MICROBIAL DIVERSITY OF A FLUIDIZED-BED BIOREACTOR TREATING DIESEL-CONTAMINATED GROUNDWATER (VEGA BAJA, PUERTO RICO)

By

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ABSTRACT

Culture and culture-independent techniques were used to characterize the microbial community structure within a fluidized bed reactor (FBR) used to remediate a diesel-contaminated aquifer. Under normal operating conditions, greater than 98% of total hydrocarbons were constantly removed. Over 25 different cultures were isolated, 92% utilized diesel constituents as carbon source and 20% were denitrifiers. Analysis of 16S rDNA demonstrated ample diversity with most cultures related to the *Proteobacteria* group. In order to better understand the dominant community structure, 16S rDNA clone libraries, Terminal Restriction Fragment Length Polymorphism (T-RFLP), and Functional Gene Microarrays (FGA) were analyzed from total community DNA samples. Clone libraries revealed at 61-days that the community was composed of 75% ß-proteobacteria, 17% γ-proteobacteria and 8% α-proteobacteria while at 212-days was dominated by 77% γ-proteobacteria and 23% of ß-proteobacteria members. T-RFLP and FGA analysis revealed a core community structure with successional changes leading toward higher levels of richness and diversity as indicated by Shannon, Jaccard, and Schao statistical indexes. A total of 270 genes for organic contaminant degradation (including naphthalene, toluene [aerobic and anaerobic], octane, biphenyl, pyrene, xylene, phenanthrene, and benzene); and 333 genes involved in metabolic activities (nitrite and nitrous oxide reductases [*nir*S, *nir*K, and *nos*Z], dissimilatory sulfite reductases [dsr*AB*], potential metal reducing C-type cytochromes, and methane monooxygenase [*pmo*A]) were constantly detected. Genes for the degradation of MTBE, explosives, and chlorinated compounds were also present, indicating the broad catabolic potential of the microbial community present in the FBR unit.

RESUMEN

Técnicas dependientes e independientes de cultivo fueron utilizadas para caracterizar la estructura de la comunidad microbiana en un reactor de lecho fluidizado (FBR, por sus siglas en inglés) empleado en la restauración de un acuífero contaminado con diesel. Bajo condiciones normales, más del 98% de los hidrocarburos totales fueron removidos de manera sostenible. De las 25 poblaciones de bacterias aisladas, el 92% utilizaron componentes de diesel como fuente de carbono mientras el 20% resultaron ser denitrificadores. El análisis del 16S rDNA demostró una amplia diversidad con la mayoría de las poblaciones relacionadas al grupo *Proteobacteria*. Para entender mejor la estructura de la comunidad dominante, muestras de DNA total de la comunidad fueron analizadas mediante genotecas de 16S rDNA, patrones de restricción derivados del fragmemto 16S rDNA terminal (T-RFLP, por sus siglas en inglés), y microarreglos construídos para detectar genes funcionales (FGA, por sus siglas en inglés). Las genotecas de 16S rDNA revelaron que la composición de la comunidad 61-días fue de 75% ß-*proteobacteria*, 17% γ-*proteobacteria* y el 8% α-*proteobacteria*, mientras que a los 212-días la comunidad estuvo dominada por 77% γ-*proteobacteria* y el 23% de los miembros ß-*proteobacteria*. El análisis de T-RFLP y FGA revelaron una estructura pilar en la comunidad con cambios graduales que conducían hacia niveles más altos de riqueza y diversidad según lo indicado por los índices estadísticos de Shannon, Jaccard, y Schao. Un total de 270 genes para la degradación orgánica del contaminante (incluyendo naftalina, tolueno [aerobio y anaerobio], octano, bifenil, pireno, xileno, fenantreno, y benceno); y 333 genes implicados en actividades metabólicas de respiración celular (reductasas de nitrito y de óxido nitroso [*nir*S, *nir*K, y *nos*Z], reductasas disimilatorias de

azufre [*dsr*AB], potencial reductor de citocromos del tipo-C, y monooxigenasas de metano [*pmo*A]) fueron detectados constantemente. Los genes para la degradación de explosivos, MTBE y compuestos clorinados estaban igualmente presentes, indicando el amplio potencial catabólico de la comunidad microbiana presente en la unidad del FBR.

DEDICATORY

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INTRODUCTION

Puerto Rico has abundant groundwater and surface-water resources due to relatively heavy rainfall over the mountainous interior of the island and receptive, sedimentary rocks around the island's periphery. These alluvial and limestone formations form an extensive artesian aquifer system on the north coast. Water-table aquifers overlie the north coast artesian aquifer and occur at shallow depths along most of Puerto Rico's coastline. Man-made reservoirs located on principal water courses collect runoff and are used for water supply, flood control, and limited hydroelectric power generation. Groundwater accounts for about 37% of the total amount of water used in Puerto Rico (Zack and Larsen, 1994).

During the past few decades, the effects of high population density, and the conversion of tropical forest to agricultural, industrial and residential use has significantly impacted the quality and availability of water for island residents. Some of these effects include: the over-utilization water supplies, filling of public-supply reservoirs with sediment, and contamination of surface and groundwaters.

Environmental contamination due to spills and leaks of petroleum hydrocarbons from storage facilities and distribution systems has resulted in the contamination of soil and water environments worldwide. Because of the threat they represent to public health, environmental regulations and the need for the safe use of renewable and non-renewable resources, multiple cleanup strategies for contamination due to petroleum products have been developed (Kamnikar, 1992; Hicks and Caplan, 1993; Weymann, 1995).

Bioremediation is a powerful technical and scientific approach to alternatively deal with contaminated sites. This process involves the use of microorganisms to degrade organic pollutants such as hydrocarbons, to concentrations that are undetectable or below the limits established as safe to all the living organisms and the environment. For groundwater, a pump and treat system is one engineering approach designed for optimum biological operation in a given situation.

Diesel fuel consists of a large variety of hydrocarbons (Appendix 1) that can be degraded either under aerobic or anaerobic conditions (Bregnard *et. al.* 1996). As groundwater pollutants, aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylene are of major concern because of their relatively high water solubility and toxicity. During the last few years, many bacterial cultures have been isolated with the ability to degrade the different diesel constituents under aerobic, anaerobic, denitrifying, iron-reducing and sulfur-reducing conditions (Evans *et.al*., 1991; Rabus *et.al*. 1993; Rabus and Widdel, 1995). For example, Hess and collaborators (1997) characterized bacterial isolates obtained from a diesel fuel-contaminated aquifer (Menziker, Switzerland) which had the capacity to degrade toluene and/or *m*-xylene under denitrifying conditions. Five bacterial strains isolated from aquifer samples were able to grow on toluene while nine strains grew on toluene and *m*-xylene under denitrifying conditions in a laboratory constructed aquifer. Under aerobic conditions, all isolates grew on toluene but none on *m*-xylene. The 16S rRNA-targeted oligonucleotide probes (Azo644 and Azo1251) showed that two of the experimental isolates were closely related to the *Azoarcus tolulyticus* group and *Azoarcus evansii* previously reported as hydrocarbon-degraders (Chee-Sanford *et. al.*, 1996). In laboratory aquifer columns, ß-

Proteobacteria were numerically dominant in both the aerobic zone (80-87%) and in the anaerobic zone (66%). The remaining bacterial groups belonged to the γ-*Proteobacteria* only with 10% and 16% in the aerobic and anaerobic zones respectively.

