Some of these biases, such as the formation of chimeric proteins, can be detected while others can be minimized by improved PCR conditions and techniques. Inherent cell characteristics, such as the size and number of genomes within a cell as well as the organization and number of rRNA genes, may also introduce bias (Farrelly et al., 1995). Potentially, bias encountered while obtaining a TRF pattern can skew the number of actual actinobacteria in a community. Identifying (as accurately as possible) and quantifying bacteria responsible for the production of a specific TRF peak is important so that greater emphasis and more detailed examinations may be performed on specific individuals.

In this study, organisms belonging to the genus *Corynebacterium* were identified as potential players in TPH degradation. Although not performed in this study, a new probe for the specific subset of actinobacteria (*Corynebacteria*) could be designed to further

identify and quantify this particular subset of actinobacteria. With the use of *Corynebacteria* specific probes, other techniques such as cloning and sequencing, FISH, and qPCR could be implored to attain a more accurate count of the *Corynebacteria* present in the community. With group specific primers and the knowledge that a soil sample contains a specific strain of bacteria, it is also possible to "spike" subsets of the soil sample with known concentrations of the bacteria. Knowing the quantity of bacteria added to each subset and comparing the relative abundances of TRF peaks from each sample may also help to acquire a more accurate number of bacteria present in a community. One such study performed by Tillmann and Friedrich, 2003 used defined template mixtures of Methanogenic pure cultures and soil DNA extracts to shown how TRF analysis can be used as a tool for the precise quantification of the PCR product pool.

Once a specific strain of actinobacteria has been potentially identified and/or quantified by TRF analysis, or other previously mentioned techniques further steps can then be implemented to help confirm the identity. Selective culture techniques specific for the growth of individual strains or groups of bacteria may be used to grow and isolate the strain in question (Waksman, 1959). Knowing what organism is being attempted to be cultured would not only save time, but could also be very cost effective. Upon successful isolation and purification, TRF patterns for the isolate could be compared to the community TRF patterns from which it was isolated. Having isolated an organism that potentially plays a role in the degradation of TPH could be very beneficial. Further studies of the isolate may help identify the specific role the microorganism plays in the community degradation of TPH.

One interesting development was observed when performing the H2-2, H2-3, G3-3 and the H2-2, G3-3 analyses. For both the H2-2, H2-3, G3-3 and the H2-2, G3-3 analyses, it was determined that a much better R^2 value was obtained when plotting physical PC1 against *Hha*I PC2 rather than *Hha*I PC1. When these same analyses were performed with data collected using the *Taq*I enzyme, the best R^2 values were always shown when physical PC1 was plotted against *Taq*I PC1. Although good R^2 values were obtained when plotting physical PC1 against *Hha*I PC2 for both the H2-2, H2-3, G3-3 and H2-2, G3-3 analyses, it must be noted that *Hha*I PC2 only accounts for only 12 % and 19 % of the variation, respectively. Two possible explanations for obtaining a better R^2 value for *Hha*I PC2 rather than *Hha*I PC1 are that (1) the digest may not have been completely successful and (2) there were lots of actinobacteria which were uncut by the *Hha*I enzyme. Either one of these situations could potentially explain the excessive loadings found in *Hha*I TRF peaks 579-81 and 576-8. The excessively large loadings of these two peaks may be responsible for skewing the data so much that it allowed *Hha*I PC2, rather than *Hha*I PC1, to correlate better with physical PC1. Due to the low percent coverage obtained when using *Hha*I, there is potentially an increased noise factor as compared to that of *Taq*I. The potentially high noise factor and undigested or uncut actinobacterial rDNA that may have resulted from the use of *Hha*I, could indicate *Taq*I is the better enzyme to use when analyzing actinobacteria communities with the F243 and 814R primers.

It has been shown that actinobacteria are one group of microorganisms that actively partake in bioremediation (Stackebrandt et al., 1997). Many studies have conclusively shown that specific actinobacteria are capable of degrading higher molecular mass PAH's (Grosser et al.,1991; Boldrin et al., 1993; Kleespies et al., 1996; Bottger et al., 1997),

simple hydrocarbons (Goodfellow and Minnikin, 1981), chlorinated hydrocarbons, numerous aliphatic and aromatic hydrocarbons, chlorinated polycyclic aromatics such as polychorinated biphenyls (Warhurst and Fewson, 1994; Iwabuchi et al., 2002), and many other compounds commonly associated with petroleum products (Kummer et al., 1999; Xue et al., 2003; Linos et al., 2002; Kim et al., 1999; Yoon et al., 2000). The goal of this experiment was to specifically identify actinobacteria associated with TPH degradation at the Guadalupe Dunes natural attenuation site. In a natural environment it is difficult to determine exactly what role a microorganism plays in TPH degradation, but this study showed a clear correlation between certain actinobacteria TRF peaks and key indicators of TPH degradation. It was thought that the use of actinobacteria specific primers would help elucidate the identification of the important anaerobic and aerobic actinobacteria in groundwater. However, it is quite apparent that the anaerobic actinobacterial community is poorly characterized and that the TRF data gathered in this study served to point out just how little we actually know about these complex microbial communities. To understand the microbiology of TPH degradation in groundwater at Guadalupe, it is necessary to further characterize these anaerobic actinobacteria. Information gathered from this study, such as correlations of specific TRF peaks to various physical/chemical parameters, may eventually be used to design methods for isolating these bacteria and characterizing their physiology with particular attention to TPH degradation.

Conclusions

- Primers F243 and 814R can distinguish between actinobacteria and non-actinobacteria rDNA

- Using primers F243 and 814R, TRF can detect differences in actinobacterial community structure

- TRF peaks present in the soil isolate TRF patterns were identified in the community TRF patterns from which they were isolated

- Sequences from soil PCR with these primers were 90% actinobacterial

- Some TRF peaks present in community TRF patterns were not identified in any of the isolate TRF patterns

- It is difficult to identify specific organisms with TRF patterns due to the highly conserved regions of many actinobacteria. Adding to the difficulty is the database used for identification, which is severely lacking in anaerobic actinobacterial sequences

- Many actinobacterial TRF peaks correlated well with TPH, dFe, CH_4, and CO_2 in samples of anaerobic ground water

- The next step in this study would be to use species-specific primers along with TRF analysis and alternative techniques such as cloning and sequencing, FISH, and qPCR to help confirm the identity of organisms potentially involved in TPH degradation

- Identification of an organism could also help one to either create or decide on a selective media which would increase the chances of isolation and cultivation

- Upon successful isolation, further tests could be performed with the isolate to help explain its specific role in TPH degradation

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Appendix A

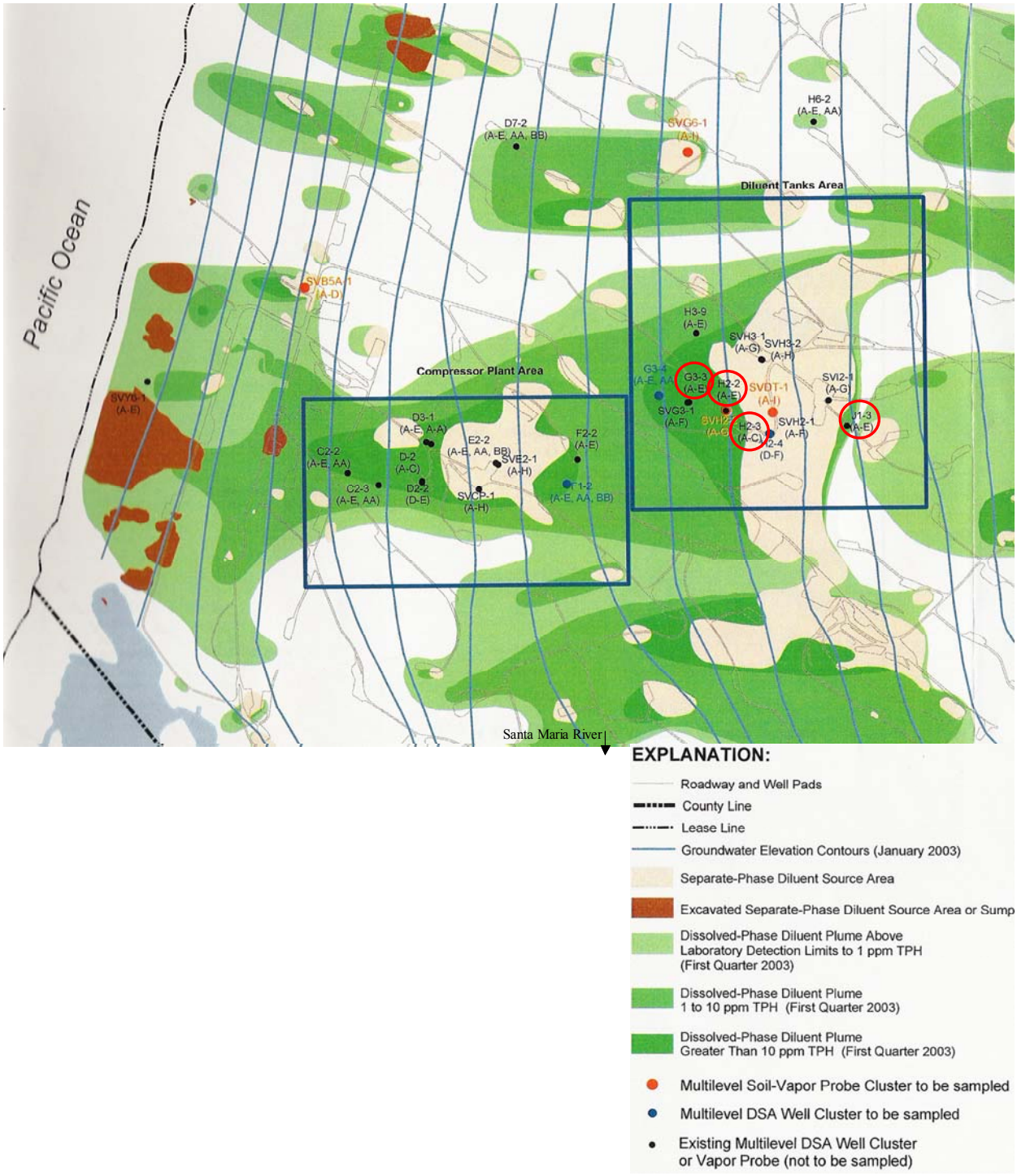

Figure 1. This map of the former Guadalupe oil field in San Luis Obispo County shows some of the monitoring wells sampled for the EBI natural attenuation field studies. Samples used for the application of actinobacteria specific primers (F243 and 814R) were collected from the wells circled in red. Map courtesy of Unocal Corporation.

Table 1. This table shows a list of all the actinobacterial strains used to test the primer pair F243 and 814R. The table is broken up into strain or ID number, the name of the organism (if known) and whether or not it amplified with PCR.

Strain ID	Blast Identification	PCR ?
BA166*	*Streptomyces sampsonii*	Yes
BA834*	Actinobacteria	Yes
BA1160*	Actinobacteria	Yes
BA1182*	Actinobacteria	Yes
BA1220*	Actinobacteria	Yes
BA1357*	Actinobacteria	Yes
BA1801*	*N.asteroides*	Yes
BA1818*	*Streptomyces sp. JL164*	Yes
BA1825*	*N.asteroides*	Yes
BA1832*	*Streptomyces tendae*	Yes
BA1860*	*N.nova*	Yes
BA1866*	*N.vaccinii*	Yes
BA1869*	*S.bikiniensis*	Yes
BA1872*	*N.asteroides (ATCC 23824)*	Yes
BA1897*	Actinobacteria	Yes
BA1900*	Actinobacteria	Yes
BA1907*	Actinobacteria	Yes
BA1920*	Actinobacteria	Yes
BA1931*	Actinobacteria	Yes
BA1937*	Actinobacteria	Yes
BA1952*	Actinobacteria	Yes
BA7176*	Actinobacteria	Yes
EA557**	*S.hygroscopicus*	Yes
EA574**	*M fulvoviolaceus*	Yes
EA581**	*M. fulvoviolaceus*	Yes
EA695**	Actinobacteria	no
EA744**	Actinobacteria	no
EA764**	*D.salmoneum*	no
EA765**	*D.thailandense*	no
EA766**	*D. salmoneum*	no
EA774**	*Streptomyces sp.strain254*	Yes
EA783**	*Streptomycetaceae*	no
EA784**	*S. intermedius (DSM) 40372T*	Yes
EA838**	*M. fulvoviolaceus*	no
EA873**	*S.mutabilis DSM 43853*	Yes
	Identification	
HA6602***	Actinobacteria	Yes
HA6603***	Actinobacteria	Yes
HA6604***	Actinobacteria	Yes