Bioreactors as a useful tool in environmental restoration

Bioreactors have been commonly developed and implemented for bioremediation processes. The goal of bioreactor treatment stratagies is to optimize degradation by microbial communities in biofilm or suspended systems in artificially constructed units that allow tightly controlled growth conditions. In suspended growth systems, such as activated sludge, or sequencing batch reactors, the contaminated water is circulated in an aeration basin where microbial populations aerobically degrade the organic matter while CO_2, H_2O and new cells are produced as degradation products. The cells form sludge, which are settled out in a clarifier unit, and are then either recycled to the aeration basin or disposed of. In attached growth systems, such as upflow fixed film bioreactors, rotating biological contactors (RBCs), trickling filters, and fluidized bed reactors (FBRs) microorganisms attach to an inert support matrix to promote degradation of water contaminants.

Fluidized bed reactors (FBR) have been in use since the 1970's for the biological treatment of nitrate in wastewater (Sutton and Mishra, 1994). Since this time, FBR systems have been successfully used for the aerobic and anaerobic treatment of a variety of chemicals in waters including petroleum hydrocarbons, pentachlorophenol, and organic chemicals from the pharmaceutical industry (LaPara *et. al.*, 2000). Fluidized bed reactors consist of a reactor vessel containing media (usually sand or activated carbon)

that is colonized by bacterial biomass. This media is "fluidized" by the upward flow of wastewater or groundwater into the vessel, with the lowest density particles moving to the top. A control system is used at the top of the reactor to remove excess biomass, and also control the expanded media bed (Qureshi *et .al*., 2005). When combined with fixed film microbial growth, such strategies have shown effectiveness in the processing of sewage and contaminated groundwater (Sutton and Mishra, 1994; Massol *et. al.*, 1995; Hatzinger *et. al.*, 2000). The microbial populations that colonize such systems can be derived either from the contaminant source zone or from an inoculum of outside organisms. Nutrients are often added to the bioreactors to support the growth of microorganisms and physical parameters are monitored and controlled during the process.

In comparison to conventional mechanically stirred reactors, FBRs provide a much lower attrition rate of solid particles. Thus the level of biocatalyst can be significantly higher and washout limitations of free cell systems can be overcome. In comparison to packed bed reactors, FBRs can be operated with smaller size particles and without the drawbacks of clogging, high liquid pressure drop, channeling and bed compaction. The smaller particle size facilitates higher mass transfer rates and better mixing. The volumetric removal attained in FBRs is usually higher than in stirred tank and packed bed bioreactors (Qureshi *et. al*., 2005).

The surface adhesion of microbes, local growth and exopolymer production in FBRs leads to the formation of robust biofilm communities (Zhou *et. al.*, 1999; Tolker-Nielsen *et. al.*, 2000). The study of biolfilm communities in fluidized bed bioreactors has provided important knowledge for better design and more cost effective operation of

engineered restoration strategies. However a better understanding of these systems, especially in tropical environments, could help devise improved restoration techniques.

Hazinger *et. al*. (2000) conducted a laboratory study with a fluidized bed reactor containing sand as a support media for the treatment of ammonium perchlorate (NH_4ClO_4). Ammonium perchlorate has been used for decades as an oxidizer in solid propellants and explosives. The discharge of effluents from manufacturing plants and from the replacement of outdated fuels in military missiles and rockets has resulted in measurable perchlorate concentrations in groundwater in several states such as California, Utah, Nevada and Texas. Two units were used in those experiments: a pilot-scale system used in the laboratory, and a full-scale system containing granular active carbon as the fluidization media. The two systems were fed with ethanol as the electron donor. Ammonia and phosphate were supplemented to enhance bacterial growth. At the end of treatment, the perchlorate was reduced by 99.9% and effluent levels of perchlorate below acceptable established limits.

Segar *et. al.* (1997) studied the use of fluidized bed reactors for the treatment of chlorinated solvents. They designed two laboratory scale FBRs using sand as the attached media, with some modifications to obtain TCE degradation by the cometabolic activity of mixed cultures of phenol-utilizing microorganisms (*Pseudomonas putida* was the dominant organism). Trichloroethene (TCE) is a common groundwater contaminant, but in the presence of some growth substrates such as aromatic compounds and phenol, aerobic microorganisms can cometabolically transform TCE to CO_2, HCl and water. Their results showed that phenol-utilizing bacteria colonized the FBR's over a period of two weeks with TCE removal efficiencies reaching a maximum of 60% when the inlet

TCE concentration was 100-200 µg/µl. Although the TCE concentration removals were lower in comparison with previous studies, their results were useful to understand the contaminant degradation bioprocess and enhanced the development of new strategies to keep the optimal parameters to be apply in full-scale FBR's in the field.

Microbial community consortiums and hydrocarbon degradation

Numerous studies have shown the effectiveness of microbial biofilms for the biodegradation of a wide range of hydrocarbon contaminants in different environments such as actived sludge, aquifers, soils and extreme habitats.

The use of natural attenuation, or intrinsic bioremediation, as a clean-up method for underground storage tank sites with petroleum-contaminated soil and groundwater has increased over the last few years. Since 1995, natural attenuation has been the most common treatment for contaminated groundwater and the second most common treatment for contaminated soil (Dojka *et. al*., 1998). However, different studies are necessary to demonstrate biological activity at the contaminated site. These include: demonstration of electron acceptor depletion, microcosm studies, growth in situ, the description of the bacterial community at the site, and identification the organisms responsible for the contaminant degradation.

Dojka and collaborators (1998) used culture-independent molecular phylogenetic techniques to describe the microbial community in an aquifer contaminated with jet fuel and chlorinated solvents undergoing intrinsic bioremediation. Their results for the restriction fragment length polymorphism (RFLP) and 16S rDNA clone libraries showed that the majority of the bacterial sequences were phylogenetically associated with 10

recognized divisions (including a wide range of *Proteobacteria*, *Archaea* and recent divisions that are yet to be cultivated). For example, a syntrophic bacterial associations between microorganisms with sequences related to *Syntrophus* and *Methanosaeta* were proposed as important in the main initial step in the degradation of petroleum hydrocarbons in an aquifer. These findings demonstrated how a diversity of interacting microorganisms are actively involved in the attenuation of hydrocarbons in natural environments.