HA6621***	Actinobacteria	Yes
HA6625***	Actinobacteria	Yes
HA6627***	Actinobacteria	no
HA6666***	Actinobacteria	Yes
HA6674***	Actinobacteria	Yes
HA6891***	Actinobacteria	Yes
HA6925***	Actinobacteria	Yes
HA6965***	Actinobacteria	no
HA6996***	Actinobacteria	Yes
HA7074***	Actinobacteria	no
HA7137***	Actinobacteria	no
HA7150***	Actinobacteria	Yes
HA7154***	Actinobacteria	Yes
HA7157***	Actinobacteria	Yes
HA7166***	Actinobacteria	Yes
HA7183***	Actinobacteria	Yes
HA7187***	Actinobacteria	Yes
HA7208***	Actinobacteria	no
HA7220***	Actinobacteria	Yes
HA7221***	Actinobacteria	Yes
HA7228***	Actinobacteria	Yes
HA7229***	Actinobacteria	no
(1) NPS000262****	Streptomyces sp. IM-6973	Yes
(2) NPS001583****	Streptomyces sacchari 16S	Yes
(3) NPS011852****	Streptomyces sp. SNG9 16S	Yes
(4) NPS008866****	Streptomyces sp. IM-6840	Yes
(5) NPS008882****	Streptomyces sp. VTT E-99-1333	Yes
(6) NPS008916****	Streptomyces maritimus 16S	Yes
(7) NPS008917****	Streptomyces sp. IM-7556	Yes
(8) NPS008919****	Streptomyces sp. VTT E-99-1333	Yes
(9) NPS008922****	Streptomycetaceae 16S rRNA	Yes
(10) NPS008924****	Streptomyces sp. SNG9 16S	Yes
(11) NPS008926****	Streptomyces sp. IM-7556	Yes
(12) NPS008934****	Streptomyces sp. IM-7556	Yes
(13) NPS008945****	Glacial ice bacterium G200-C1 16S	Yes
(14) NPS009211****	Streptomyces sp. IM-6826	Yes
(15) NPS009215****	Streptomyces sp. IM-6840	Yes
(16) NPS009398****	Streptomycetaceae 16S rRNA	Yes
(17) NPS009377****	Streptomyces thermocoprop	Yes
(18) NPS009419****	Streptomyces sp. IM-6840	Yes
(19) NPS009423****	Streptomycetaceae 16S rRNA	Yes
(20) NPS009431****	Streptomyces sp. EF-25 16S ribosome	Yes
(21) NPS009432****	Actinomadura mexicana 16S	Yes
(22) NPS009399****	Streptomyces sp. NK528 16S ribosome	Yes
(23) NPS009444****	Streptomyces sp. VTT E-99-1333	Yes
(24) NPS009450****	Streptomyces sp. NK1057 16S ribosome	Yes
(25) NPS009452****	Streptomyces sp. SNG9 16S	Yes

(26) NPS009453****	Streptomyces sp. SNG9 16S	Yes
(27) NPS009457****	Streptomyces sp. SNG9 16S	Yes
(28) NPS009459****	Streptomyces sp. IM-7585	Yes
(29) NPS009460****	Streptomyces sp. SNG9 16S	Yes
(30) NPS009563****	Streptomyces sp. IM-7585	Yes
(31) NPS009571****	Nonomuraea turkmeniaca 16S	Yes
(32) NPS009577****	Streptomyces speibonae 16S ribosome	Yes
(33) NPS009710****	Streptomyces sp. IM-7585	Yes
(34) NPS009797****	Streptomyces sp. NK1057 16S ribosome	Yes
(35) NPS009800****	Spirillospora rubra 16S rRNA	Yes
(36) NPS009855****	Streptomyces sp. SNG9 16S	Yes
(37) NPS009856****	Salinospora sp. CNH898 16S ribosome	Yes
(38) NPS009948****	Micromonospora sp. CNH394 16S ribosome	Yes
(39) NPS010114****	Micromonospora sp. CNH512 16S ribosome	Yes
(40) NPS010479****	Micromonospora sp. IM-741	Yes
(41) NPS010523****	Nonomuraea rubescens 16S ribosome	Yes
(42) NPS010530****	Streptomyces sp. NK528 16S ribosome	Yes
(43) NPS011072****	Streptomyces sp. IM-7556	Yes
(44) NPS011040****	Streptomycetaceae 16S rRNA	Yes
(45) NPS011054****	Verrucosispora sp. IM-690	Yes
(46) NPS011055****	Streptomyces sp. VTT E-99-1336	Yes
(47) NPS011135****	Streptomyces sp. SNG9 16S	Yes
(48) NPS011143****	Streptomyces sp. NK528 16S ribosome	Yes
(49) NPS011156****	Streptomyces sp. NK528 16S ribosome	Yes
(50) NPS011203****	Streptomyces sp. SNG9 16S	Yes
(51) NPS011234****	Streptomyces sp. IM-7585	Yes
(52) NPS011331****	Unidentified eubacteriu	Yes
(53) NPS011361****	Micromonospora sp. CNH512 16S ribosome	Yes
(54) NPS011370****	Micromonospora sp. CNH512 16S ribosome	Yes
(55) NPS011428****	Glacial ice bacterium G200-C1 16S	Yes
(56) NPS011448****	Streptomyces sp. IM-7585	Yes
(57) NPS011483****	S.caesia gene for 16S ribosome	Yes
(58) NPS011644****	Streptomyces thermocoprop	Yes
(59) NPS011664****	Streptomyces sp. R25 16S ribosome	Yes
(60) NPS011745****	S.caesia gene for 16S ribosome	Yes
(61) NPS011507****	Glacial ice bacterium G200-C1 16S	Yes
(62) NPS011524****	Unidentified eubacteriu	Yes
(63) NPS011526****	Micromonospora sp. CNH512 16S ribosome	Yes
(64) NPS011544****	Streptomyces sp. IM-6840	Yes
(65) NPS011561****	Streptomyces sp. IM-7585	Yes
(66) NPS011571****	Streptomycetaceae 16S rRNA	Yes
(67) NPS011572****	Streptomyces sp. IM-7585	Yes
(68) NPS011575****	Streptomycetaceae 16S rRNA	Yes
(69) NPS011598****	Streptomyces sp. SNG9 16S	Yes
(70) NPS011614****	Streptomyces sp. SNG9 16S	Yes
(71) NPS011631****	Streptomyces sp. A3 16S ribosome	Yes
(72) NPS011748****	Streptomyces sp. IM-7585	Yes

(73) NPS011797****	Streptomyces sp. SNG9 16	Yes
(74) NPS011843****	NA	Yes
(75) NPS011849****	Nocardiopsis halototer	Yes
(76) NPS011857****	Streptomyces sp. IM-7585	Yes
(77) NPS011862****	Streptomyces sp. IM-7585	Yes
(78) NPS011881****	Streptomyces sp. F50970 16S ribosome	Yes
(79) NPS011892****	Streptomyces sp. IM-7585	Yes
(80) NPS011926****	Streptomyces sp. IM-7585	Yes
(81) NPS011936****	Streptomyces sp. IM-6973	Yes
(82) NPS011938****	Streptomyces sp. SNG9 16S	Yes
(83) NPS011939****	Streptomyces sp. SNG9 16S	Yes
(84) NPS011985****	Streptomycetaceae 16S rRNA	Yes
(85) NPS011893****	Unidentified eubacteriu	Yes
(86) NPS011903****	Streptomyces sp. SNG9 16S	Yes
(87) NPS012140****	Streptomyces sp. SNG9 16S	Yes
(88) NPS012074****	Streptomyces sp. IM-6840	Yes
(89) NPS012102****	Streptomyces sp. IM-7585	Yes
(90) NPS012129****	Streptomyces sp. SNG9 16S	Yes
(91) NPS012132****	Unidentified eubacteriu	Yes
(92) NPS012133****	Streptomyces sp. IM-7585	Yes
(93) NPS012135****	Streptomyces sp. IM-7585	Yes
(94) NPS012136****	NA	no
(95) NPS012137****	Streptomyces sp. IM-7585	Yes
(96) NPS012167****	Streptomycetaceae 16S rRNA	Yes
	Morphological Identification	
EBI4071cy#	Actinobacteria	Yes
EBI4072cy#	Actinobacteria	Yes
EBI4073cy#	Actinobacteria	Yes
EBI4074cy#	Actinobacteria	Yes
EBI4075cy#	Actinobacteria	Yes
EBI4077cy#	Actinobacteria	Yes
EBI4079cy#	Actinobacteria	Yes
EBI4080cy#	Actinobacteria	Yes
EBI4081cy#	Actinobacteria	Yes
EBI4084sc#	Actinobacteria	Yes
EBI4086sc#	Actinobacteria	Yes
EBI4088sc#	Actinobacteria	Yes
EBI4090sc#	Actinobacteria	Yes
EBI4091sc#	Actinobacteria	Yes
EBI4095sc#	Actinobacteria	Yes
EBI4096sc#	Actinobacteria	Yes
EBI4100sc#	Actinobacteria	Yes
EBI4102sc#	Actinobacteria	Yes
EBI4103sc#	Actinobacteria	Yes
EBI4104sc#	Actinobacteria	Yes
EBI4105sc#	Actinobacteria	Yes
EBI4108sc#	Actinobacteria	Yes

EBI4111sc#	Actinobacteria	Yes
EBI4112sc#	Actinobacteria	Yes
EBI4115sc#	Actinobacteria	no
EBI4116sc#	Actinobacteria	Yes
EBI4117sc#	Actinobacteria	Yes
EBI4118sc#	Actinobacteria	Yes
EBI4119sc#	Actinobacteria	Yes
EBI4120sc#	Actinobacteria	Yes
EBI4121sc#	Actinobacteria	no
EBI4122sc#	Actinobacteria	Yes
EBI4123sc#	Actinobacteria	no
EBI4124sc#	Actinobacteria	Yes
EBI4125sc#	Actinobacteria	no
EBI4126sc#	Actinobacteria	Yes
EBI4127sc#	Actinobacteria	Yes
EBI4128sc#	Actinobacteria	Yes
EBI4129sc#	Actinobacteria	no
EBI4130cy#	Actinobacteria	Yes
EBI4131cy#	Actinobacteria	no
EBI4132cy#	Actinobacteria	Yes
EBI4133cy#	Actinobacteria	Yes
EBI4134cy#	Actinobacteria	Yes
EBI4136cy#	Actinobacteria	Yes
EBI4138cy#	Actinobacteria	Yes
EBI4140cy#	Actinobacteria	Yes
EBI4141cy#	Actinobacteria	Yes
EBI4142cy#	Actinobacteria	Yes
EBI4143cy#	Actinobacteria	no
EBI4144cy#	Actinobacteria	Yes
EBI4145cy#	Actinobacteria	Yes
EBI4146cy#	Actinobacteria	Yes
EBI4148cy#	Actinobacteria	Yes
EBI4151cy#	Actinobacteria	Yes
EBI4153cy#	Actinobacteria	Yes
EBI4154cy#	Actinobacteria	Yes
EBI4155cy#	Actinobacteria	Yes
EBI4156cy#	Actinobacteria	Yes
EBI4157cy#	Actinobacteria	Yes
EBI4159cy#	Actinobacteria	Yes
EBI4160cy#	Actinobacteria	no
EBI4163cy#	Actinobacteria	Yes
EBI4165sc#	Actinobacteria	Yes
EBI4166sc#	Actinobacteria	Yes
EBI4168sc#	Actinobacteria	Yes
EBI4170sc#	Actinobacteria	Yes
EBI4171sc#	Actinobacteria	Yes
EBI4175Asc#	Actinobacteria	no

EBI4175Bsc#	Actinobacteria	no
EBI4177sc#	Actinobacteria	Yes
EBI4178sc#	Actinobacteria	no
EBI4179sc#	Actinobacteria	no
EBI4182sc#	Actinobacteria	Yes
EBI4184sc#	Actinobacteria	Yes
EBI4185sc#	Actinobacteria	Yes
EBI4186sc#	Actinobacteria	no
EBI4187sc#	Actinobacteria	Yes
EBI4188sc#	Actinobacteria	Yes
EBI4190sc#	Actinobacteria	Yes
EBI4191sc#	Actinobacteria	Yes
EBI4192sc#	Actinobacteria	Yes
EBI4194sc#	Actinobacteria	Yes
EBI4195sc#	Actinobacteria	Yes
EBI4201sc#	Actinobacteria	Yes
EBI4202sc#	Actinobacteria	no
EBI4203cy#	Actinobacteria	Yes
EBI4205cy#	Actinobacteria	Yes
EBI4207cy#	Actinobacteria	Yes
EBI4208cy#	Actinobacteria	Yes
EBI4209cy#	Actinobacteria	Yes
EBI4210cy#	Actinobacteria	Yes
EBI4212cy#	Actinobacteria	Yes
EBI4213cy#	Actinobacteria	Yes
EBI4215cy#	Actinobacteria	Yes
EBI4218cy#	Actinobacteria	Yes
EBI4219cy#	Actinobacteria	Yes
EBI4221cy#	Actinobacteria	Yes
EBI4223cy#	Actinobacteria	Yes
EBI4224cy#	Actinobacteria	Yes
EBI4225cy#	Actinobacteria	Yes
EBI4226cy#	Actinobacteria	Yes
EBI4227cy#	Actinobacteria	Yes
EBI4228cy#	Actinobacteria	Yes
EBI4229cy#	Actinobacteria	Yes
EBI4230cc#	Actinobacteria	Yes
EBI4237cc#	Actinobacteria	Yes
EBI4238cc#	Actinobacteria	Yes
EBI4239cc#	Actinobacteria	Yes
EBI4242cc#	Actinobacteria	Yes
EBI4243cc#	Actinobacteria	Yes
EBI4244cc#	Actinobacteria	Yes
EBI4245cc#	Actinobacteria	Yes
EBI4247cc#	Actinobacteria	Yes
EBI4248cc#	Actinobacteria	Yes
EBI4250cc#	Actinobacteria	Yes