Rooney-Varga *et. al.* (1999), described the composition of microbial communities associated with benzene oxidation under intrinsic *in situ* reducing conditions in a petroleum-contaminated aquifer located in Bemidji, Minnesota. BTEX (benzene/toluene, ethylbenzene/xylene) is rapidly degraded under aerobic conditions, but the low aqueous solubility of oxygen limits it's degradation in groundwater environments. In anaerobic conditions, Fe(III) is the most abundant terminal electron acceptor in aquifers. Degradation of BTEX compounds under Fe(III)-reducing conditions has the potential to be an effective natural attenuation process. Denaturing gradient gel electrophoresis (DGGE) of the 16S rDNA was used to asses the differences in the composition of the microbial communities associated with benzene oxidation under *in-situ* Fe(III) reducing conditions in a petroleum-contaminated aquifer. Comparisons of the DGGE results with the phospolipid fatty acid analysis and most-probable-number PCR enumeration support the possible relation between anaerobic benzene oxidation and the abundance of members of the family *Geobacteraceae* (*Geobacter sp*.). Microbial community analysis by molecular techniques has suggested that a functional role in anaerobic benzene degradation is played by members of the family *Geobacteraceae* under Fe(III)-reducing

conditions (Chakraborty and Coates ,2005). Recently two *Dechlorosomas* strains were isolated from different environments as the first two organisms capable of anaerobic benzene degradation (Chakraborty and Coates, 2005).

Microbial sulfate reduction is another important metabolic activity in petroleum-contaminated aquifers. This process is mediated by a diverse group of microorganisms collectively known as sulfate reducing bacteria (SRB). This microbial group has been found to grow on petroleum constituents such as benzene, toluene, xylenes, naphthalene and others. Kleikemper *et. al*. (2000) assessed the SRB diversity in a PHC-contaminated aquifer in Studen, Switzerland by using macroscopic measurements (carbon source quantification and SO_4^{2-} reduction) and molecular analyses (FISH [Fluorescent in-situ hybridization] and DGGE). Hybridizations with the genus-specific SRB probes showed that all targeted genera were present (*Desulfobulbus sp.*, *Desulfovibrio sp*., *Desulfobacter sp*.) and confirmed the DGGE results. Different carbon sources were consumed by microbial activity while lactate enhanced SO_4^{2-} reduction thus demonstrating the important role of SRB. The combination of macroscopic and molecular techniques complement each other and have provided valuable insights into microbial processes involved in sulfate reducing zone at petroleum-contaminated sites (Rabus *et. al*., 1993, Rabus *et. al*., 1996, Ming-So and Young, 1999).

Kanaly and collaborators (2000) recovered a microbial consortium able to the rapid mineralization of benzo[*a*]pyrene from soil. Diesel fuel was used as a complex mixture of hydrocarbons that helped to make the benzo[*a*]pyrene more accessible for biodegradation. The consortium-degrading tests were combined with the rDNA based DGGE and cloning and phylogenetic analysis. The degrading tests showed that higher

levels of diesel fuel (0.2% wt/vol) promoted 75% mineralization of benzo[*a*]pyrene [10mg/liter] within 3 weeks. The DGGE and sequence analysis showed the dominant consortium members with high molecular weight PAH's and PAH's degraders to be *Sphingomonas, Mycobaterium, Alcaligenes* and *Burkholderia*.

Microbial communities and biofilm development

Studies of a wide variety of natural habitats have shown dominance of attached microbial growth to surfaces, rather than free-floating living organisms (Costerton *et. al*., 1995). Biofilms are complex communities of microorganisms attached to abiotic and biotic surfaces acting as a cooperative consortium. These microbial communities are often composed of multiple species that interact with each other and their environment. The biofilm architecture, particularly the spatial arrangement of cells relative to one another has profound implications for the function of these complex communities (Massol *et. al.*, 1994).

Bacterial biofilms play key roles in the degradation of organic matter including many environmental pollutants. Biofilms play a central role in natural cycles such as the carbon, nitrogen, sulfur and many metals that are indispensable for all the living organisms (Costerton *et. al*., 1995; Kanaly *et. al.*, 2002). There are some ecological advantages for microbes living in biofilms rather than free-living in the environment. Understanding these traits can lead to the improvement of strategies that will prevent contamination and aid in the restoration of polluted environments. When bacteria arranged in biofilms, they form a special matrix of a mixture of proteins, nucleic acids and special polysaccharides that surrounds their community. It functions as a protective

coat from a variety of environmental stresses and might play an important role in the stability of the biofilm on a given surface (Daffoncio *et. al*., 1995; Thaveesri *et. al*., 1995; González-Gil *et. al*., 2001). Biofilms allow improved nutrient flux and metabolic cooperativity within multi-species consortia. Substrates (e.g., hydrocarbons) could be more accessible thus enhancing their degradation rate (Massol *et. al.*, 1995, 1997). Finally, this structure could facilitate acquisition of new genetic traits by horizontal gene transfer, an important process in the evolution and genetic diversification of natural microbial communities (Macnaughton *et. al*, 1999; Davey and Toole, 2000; Rölling, *et. al*., 2002; Top and Springael, 2003).

Numerous new experimental approaches and methodologies have been developed in order to explore metabolic interactions, phylogenetic groupings, and competition among members of biofilms. To complement this broad view of biofilm ecology, individual organisms have been targeted in molecular genetic investigations in order to identify the genes required for biofilm development and to understand the regulatory pathways that control the plankton-to-biofilm transition. These molecular genetic studies have led to the emergence of the concept of biofilm formation as a novel system for the study of bacterial development. The recent explosion in the field of biofilm research has led to exciting progress in the development of new technologies for studying these communities, advancement in our understanding of the ecological significance of surface-attached bacteria, and new insights into the molecular genetic basis of biofilm development.

Characterization of microbial communities by molecular techniques

Changes in the microbial community structure in responce to environmental disturbances are poorly understood. Previously existing methodology in microbial ecology has been dependent on culture dependent methods or lacked the sensitivity to detect changes in response to known biological stressors. New methods that allow detection of changes in microbial community structure and function will provide novel information about the potential of ecosystems to withstand stresses and decompose toxins.

Cultured-based methods are useful for understanding the physiological potential of isolated organisms but do not necessarily provide comprehensive information on the composition of microbial communities. This difference between cultivable and *in-situ* diversity techniques has resulted in difficulties to assess the ecological and environmental significance of cultured members in microbial communities. Recent studies have employed culture-independent molecular techniques to show that cultivated microorganisms from different environments could represent only a small fraction of the microbial community *in situ* (Marsh *et. al*., 2000; Forney *et. al.*, 2004). Most previous work on molecular characterization of microorganisms has been based on the 16S rDNA molecule. The 16S rDNA gene is used for identification and characterization of microbial communities because of it is ubiquity, conserved function, it is easy to sequence, reasonable resolution of different microbial groups, and a large growing database is available for sequence alignment and identification (RDP/ http://rdp.cme.msu.edu/).

The 16S rDNA approach combined with other molecular techniques have a lot of important advantages including the ability to; (i) rapidly evaluate gross similarities and differences within microbial communities, (ii) provide a rapid means of identifying bacterial isolates, (iii) detect and identify those biofilm bacteria that are no longer viable or culturable, and (iv) identify the presence of individual uncultured bacterial species within a complex biofilm community. These advancements have been possible by employing molecular techniques such as gene cloning, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and DNA microarrays to show that cultivated microorganisms from different environments represent only a minor component of the microbial community and attempt to further understand the importance of this non-cultivated component.

Bioremediation as a technical solution for groundwater restoration in Puerto Rico

According to the Environmental Quality Board (EQB), over 800 sites in Puerto Rico are being evaluated due to significant risks of groundwater pollution by leaks of hydrocarbons from underground storage tanks (USTs) and distribution systems. Therefore, cost effective solutions are necessary such as bioremediation strategies.

Many studies had demonstrated the use of different bioprocesses to achieve the goal of organic contaminant removal by enhancing the natural biodegradation potential of natural communities contaminated with petroleum hydrocarbons. In September of 2004, a FBR unit was installed to remediate groundwater contaminated with diesel fuel at the Puerto Rico Energy Power Authority (PREPA, Vega Baja, PR). A biological treatment

unit was designed and constructed for this remediation project by UPRM scientists (Biology Department).

Total community samples from the reactor were routinely collected to perform both cultured-based and culture independent molecular genetic profiles of the microbial community (Marsh *et. al*., 2000; Forney *et. al.*, 2004). Although these analysis are not required for the operation of the treatment unit or regulatory agencies, the information will allow us to better understand the active microbial community and improve system design and operation for future applications. To study bacterial community structure in the fluidized bed reactor used to remediate this site, culture-dependent approaches were complemented with culture-independent techniques to improve our understanding of biofilm communities in tropical environments.

OBJECTIVES

1. To isolate and characterize diesel-degrading bacteria from a fluidized bed treatment unit used to remediate a diesel contaminated aquifer in Puerto Rico.
2. To asses the degree of diversity among the isolated strains by Amplified Ribosomal DNA Restriction Analysis (ARDRA).
3. To establish phylogenetic relationships of diesel degrading cultures by 16S rDNA sequence analysis.
4. To evaluate how the community structure relates to the function of the FBR integrating total community genetic profiling techniques such as 16S rDNA clone libraries, Terminal Restriction Fragment Length Polymorphism (T-RFLP), and Functional Gene Microarrays.

LITERATURE REVIEW

Analysis of microbial communities involve in *in-situ* hydrocarbon biodegradation activities has been a challenge to microbiologists. One major difficulty is that less than 0.1% of the species making up competent degrading communities do not form colonies when cultured in the laboratory (Macnaughton *et. al*., 1999). Nucleic acid-based molecular techniques (DNA and RNA) provide powerful tools for elucidating the microbial ecology of active bioremediation communities. Culture-independent techniques such as 16S rDNA cloning, denaturing gradient gel electrophoresis (DGGE), fluorescent *in-situ* hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP) and DNA microarrays can provide important insights of the microbial community such as viable biomass, community structure (microbial population genotypic profiles), nutritional and physiological status and metabolic activities.

Massol *et. al*. (1997) studied the composition and succession of microbial biofilms in three fluidized-bed reactors (FBR) with a mixture of aromatic hydrocarbons (toluene, benzene, and *p*-xylene (BTX) as feeds. The reactors contained granular activated carbon as the biomass carrier for the development of the microbial biofilm. Twelve different toluene-degrading populations were isolated and characterized by REP-PCR (Louws *et. al.*, 1994; Versalovic *et. al.*, 1994) and ARDRA (Amplified Ribosomal DNA Restriction Analysis) and all of them showed unique patterns. Partial sequencing and phylogenetic analysis showed three dominant strains affiliated to the *Proteobacteria*. The genetic pattern of the isolates was similar to well-known biodegraders. One of the strains was related to *Comamonas testosteroni* in the beta subdivision. The other two

strains were closely related to *Pseudomonas putida* (gamma subdivision), and *Erythrobacter longus*/*Zymomonas mobilis*, respectively. The bacterial community genetic profiles were assessed by ARDRA fingerprints. Comparisons between microbial communities for the three reactors showed that seeded toluene reactors and naturally colonized toluene reactors had the same ARDRA patterns. Similar results were observed for the BTX seeded and naturally colonized BTX reactors, but differences between toluene and BTX fed communities were observed. Based in the finding of a common community among the different treatments over the timecourse of the study, it can be concluded that a core community was selected with a strong potential to biodegrade the hydrocarbons. In addition, alpha, beta and gamma *Proteobacteria* were found to be dominant members and effective competitors for aromatic hydrocarbon degradation.

Fries and collaborators (1997) perfomed a similar study based on the genetic characterization of microbial community composition from an aquifer amended with phenol, toluene, and chlorinated aliphatic hydrocarbons to stimulate trichloroethene (TCE) removal. They focused on microbial succession as a result of injection of different aromatic substrates in the aquifer. The community composition was described by means of 16S rDNA restriction digestions patterns. Their results showed high similarity of band patterns from the original community and communities obtained under different carbon sources supporting the hypothesis that the structure of the community did not suffer major change during treatment. Comparisons between the phylogenetic analysis of ARDRA results from pure isolates and directly from the microbial communities correlated together and showed six dominant hydrocarbon-degrader clusters related to *Comamonas*, *Azoarcus*, *Burkholderia*, an unknown gram-positive groups, *Nocardia* and

Bacillus. These studies demonstrated the utility of ARDRA as an alternative method to evaluate microbial community structure.