EBI4251cc#	Actinobacteria	Yes
EBI4253cc#	Actinobacteria	Yes
EBI4254cc#	Actinobacteria	Yes
EBI4255cc#	Actinobacteria	Yes
EBI4256cc#	Actinobacteria	Yes
EBI4257cc#	Actinobacteria	Yes
EBI4258cc#	Actinobacteria	Yes
EBI4260cc#	Actinobacteria	no
EBI4261cc#	Actinobacteria	Yes
EBI4263cc#	Actinobacteria	Yes
EBI4264cc#	Actinobacteria	Yes
EBI4265cc#	Actinobacteria	Yes
EBI4266cc#	Actinobacteria	Yes
EBI4267cc#	Actinobacteria	Yes
EBI4268cc#	Actinobacteria	Yes
EBI4269cc#	Actinobacteria	Yes
EBI4271cc#	Actinobacteria	Yes
EBI4272cc#	Actinobacteria	Yes
EBI4274cc#	Actinobacteria	Yes
EBI4275cc#	Actinobacteria	Yes
EBI4276cc#	Actinobacteria	Yes
EBI4277cc#	Actinobacteria	Yes
EBI4279cc#	Actinobacteria	Yes
EBI4281cc#	Actinobacteria	no
	ID	
EBI345	*Streptomyces*	no
EBI596	*Streptomyces*	yes
EBI391	*Nocardia*	yes
EBI545	*Nocardia*	yes
No EBI Stock #	*Streptomyces coelicolor*	yes
No EBI Stock #	*Corynebacterium xerosis*	yes
No EBI Stock #	*Micrococcus roseus*	no
No EBI Stock #	*Micrococcus luteus (4698)*	yes
No EBI Stock #	*Brevibacterium linens (9172)*	yes
No EBI Stock #	*Mycobacterium phlei*	yes

*BA strains – Biolead strains isolated from Indonesia, ** EA strains – Strains isolated from Ecuador, *** HA strains – Homer Threshner strains (Currently all BA, EA, and HA strains belong to microcide), ****NPS – Nereus Pharmaceutical Strains, # - Soil isolate
EBI – Environmental Biotechnology Institute stock number, sc – isolated on starch-casein agar, cc – isolated on colloidal chitin agar, cy – isolated on czapek yeast agar

Table 2. This table shows a list of all the non-actinobacterial strains used to test the primer pair F243 and 814R. The table gives the strain name and number if available as well as the result of PCR amplification.

Non Actinobacteria	PCR?
Aeromonas hydrophilia (7966)	No
Bacillus subtilis (6633)	No
Bacillus licheniformis	No
Bacillus megaterium	No
Bacillus subtilus (6633)	No
Bacillus aureus	No
Bacillus thuringensis	No
Citrobacter freundii (8090)	yes (light band)
Enterobacter aerogenes (13048)	No
Enterococcus faecalis (19433)	No
E. coli	No
Haloferax vulcanii	No
Klebsiella pneumoniae	No
Lactobacillus arabinosis	No
Lactobacillus acidophilous	No
Proteus mirabalous	No
Proteus vulgaris (13315)	No
Pseudomonas aeruginosa	No
Pseudomonas fluorescens (13525)	No
Serratia marcescebs (13880)	No
Staphylococcus epidermidis (155)	No
Streptococcus faecalis	No
Cal Poly env. Sample 4-401 stock	No
Cal Poly env. Sample 4-711a stock	No
Cal Poly env. Sample 4-711b stock	No
Cal Poly env. Sample 4-710a stock	No
Cal Poly env. Sample 4-410b stock	No
Cal Poly env. Sample 4-402 stock	No
1#	No
2#	No
3#	No
4#	No
5#	No
6#	No
7#	No
8#	No
9#	No
10#	No
11#	No
12#	No
13#	No
14#	No
15#	No
16#	No
17#	No
18#	No
19#	No
20#	No

- Soil isolate

Table 3. This table shows the TRF peaks for different isolates (with both *Taq*I and *Hha*I enzymes) as well as the soil samples from which they were isolated. The table also shows the percent in which each of the soil isolate peaks was present in the community TRF pattern.

Soil Sample #	Isolate (EBI Sample #)	*Hha*I TRF Peak	% Observed In *Hha*I Community TRF	*Taq*I TRF Peak	% Observed In *Taq*I Community TRF
HAC8CS-6-6.0-060701	EBI4190sc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4191sc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4192sc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4194sc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4195sc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4201sc			390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4203cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4205cy			390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4207cy			351, 353	2.1% (352)
HAC8CS-6-6.0-060701	EBI4208cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4209cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4210cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4212cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4213cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4215cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4218cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4219cy			390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4221cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4223cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4224cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4225cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4226cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4227cy	256	5.00%		
HAC8CS-6-6.0-060701	EBI4228cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4229cy	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4245cc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4247cc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4248cc	256	5.00%		
HAC8CS-6-6.0-060701	EBI4250cc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4251cc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4253cc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4254cc	256	5.00%	390,392	15.4% (391)
HAC8CS-6-6.0-060701	EBI4255cc	256	5.00%	390,392	15.4% (391)
HAC8HDW-5-1.0-060701	EBI4153cy	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4154cy	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4155cy	156	5.60%	557	8.1% (557)
HAC8HDW-5-1.0-060701	EBI4156cy	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4157cy	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4159cy			390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4163cy	156	5.60%		
HAC8HDW-5-1.0-060701	EBI4165sc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4166sc	147	20.50%	557	8.1% (557)
HAC8HDW-5-1.0-060701	EBI4168sc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4170sc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4171sc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4177sc	256	1.90%	390,392	12.8% (391)