It is well known that single species of bacteria are often able to degrade a limited number of contaminants, but that consortium composed of many different bacterial species is usually involved in fuel and oil degradation (Massol *et. al*., 1995, 1997; Bregnard *et. al*., 1996; Da Silva *et. al*., 2004; Ericksson *et. al*., 2004). It had been proved that nutrient level (nitrogen and phosphorus) is a limiting factor essential for microbial growth. To better understand how nutrient levels affect bioremediation progress in the field, Rölling *et. al*. (2002) employed two molecular methods (16S rDNA denaturing gradient gel electrophoresis (DGGE) and clone libraries) to characterize microbial communities in microcosms with different nutrients levels. DGGE patterns showed distinctive band profiles over time within treatments and between treatments, thus demonstrating that variation in the nutrient treatments led to clearly different microbial communities. Communities were highly similar at the beginning of the experiment but then unique microbial communities were selected for each microcosm. To obtain a detailed picture of the diversity of microbial populations 16S rRNA clone libraries were screened by ARDRA. Phylogenetic analysis showed members of the γ-*Proteobacteria* dominating in oil microcosm while 4% N-NH_3 was dominated by α-*Proteobacteria* including *Erythrobacter longus* and *E. citreus* both members of the anoxygenic phototrophic bacteria group. Dominant clones closely related to the alkane-degrading *Alcanivorax*/*Fundibacter* group were found as well. Both molecular methods were effective and led to the conclusion that N and P availability selected different microbial

communities dominated by the organisms most capable of utilizing the inorganic nutrients at the level added to the polluted habitat.

Rölling *et. al*. (2004) characterized the bacterial community dynamics and the hydrocarbon degradation potential during a field scale bioremediation project on a mudflat beach contaminated with buried oil. Bacterial community structure was determined by 16S rRNA DGGE patterns and 16S rRNA clone libraries. Their results showed that treatment with a slow-release of fertilizer, led to rapid changes in the microbial community in comparison with the non-treated reference soil. The phylogenetic analysis for the clone libraries correlated for the non-oil and the only-oil treatments had no major changes in the community profiles. Clones were screened by ARDRA and those more common were sequenced. These dominant clones were closely related to *Pseudomonas stutzeri* and *Alcanivorax borkumensis* with 99.7% and 99.9% similarity, respectively. Sequencing data for the DGGE dominant bands in slow-release fertilizer treatment let to the finding of a DNA fragment which exhibited 96% identity with the gamma-proteobacterium *Idiomarina loihiensis* and another 16S rRNA fragment with 90.2% identity with the sequences of the γ-proteobacterium *Microbulber hydrolyticus* and *Serratia plymythica*.

Terminal restriction fragment length polymorphism (T-RFLP) has also been shown to be an effective tool to discriminate among microbial communities in a wide range of environments. This is a rapid method for finding major differences between communities (Blackwood *et. al*., 2003). T-RFLP is a modern modification of the commonly used ARDRA that relies in the position of restriction sites among sequences and determination of the length of fluorescently labeled terminal restriction fragments by

high-resolution gel electrophoresis on automated DNA sequencers. The automated capillary electrophoresis system permits high throughput sample analysis with high precision for determinating fragments lengths. The data can be compared with data from *in silico* analyses of sequence databases or clone libraries derived from the samples themselves to infer the taxonomic composition of samples (Forney *et. al.*, 2004). It is a highly reproducible method that allows the semi-quantitative analysis of the diversity of a particular gene in a community (Grüntzig *et. al.*, 2002). Various statistical methods, such as similarity indices, hierarchical clustering algorithms and principal-component analyses can be used to analyze T-RFLP data.

As part of the rapid advancement in bioinformatics, Marsh and collaborators (2000) described a web-based research tool for microbial community analysis using terminal restriction fragment length polymorphism (TAP) as a method for 16S ribosomal DNA community characterization. This web-program is located in the Ribosomal Database Project web site (http//www.cme.msu.edu/RDP/html/analyses.html). The tools allow the investigator to address important questions when performing T-RFLP analysis from microbial communities. Important experimental parameters can be examined using the web-program such as: (i) the type of restriction enzyme that provides the most discrimination for estimating population diversity; (ii) the enzyme that will provide the best resolution for the phylogenetic group(s) of interest; and (iii) the optimal primer-enzyme combination for the community under study. TAP provides the investigators a novel strategy to stimulate T-RFLP with the entire RDP as the surrogate community, to access the most recent release of the RDP prokaryote database with names and

sequences, and the option of performing multiple single digests or a single multiple enzyme digests.

Frequently, small-subunit (SSU) rRNA genes are used as phylogenetic markers, and T-RFLP assay results useful to investigate a broad range of different lineages in natural ecosystems including *Bacteria*, *Eukarya* and *Archaea*. However, functional genes can also be used in T-RFLP as molecular markers to specifically target functional guilds of microorganism (Lueders *et. al.*, 2003). Functional gene markers encode enzymes for metabolic pathways in particular allowing an affiliation of microorganisms detected to their function in the environment. As a result T-RFLP techniques can perform community profiles for functional genes such as: mercury-resistance, nitrogen fixation, denitrification, and methanogenesis.

Lueders and Friedrich (2003) used T-RFLP's for *mrc*A genes present in a known mixture of methanogenic cultures to assess the efficiency of the technique. The bacterial community consisted of four genomic DNAs of pure cultures mixed: *Methanobacterium bryantii*, *Methanosaeta concilii*, *Methanospirillum hungaeti* and *Methanococcus jannaschii*. The SSU rRNA genes for this community were also amplified and processed. The results showed that variations in the annealing temperature for the SSU rRNA genes did not affect the T-RFLP profiles. In contrast annealing temperature variations in the amplification of *mrc*A genes in the community resulted in a dramatic effect in the T-RFLP's amplicon patterns. This result could be found because of the use of highly degenerate primers often used for functional marker genes to cover a wide phylogenetic range. T-RFLPs thus proved to be a powerful technique to determine archaeal template

ratios, but also elucidated some PCR bias problems to be considered in this kind of experiments.

Fennell *et. al*. (2004) performed a T-RFLP analysis of reverse-transcribed 16S rRNA for the detection and characterization of dehalogenating microorganisms in a sulfidogenic 2-bromophenol (2-BP) enrichment. Their research aimed to identify organisms capable of dehalogenation, a critical step for developing appropriate methods for site-specific treatment. A coculture was developed from an original sulfidogenic enrichment and use to isolate dehalogenating bacteria with various substrates. T-RFLP fingerprints for the communities revealed approximately 12 restriction fragments representing the more abundant bacteria present in the consortium for the different treatments. The banding patterns for the different treatments showed unique peaks when compared with the original enrichment, suggesting selection for what were minor populations in the original enrichment. A good correlation was found when T-RFLP results were compared with phylogenetic sequence analysis of a clone library. The most interesting finding was that the 16S rRNA sequence for the most relevant T-RFLP fragment was also the most abundant clone (2-BP-48) in the library being 97% similar with a dehalogenating-representative of the genus *Desulfovibrio* (*D. gracilis*).