HAC8HDW-5-1.0-060701	EBI4182sc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4184sc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4185sc	256	1.90%		
HAC8HDW-5-1.0-060701	EBI4187sc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4188sc	256	1.90%	351,353	3.3% (352)
HAC8HDW-5-1.0-060701	EBI4237cc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4238cc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4239cc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4242cc	147	20.50%	557	8.1% (557)
HAC8HDW-5-1.0-060701	EBI4243cc	256	1.90%	390,392	12.8% (391)
HAC8HDW-5-1.0-060701	EBI4244cc	256	1.90%	390,392	12.8% (391)
HAC8MDC-1-1.0-061101	EBI4102sc	454	23.90%	351,353	5.0% (352)
HAC8MDC-1-1.0-061101	EBI4103sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4105sc			390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4108sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4111sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4112sc	454	23.90%	351,353	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4116sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4117sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4118sc	256	4.30%	389,391	10.1% (390)
HAC8MDC-1-1.0-061101	EBI4119sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4120sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4122sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4124sc	256	4.30%		
HAC8MDC-1-1.0-061101	EBI4126sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4127sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4128sc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4130cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4132cy	256	4.30%	351,353	5.0% (352)
HAC8MDC-1-1.0-061101	EBI4133cy	229	6.10%	391,393	28.4%, 2.1% (391,393)
HAC8MDC-1-1.0-061101	EBI4136cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4138cy			557	8.0% (557)
HAC8MDC-1-1.0-061101	EBI4140cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4141cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4142cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4144cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4145cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4146cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4148cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4151cy	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4256cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4257cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4258cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4261cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4263cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4264cc	256	4.30%	351,353	5.0% (352)
HAC8MDC-1-1.0-061101	EBI4265cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4266cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4267cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4268cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4269cc	256	4.30%		
HAC8MDC-1-1.0-061101	EBI4271cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4272cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4274cc	256	4.30%	557	8.0% (557)
HAC8MDC-1-1.0-061101	EBI4275cc	256	4.30%	390,392	28.4 % (391)

HAC8MDC-1-1.0-061101	EBI4276cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4277cc	256	4.30%	390,392	28.4 % (391)
HAC8MDC-1-1.0-061101	EBI4279cc	256	4.30%	390,392	28.4 % (391)
HAC8MDW-5-6.5-061201	EBI4071cy	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4072cy	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4073cy	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4074cy	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4075cy	256	36.90%		
HAC8MDW-5-6.5-061201	EBI4077cy	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4079cy	256	36.90%	557	3.3% (557)
HAC8MDW-5-6.5-061201	EBI4080cy	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4081cy	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4084sc	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4086sc	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4088sc	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4090sc	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4091sc	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4095sc	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4096sc	256	36.90%	390,392	0
HAC8MDW-5-6.5-061201	EBI4100sc	256	36.90%	389,391	42.7% (390)
HAC8MDW-5-6.5-061201	EBI4230cc	256	37%		

All samples in the above table (#3) are soil isolates. EBI – Environmental Biotechnology Institute stock number, sc – isolated on starch-casein agar, cc – isolated on colloidal chitin agar, cy – isolated on czapek yeast agar

Table 4A. Table shows the results of the cloning and sequencing from soil sample O13 Soil ID compA18-9.0-111302.

Clone #	Blast ID	Actino?	% Match	Score (Bits)	E-Value
O13-8	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	94%	817	0
O13-10	Uncultured actinobacterium 16S ribosomal RNA gene, partial sequence	y	99%	1027	0
O13-11	Uncultured Rubrobacteridae bacterium clone glen99_12 16S ribosomal RNA gene	y	94%	773	0
O13-12	Rhizosphere soil bacterium clone RSC-II-86, 16S rRNA gene (partial)	n	89%	579	e-162
O13-14	Uncultured actinobacterium isolate T15 16S ribosomal RNA gene, partial sequence	y	92%	694	0
O13-17	Streptomyces coeruleorubidus partial 16S rRNA gene, strain ISP 5145	y	98%	1033	0
O13-18	Uncultured actinobacterium clone ASc02 16S ribosomal RNA gene, parial sequence	y	95%	841	0
O13-20	Uncultured Verrucomicrobia bacterium YNPRH34A 16S ribosomal RNA gene	n	91%	718	0
O13-22	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	92%	666	0
O13-23	Arthrobacter sp. 12E 16S ribosomal RNA gene, partial sequence	y	98%	961	0
O13-27	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	96%	864	0
O13-28	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	92%	660	0
O13-29	Uncultured actinobacterium 16S ribosomal RNA gene, partial sequence	y	94%	779	0
O13-30	Rhizosphere soil bacterium clone RSC-II-86, 16S rRNA gene (partial)	n	88%	561	e-157
O13-34	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	95%	829	0
O13-35	Streptomyces avermitilis 16S ribosomal RNA gene, complete sequence	y	96%	900	0
O13-37	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	92%	714	0
O13-38	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	94%	813	0
O13-45	Nocardiopsis sp. 10035 16S ribosomal RNA gene, partial sequence	y	92%	720	0
O13-46	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	95%	809	0
O13-47	Bacterium Ellin347 16S ribosomal RNA gene, partial sequence	y	97%	938	0
O13-48	Rhodococcus sp. BDC14 16S ribosomal RNA gene, partial sequence	y	97%	966	0
O13-49	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	93%	731	0
O13-55	Uncultured actinobacterium 16S ribosomal RNA gene, partial sequence	y	94%	829	0
O13-58	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	87%	413	e-112
O13-60	Streptomyces sp. EF-76 16S ribosomal RNA gene, partial sequence	y	91%	545	e-152
O13-62	Uncultured Rubrobacteridae bacterium clone glen99_12 16S ribosomal RNA gene	y	94%	846	0
O13-63	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	93%	767	0

O13-64	Uncultured bacterium partial 16S rRNA gene, clone Gitt-KF-183	y	96%	916	0
O13-70	Arthrobacter sp. 'SMCC G960' 16S ribosomal RNA gene, partial sequence	y	98%	981	0
O13-72	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	97%	969	0
O13-74	Uncultured Rubrobacteridae bacterium clone WYO30C 16S ribosomal RNA gene	y	96%	553	e-154
O13-77	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	96%	880	0
O13-78	Uncultured verrucomicrobium DEV045 partial 16S rRNA gene, clone DEV045	n	91%	765	0
O13-80	Clavibacter michiganensis insidiosum LMG 3663 16S rRNA	y	97%	946	0
O13-82	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	93%	747	0
O13-83	Rhodococcus sp. BDC14 16S ribosomal RNA gene, partial sequence	y	94%	813	0
O13-84	Mycobacterium sp. IP20010664 16S ribosomal RNA gene, partial sequence	y	96%	854	0
O13-87	Kitasatospora sp. IM-7206 16S ribosomal RNA gene, partial sequence	y	92%	690	0
O13-88	Uncultured actinobacterium isolate D35 16S ribosomal RNA gene, partial sequence	y	95%	690	0
O13-89	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	95%	860	0
O13-90	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	92%	751	0
O13-94	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	99%	1068	0
O13-96	Uncultured Rubrobacteridae bacterium clone WYO30C 16S ribosomal RNA gene	y	93%	815	0

Table 4B. Table shows the results of the cloning and sequencing from soil sample O13 Soil ID# compA82-10.0-111102.