Pérez-Jiménez and Kerkhof (2005) analyzed how sulfate-reducing bacterial (SRB) communities and their hydrocarbon biodegradation potential were distributed globally. Terminal restriction fragment length polymorphisms of the dissimilatory sulfite reductase genes (dsr*AB*) were performed. Samples from different locations included United States (California, New Jersey, New York, and Virginia), South Korea, Italy, Latvia, Venezuela, and Puerto Rico. T-RFLP results showed that the majority of

fragments were found in the western hemisphere (73.8%) and in temperate climates (51.3%) when compared with the eastern and tropical climates (26.2% and 48.7%, respectively). Ninety-four of 369 TRF's were associated with dsr*AB* genes in the GenBank database, indicating that <20% of the dsr*AB* genes from the worldwide SRB community reside in the GenBank database. Hydrocarbon biodegradation potential was also widely distributed between the different sampling locations as deliniated by means of the T-RFLP patterns and phylogenetic analysis of clone libraries for toluene, benzene, phenanthrene, naphthalene and alkane degradation. The presence of these genes demonstrates the worldwide potential for mineralization of petroleum hydrocarbons.

Recent advances in molecular techniques have also resulted in the development of DNA microarray technology. This method is now routinely used to analyze gene expression in pure cultures or tissue samples for different organisms. Adapting this novel technique for use in environmental studies has been a great challenge in molecular biology. Recently, various formats of environmental microarrays have been proposed, developed and evaluated for species detection and microbial community analyses in complex environments (Zhou, 2003). Microarrays can contain thousands to hundreds of thousands of probe targets. Many studies indicate that microarrays technologies have a great potential as specific, sensitive, quantitative and high throughput tool for microbial detection, identification and characterization directly from natural environments (Schadt, *et. al*., 2005).

Several types of microarrays have been successfully applied to study microbial communities. These arrays can be divided to at least five categories based on the genes represented: (i) phylogenetic oligonucleotide arrays (POA's) are designed based on a

conserved marker such as 16S rRNA gene to compare the relatedness of communities in different environments; (ii) community genome arrays (CGA's) contain the whole genomic DNA of cultured organisms and describe a community based on its relationship to these cultivated organisms; (iii) metagenomics arrays (MGA's) contain probes produced directly from environmental DNA itself and is applied with no knowledge of the community; (iv) whole-genome open reading frame arrays (WGA's) contain probes for all the open reading frames in one or multiple genomes and are used for comparative or functional genomic analyses; and finally, (v) functional gene arrays (FGA's) are designed to encompass the diversity of key functional genes involved in biological processes such as carbon, nitrogen and sulfur cycles and provide information about the microbial populations controlling these processes (Schadt *et. al*., 2005).

The genes encoding functional enzymes involved in biogeochemical cycles such as nitrogen, carbon and sulfur in bioremediation processes are useful signatures to monitoring the potential activities and physiological status of microbial populations and communities that drive these processes in the environment. Functional gene arrays (FGA's) are primarily used for analysis of microbial community samples in the environment. This kind of arrays contains synthetic oligonucleotides that are designed and synthesized based on sequence information from public databases. The developed microarrays can be used to obtain a broad profile of the differences or similarities between the functional capabilities of any given microbial community.

Wu and collaborators (2001) first developed a prototype FGA to examine the potential specificity, sensitivity, and quantitative nature of microarray hybridization data for use with environmental community samples. The array contained 100 functional

genes encoding enzymes for important ecosystem processes including denitrification, nitrification and methane oxidation. Community DNA from marine sediments and surface soil samples were hybridized with the functional gene arrays (FGA's). Strong signals above the background were obtained for both samples. The hybridization signals for the genes *nir*S and *nir*K were more abundant as expected because most of the functional genes in the array came from marine sediment environments. These probes also hybridized well with the community DNA from soil samples. Variations in relevant parameters such as hybridization temperature, hybridization solution volume, low and high stringency in the washings steps using different concentrations of the buffers, and DNA target concentrations were evaluated using the arrays. In conclusion, all the parameters listed above are critical when performing microarrays. The optimized protocol for this study with a reduced hybridization solution volume of 2 µl allowed for successful detection of genes from only 1ng of pure genomic DNA and 25 ng of bulk community DNA from soil.

Taroncher *et. al*. (2003), further described the development and optimization of a DNA microarray methods to detect and quantify functional gene in the environment. Two 70-mer synthetic oligonucleotide probe arrays were constructed: one containing probes from previously known functional genes representing denitrification, nitrogen fixation, and ammonia oxidation; and the other one which utilized *in-vitro* amplified DNA sequences of nitrite reductase genes (*nir*S) obtained from estuarine sediments (Taroncher-Oldenbrug *et.al*., 2003). The *nir*S microarray was constructed to assess the community composition in terms of the *nir*S diversity between two sediments samples from a river station in Choptank River, (Chesapeake Bay, Maryland). Community DNA

from both samples were labeled with Cy5 dye and hybridized with both arrays. The hybridizations showed good results in terms of detection and target concentration for the two samples. A different distribution for the *nir*S gene was observed with lower signals in the mid-river station comparing with the up-river station spot patterns. These results correlated completely with those expected because the genes printed in the arrays came from a clone library of the up-river station samples.

Rhee *et. al*. (2004) used bulk community DNA from an aromatic hydrocarbon and some heavy metals contaminated soil to further evaluate the potential of FGA hybridization for environmental analysis. Clustering analyses revealed main groups of genes such as: naphthalene degradation, and anaerobic benzoate degradation genes were different between the contaminated samples. The results showed clearly the gene profiles expected for the samples contaminated with aromatic compounds and the ones contaminated only with BTEX. It was clearly proved that probe hybridizations were representative of the microbial-gene diversity involved in the biodegradation of the contaminants. The developed functional gene array clearly has potential as demonstrated from such studies as general tool for monitoring the composition, structure, activity, and dynamic of microbial populations involved in biodegradation and metal resistance across environments.

Tiquia *et. al* (2004), also evaluated a 50-mer oligonucleotide-based functional gene arrays for potential application in environmental samples. This array targeted genes involved in nitrification, denitrification, nitrogen fixation, methane oxidation, and sulfate reduction. The microarray was constructed with 763 genes involved in the biochemical

cycles mentioned above (*nir*S, *nir*K, *nif*H, *amo*A, *pmo*A, drs*AB*) from public databases and their own collection in the Environmental Sciences Division at Oak Ridge National Laboratory, TN. To prove the sensitivity of the array when applying to environmental samples, a mixture of DNA from pure cultures was hybridized. The signal intensity was significantly higher than the background level with a detection limit of 60 ng of DNA in the presence of non-target DNA's. Later to evaluate the detection potential of these arrays for microbial populations in the environment, 5 µg of community DNA from a marine sediment sample was tested. The results showed that hybridizations were achievable with the environmental DNA from highly diverse environments. Strong signals were obtained for some nitrogenases (*nir*H), dissimilatory sulfite reductases (dsr*AB*), ammonia monooxygenases (*amo*A), methane monooxygenases (*pmo*A), and nitrite reductases (*nir*SK). This study suggested that the oligonucleotide microarray technology allowed successful detection of dominant populations involved in the different biogeochemical processes occurring in unenriched environmental samples.

One key point to consider when selecting functional genes for the fabrication of microarrays is the vast differences in available sequences data for various genes in a given pathway. They concluded that an ideal candidate gene for a FGA: (i) encodes a critical enzyme or protein in the process of interest; (ii) is evolutionary conserved but at the same time has enough sequence divergence in different microorganisms to allow probe design for individual species; and (iii) has substantial sequence data from isolates and environmental samples available in public databases (Schadt *et. al*., 2005).

Methé *et. al*. (2005) demonstrated how DNA microarrays can provide insights into environmental relevant processes such as nitrogen fixation and growth with Fe(III)

as an electron acceptor. The microarray consisted of 3,417 uniques PCR products representing coding sequences in *Geobacter sulfurreducens*. *G. sulfurreducens* was grown under strictly nitrogen-fixation conditions and Fe(III) reduction conditions to get a better knowledge about it's physiology and the genetic transcription profile. The arrays confirmed previous information (Holmes *et. al.*, 2004) about Fe(III) reduction and nitrogen fixation. Increase in transcriptional levels during growth on Fe(III) of a gene region coding for metal efflux genes and a putative *c*-type cytochrome shown to have roles in metal homeostasis and energy metabolism were observed. This cytochrome is unique to the *Geobacteraceae* suggesting that the mechanism of Fe(III) reduction in *Geobacter spp*. may be different in comparison to other prokaryotes. In addition, 30% of the genes with significant changes in transcription levels during Fe(III) reduction conditions lack homology to other prokaryotes or had unknown function, suggesting that *G. sulfurreducens* had differences in it's metal reduction physiology. It was clearly demonstrated that microarrays can provide reliable information on gene expression in a particular organism and within a natural environment.

Do to rapid advances in the printing technology of microarray slides, a main limitations for FGA's is the availability of cultures and sequence data and methods for the array construction (Schadt *et. al.*, 2005). The largest FGA published to date contained 1,662 probes for genes involved in the carbon, nitrogen, and sulfur cycles, organic contaminant degradation and metal resistance, but this FGA has been recently expanded to over 24,000 probes as described by Schadt and co-workers. This study used the most advance FGA to evaluate functional diversity within degradative communities in the bioreactor (Schadt *et. al.*, 2005).

METHODOLOGY

Site description and treatment unit. Approximately 20,800 cubic yards of soil and groundwater were contaminated by a diesel spill (approx. 45,500 L) from a storage tank split at the Hydro Gas Station in Vega Baja, Puerto Rico in 1992. A 5-liter working volume polyvinyl chloride column reactor (15 cm diameter x 274 cm long) was used in this study (Figure 1). Contaminated groundwater was pumped simultaneously from multiple extraction wells to a 3,000 L equalization tank. After collection, the contaminated groundwater was passively supplemented with ORC® (3-7 mg/L) and 0.1 g/L of a nutrient solution (NH_4Cl and KH_2PO_4) at a ratio of 30:5:1 (carbon: nitrogen: phosphorous) prior to the reactor's inlet port. The bioreactor was normally operated as a discontinuous one-pass up-flow batch system without recycle at a flow rate of 3.8 liter per min for nearly 7 months. About 11 kg (dry-weight) granular activated carbon (GAC) (Calgon Filtrasorb 300, Calgon Company, Pittsburgh, PA) with a geometric mean diameter of 0.9 mm and an overall density of 0.48 g/cc was added to the treatment column as adsorbent/biomass carrier. After treatment, the effluent was collected in one of two retention tanks and stored until chemical analysis were performed to certify removal of hydrocarbons to cleanup standards. A private laboratory, Al Chem Inc. (San Juan, PR) was responsible for the analyses. If cleanup goals were achieved, the treated water containing traces of nutrients and microorganisms were reinjected into the aquifer to accelerate *in situ* remediation of the site. To enhance the startup phase of the bioreactor, a mixed-culture from the site was grown in minimum culture media (Bushnell-Hass) amended with free diesel product from the site as the sole carbon source. Indigenous populations capable of diesel degradation under aerobic and denitrifying

conditions were selected and inoculated into the bioreactor (2.5-L). In general, two or three batches were treated on a weekly basis. To evaluate the reactor's performance, water samples from the influent and effluent sampling ports were collected for chemical analysis including dissolved oxygen, pH, temperature, electrical conductivity, turbidity (HORIBA U-10 Water Quality Checker/HACH Portable turbidimeter Model 2100P) and uptake of total petroleum hydrocarbons (UVF-3100, Site Lab, CO).

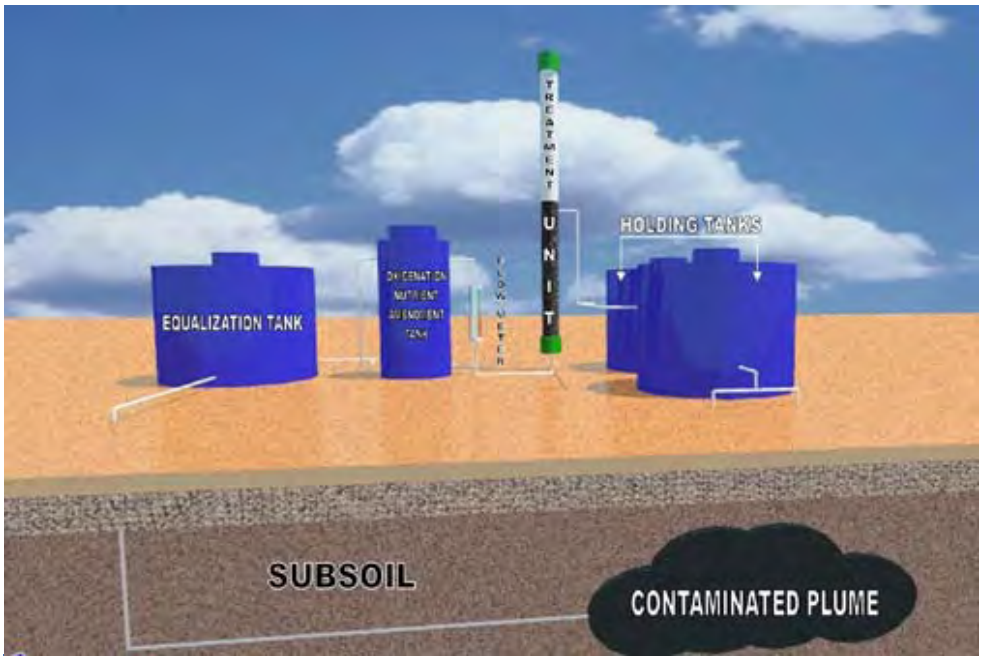


Figure 1. Schematic diagram of the fluidized bed reactor unit treating a diesel-contaminated aquifer in Vega Baja, Puerto Rico.