Clone #	Blast ID	Actino?	% Match	Score (Bits)	E-Value
O13-1	Uncultured delta proteobacterium partial 16S rRNA gene, clone JG37-AG-113	n	97%	995	0
O13-2	Streptomyces sp. 40005 16S ribosomal RNA gene, partial sequence	y	97%	954	0
O13-3	Saccharothrix longispora 16S ribosomal RNA gene, partial sequence	y	95%	844	0
O13-4	Unidentified actinomycete L43598 16S ribosomal RNA, partial sequence	y	98%	1031	0
O13-5	Agricultural soil bacterium clone SC-I-74, 16S rRNA gene (partial)	n	98%	1063	0
O13-6	Streptomyces sp. LK4-2 16S ribosomal RNA gene, partial sequence	y	99%	1078	0
O13-7	Uncultured earthworm cast bacterium clone c276 16S ribosomal RNA gene	y	98%	1023	0
O13-8	Streptomyces albulus DNA for 16S ribosomal RNA, strain:IMC S-0802	y	98%	1047	0
O13-9	Streptomyces sp. EF-25 16S ribosomal RNA gene, partial sequence	y	99%	1086	0
O13-10	Actinosynnema pretiosum subsp. pretiosum 16S ribosomal RNA gene	y	93%	587	e-165
O13-11	Streptomyces sp. IM-6887 16S ribosomal RNA gene, partial sequence	y	98%	1019	0
O13-12	Agricultural soil bacterium clone SC-I-74, 16S rRNA gene (partial)	n	92%	353	1.00E-94
O13-13	Arthrobacter sp. 12E 16S ribosomal RNA gene, partial sequence	y	97%	940	0
O13-14	Denitrifying bacterium X(A2) 16S ribosomal RNA gene, partial sequence	y	89%	184	4.00E-44
O13-15	Streptomyces sp. YIM 35551 16S ribosomal RNA gene, partial sequence	y	96%	908	0

O13-16	Streptomyces sp. A3 16S ribosomal RNA gene, partial sequence	y	97%	985	0
O13-17	Rhizosphere soil bacterium clone RSC-II-18, 16S rRNA gene (partial)	n	91%	666	0
O13-18	Streptomyces armeniacus gene for 16S rRNA, partial sequence, strain: JCM 3070, clone: 6	y	95%	452	e-124
O13-19	Streptomyces sp. YIM 80244 16S ribosomal RNA gene, partial sequence	y	92%	749	0
O13-20	Mycobacterium sp. 2333 16S ribosomal RNA gene, partial sequence	y	92%	696	0
O13-21	Mycobacterium heckeshornense 16S ribosomal RNA gene, partial sequence	y	91%	696	0
O13-22	Actinomycete species 16S rRNA gene (clone Ep_T1.11)	y	96%	763	0
O13-23	Cellulomonas sp. 16S rRNA gene, strain 1781	y	96%	906	0
O13-24	Streptomyces sp. YIM 80244 16S ribosomal RNA gene, partial sequence	y	89%	522	e-145
O13-25	Curtobacterium sp. VKM Ac-2056 gene for 16S ribosomal RNA, partial sequence	y	95%	817	0
O13-26	Uncultured bacterium clone CDH5 16S ribosomal RNA gene, partial sequence	n	98%	414	e-113
O13-27	Saccharothrix aerocolonigenes gene for 16S ribosomal RNA	y	95%	755	0
O13-28	Nocardia xishanensis 16S ribosomal RNA gene, partial sequence	y	96%	293	2.00E-76
O13-30	Streptomyces griseorubiginosus partial 16S rRNA gene, strain ISP 5469	y	97%	971	0
O13-31	Agricultural soil bacterium clone SC-I-74, 16S rRNA gene (partial)	n	96%	991	0
O13-32	Cryptosporangium arvum 16S rRNA gene, isolate YU 629-21, partial sequence	y	95%	866	0
O13-33	Streptomyces platensis strain SAFN-030 16S ribosomal RNA gene, partial sequence	y	95%	787	0
O13-34	Acidothermus cellulolyticus 16S rRNA, partial	y	97%	983	0
O13-35	Mycobacterium sp. IP20010664 16S ribosomal RNA gene, partial sequence	y	94%	757	0
O13-36	Mycobacterium cookii 16S ribosomal RNA gene, partial sequence	y	96%	801	0
O13-37	P.alni 16S rRNA gene	y	95%	856	0
O13-38	Mycobacterium sp. JLS 16S ribosomal RNA gene, partial sequence	y	98%	1011	0
O13-39	Streptomyces sp. IM-6863 16S ribosomal RNA gene, partial sequence	y	95%	912	0
O13-40	Uncultured earthworm cast bacterium clone c260 16S ribosomal RNA gene, partial seq.	y	97%	912	0
O13-41	Unidentified actinomycete L43598 16S ribosomal RNA, partial sequence	y	98%	1009	0
O13-42	Bacterium Ellin347 16S ribosomal RNA gene, partial sequence	y	98%	1047	0
O13-44	Unidentified bacterium gene for 16S rRNA, partial sequence, strain: IF015706	y	89%	289	2.00E-75
O13-45	Streptomycetaceae 16S rRNA gene, isolate SR 168	y	91%	650	0
O13-46	Streptomyces caviscabies strain SAFR-024 16S ribosomal RNA gene, partial sequence	y	97%	954	0
O13-47	Firmicutes str. ikaite c10 partial 16S rRNA gene, isolate ikaite c10	y	98%	967	0
O13-49	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF2	y	98%	1033	0
O13-50	Bacterium Ellin347 16S ribosomal RNA gene, partial sequence	y	98%	1021	0
O13-51	Streptomyces sp. IM-6438 16S ribosomal RNA gene, partial sequence	y	96%	920	0
O13-52	Uncultured actinobacterium 16S ribosomal RNA gene, partial	y	94%	817	0

	sequence				
O13-53	Uncultured actinobacterium gene for 16S rRNA, partial sequence, clone:APe2_47	y	95%	813	0
O13-55	Amycolatopsis sacchari 16S ribosomal RNA gene, partial sequence	y	95%	858	0
O13-56	Actinokineospora riparia 16S ribosomal RNA gene, partial sequence	y	92%	696	0
O13-57	uncultured bacterium partial 16S rRNA gene, clone Alt9-K62	n	96%	224	4.00E-56
O13-58	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	92%	735	0
O13-59	Mycobacterium austroafricanum isolate VM0573 16S ribosomal RNA gene, complete seq.	y	97%	946	0
O13-60	Mycobacterium cookii strain CIP 105396 16S ribosomal RNA gene, partial sequence	y	96%	581	e-163
O13-61	Actinobispora xinjiangensis 16S ribosomal RNA gene, partial sequence	y	93%	394	e-107
O13-62	Uncultured earthworm cast bacterium clone c256 16S ribosomal RNA gene, partial seq.	y	97%	416	e-114
O13-63	Actinokineospora diospyrosa 16S ribosomal RNA gene, partial sequence	y	95%	856	0
O13-64	Unidentified eubacterium RB24 16S ribosomal RNA gene, partial sequence	n	95%	443	e-122
O13-65	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	95%	900	0
O13-66	Streptomyces sp. S78 16S ribosomal RNA gene, partial sequence	y	96%	936	0
O13-67	Streptomyces tumescens strain OTP-4-2 16S ribosomal RNA gene, complete sequence	y	97%	1001	0
O13-68	Streptomyces sp. 11709 16S ribosomal RNA gene, partial sequence	y	97%	924	0
O13-69	Streptomyces sp. 16S rRNA gene, strain A46R62	y	94%	811	0
O13-70	Uncultured actinobacterium clone ccspost2208 16S ribosomal RNA gene	y	96%	866	0
O13-71	Streptomycetaceae 16S rRNA gene, isolate SR 70	y	97%	985	0
O13-72	Uncultured actinomycete partial 16S rRNA gene, clone AOF41	y	95%	872	0
O13-73	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	95%	892	0
O13-74	Glycomyces rutgersensis partial 16S rRNA gene, strain IMSNU 22074T	y	96%	957	0
O13-75	Bacterium Ellin306 16S ribosomal RNA gene, partial sequence	y	95%	837	0
O13-76	Kitasatospora sp. IM-7206 16S ribosomal RNA gene, partial sequence	y	92%	690	0
O13-77	Uncultured actinobacterium isolate D52 16S ribosomal RNA gene, partial sequence	y	98%	446	e-123
O13-78	Unidentified actinomycete L43597 16S ribosomal RNA, partial sequence	y	95%	862	0
O13-79	Streptomyces sp. LS182 16S ribosomal RNA gene, partial sequence	y	98%	1013	0
O13-80	Streptomyces sp. IM-7307 16S ribosomal RNA gene, partial sequence	y	98%	1011	0
O13-81	Mycobacterium sp. I5 16S ribosomal RNA gene, partial sequence	y	97%	920	0
O13-82	Bacterium Ellin347 16S ribosomal RNA gene, partial sequence	y	97%	944	0
O13-83	Streptomyces caviscabies strain SAFR-024 16S ribosomal RNA gene, partial sequence	y	98%	995	0
O13-84	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF2	y	97%	938	0
O13-85	Mycobacterium cookii 16S ribosomal RNA gene, partial sequence	y	97%	902	0

O13-87	Uncultured bacterium partial 16S rRNA gene, clone Gitt-KF-183	y	95%	797	0
O13-88	Mycobacterium moriokaense 16S rRNA gene, strain DSM 44221T	y	95%	700	0
O13-89	uncultured Actinobacteridae bacterium partial 16S rRNA gene, clone ACF37	y	91%	660	0
O13-90	M.multipartita 16S rRNA gene	y	92%	688	0
O13-91	Uncultured earthworm intestine bacterium clone ew31 16S ribosomal RNA gene	y	94%	783	0
O13-92	Pseudonocardia spinospora partial 16S rRNA gene, isolate LM 141T	y	97%	975	0
O13-93	Mycobacterium phlei 16S ribosomal RNA gene, partial sequence	y	95%	839	0
O13-94	Bacterium Ellin347 16S ribosomal RNA gene, partial sequence	y	97%	940	0
O13-95	Uncultured actinobacterium clone CSb01 16S ribosomal RNA gene, partial sequence	y	96%	789	0
O13-96	Bacterium Ellin347 16S ribosomal RNA gene, partial sequence	y	97%	950	0

Table 4C. Table shows the results of the cloning and sequencing from soil sample Nereus soil ID #13-3-2

Clone #	Blast ID	Actino?	% Match	Score (Bits)	E-Value
NPS-1	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	94%	609	e-171
NPS-2	Uncultured delta proteobacterium partial 16S rRNA gene, clone Hyd89-23	n	92%	700	0
NPS-3	uncultured High G+C Gram-positive bacterium Sva1007 16S rRNA gene, partial	y	96%	823	0
NPS-5	Uncultured gram-positive bacterium TIHP368-02 gene for 16S rRNA, partial sequence	y	98%	827	0
NPS-6	Uncultured delta proteobacterium partial 16S rRNA gene, clone Hyd89-23	n	93%	793	0
NPS-7	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	96%	876	0
NPS-10	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	95%	797	0
NPS-11	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	92%	531	e-148
NPS-12	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	97%	983	0
NPS-13	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	97%	930	0
NPS-14	Unidentified bacterium DNA for 16S rRNA, strain rJ7	y	93%	776	0
NPS-17	Uncultured Verrucomicrobia bacterium clone 12-30 16S ribosomal RNA gene, partial sequence	n	89%	285	9.00E-74
NPS-18	Rhizosphere soil bacterium clone RSC-II-18, 16S rRNA gene (partial)	y	89%	617	e-174
NPS-21	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	96%	856	0
NPS-24	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	96%	696	0
NPS-27	Uncultured bacterium clone pIR3BC03 16S ribosomal RNA gene, partial sequence	y	96%	820	0
NPS-29	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	97%	787	0
NPS-30	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	97%	920	0
NPS-34	Streptomyces purpurascens partial 16S rRNA gene, type strain DSM 40310	y	97%	954	0
NPS-35	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	96%	727	0
NPS-40	Uncultured delta proteobacterium partial 16S rRNA gene, clone Hyd89-23	n	94%	668	0
NPS-42	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	96%	862	0
NPS-43	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	96%	884	0
NPS-48	Uncultured delta proteobacterium partial 16S rRNA gene, clone Hyd89-23	n	93%	525	e-146
NPS-55	Unclassified bacterial species 16S rRNA gene, isolate koll13	y	96%	777	0

NPS-57	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	96%	805	0
NPS-62	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	98%	653	0
NPS-64	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	95%	660	0
NPS-66	Unidentified bacterium DNA for 16S rRNA, strain rJ7	y	94%	809	0
NPS-67	Uncultured gram-positive bacterium TIHP368-02 gene for 16S rRNA, partial sequence	y	96%	795	0
NPS-73	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	96%	890	0
NPS-75	Uncultured bacterium clone pIR3BC03 16S ribosomal RNA gene, partial sequence	y	95%	862	0
NPS-78	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	97%	902	0
NPS-80	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	97%	888	0
NPS-81	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	95%	858	0
NPS-82	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	95%	565	e-158
NPS-84	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	96%	866	0
NPS-90	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	97%	884	0
NPS-91	Uncultured actinobacterium clone SDe09 16S ribosomal RNA gene, partial sequence	y	97%	942	0
NPS-95	uncultured High G+C Gram-positive bacterium Sva0389 16S rRNA gene, partial	y	97%	874	0
NPS-96	Uncultured bacterium partial 16S rRNA gene (clone group A38t)	n	95%	449	e-123