Isolation of diesel-growing bacteria. Biofilm samples were collected aseptically from the bottom 30% of the column sampling port every month for the duration of reactor operation. Cells were removed and homogenized as previously described (Massol *et. al.*, 1997). Approximately one gram of biological activated carbon (BAC) was diluted 1/100 in a sterile saline solution (0.85% NaCl). Culture enrichments were prepared by inoculating 0.1 ml of the diluted samples in 500 ml flasks containing Bushnell Hass

medium. Diesel [10 mg/L] was added to the liquid phase as the sole source of carbon. The bottles were incubated in a shaker at room temperature (25°C) for 7 days. After the incubation period, 1 ml of the enrichment culture was submitted to serial dilutions (10^{-2} to 10^{-5}) and incubated at room temperature in plates containing R2A medium (Difco, Detroit, Mich.). Discrete colonies were isolated and further purified by repetitive plating in R2A. The isolates strains were characterized based on their phenotypic and biochemical features.

***BAC bacterial enumeration*.** Cells were removed from the GAC monthly samples and homogenized by extracting approximately 1 gram of sample in 99 ml of cell extraction buffer (0.001M EDTA, 0.0004M Tween 20, 0.01% peptone, 0.007% yeast extract, 1.3mM sodium chloride, 100mM sodium phosphate [Massol-Deyá *et. al*., 1995]) for 5 minutes in a rotary shaker at 300 rpm. This extraction protocol resulted in a higher efficiency for viable cell recovery. Viable bacterial numbers were detected in duplicates by using R2A medium which was designed to improved recovery of environmental heterotrophs. R2A plates were incubated at 30°C for 7 days before estimating the most probable number (MPN).

***Phenetic and biochemical characterization of pure bacterial isolates*.** Cultures isolated from the treatment unit were examined for the color, texture and shape of their colonies in R2A medium. Their morphology and gram reaction were evaluated by light microscopy. To determine the ability to reduce nitrate (NO_3) to nitrites (NO_2) or beyond the molecular nitrogen stage (N_2), the isolates were grown in Nitrate Broth (0.1% KNO_3,

peptone and yeast extract) and incubated at 37°C for 24-48 hrs. The nitrate colorimetric test was done following conventional microbiology techniques to confirmed nitrate reduction capacity of isolates (Capuccino and Sherman, 1992). The diesel utilization potential for each isolate was determined as follows: (i) cells were grown on R2A plates, washed and resuspended in phosphate buffer, and transferred to sterile tubes containing minimum media with a thin layer of diesel fuel (Bushnell-Hass/Diesel [10 ml/L], (ii) the inoculated glass tubes were sealed and incubated in a rotary shaker for 5 days, and (iii) growth was measured daily by optical density using the HACK spectrophotometer (DR/4000U model) at 660 nm. A semiquatitative growth scale was assigned to the isolates relative to the non-diesel control tube.

DNA fingerprinting of bacterial isolates. Isolates representing populations capable of using diesel as a sole carbon source were further characterized by partial 16S rRNA gene sequence analyses and amplified ribosomal DNA restriction analysis (ARDRA).

DNA was extracted from biomass material collected by centrifugation. Lyses were performed using 25% Sucrose TE buffer, [5 mg/ml] lyzozyme, 0.25M EDTA, 10% SDS, and [10 mg/ml] Proteinase K. The DNA was precipitated using 5M Sodium Chloride, 8M Potassium Acetate and 95% cold ethanol. Finally, the DNA was recovered and desalted using 70% cold ethanol and resuspended in 50 µl of TE Buffer, pH 8.0. DNA concentrations were estimated by spectrophotometric measurements at 260nm and 280nm. The 260/280 and 260/230 ratios were calculated to assured the range of purity of the DNA samples prior to the amplification of the 16S rDNA gene.

The bacterial DNA was used as template for the amplification of the 16S ribosomal DNA gene using the following universal primers: forward primer 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1392R (5'-ACGGGCGGTGTGTACA-3'). The total volume for each PCR reaction was 50 µl using 5 µl of 10X *Taq* polymerase reaction buffer B, 6.0 µl of 25mM $MgCl_2$, 1.0 µl of dNTP's mix [2.5mM (1:1:1:1:1 proportion)], 1.0 µl of each 16S rDNA primer [50 pmol/µl] and 0.5 µl (2.5U) of *Taq* DNA polymerase (Promega®), 100 ng of DNA and ddH_2O. Amplification was performed using a Perkin Elmer Gene Amp PCR System 2400. The cycling parameters were: denaturation at 95°C for 1 minute, followed by 35 cycles of melting at 94°C for 1 minute, annealing at 52°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 7 minutes. For ARDRA, aliquots of 12 µl of each PCR products were digested separately with *Hae*III, *Rsa*I and *Hinf*I. Each reaction was prepared by adding 1.5 µl of 10X Reaction buffer, 1.0 µl of ddH_20 and 0.5 µl (1.0U) of each enzyme to the amplified products for a total volume of 15 µl per reaction incubated at 37°C for 3 hours. A 3% Metaphor (1X TAE buffer) agarose gel was used for analyzing the resulting DNA fragments. After gel electrophoresis, image and cluster analysis were performed using the pGEM DNA molecular marker and well-characterized hydrocarbons-degrader strains isolated from the same treatment unit for the restoration of a gasoline-contaminated groundwater using the computer programs Gel Pro Analyzer 3.1 and SYSTAT®9.0.

Sequence analysis of the BAC isolates. A 900 bp 16S rDNA gene product was obtained from the pure BAC cultures using the primers UNIV 519F (5'-CAGCMGCCGCGGTAATWC-3') and the reverse universal primer UNIV 1392R (5'-ACGGGCGGTGTGTRC-3'). A total of 50 µl of PCR reaction was prepared as followed: 5.0 µl of 10X *Taq* polymerase buffer B, 6.0 µl of 25mM $MgCl_2$, 1.0 µl of dNTP's mix [2.5mM each (1:1:1:1 proportion)], 0.75 µl of [20 mg/ml] BSA, 1.0 µl of each primer [50pM/µl], 0,5 µl of *Taq* polymerase enzyme (2.5U) (Promega®), the ddH_20 volume was adjusted by the amount of DNA template (100 ng) added. The PCR cycling parameters were: denaturation at 94°C for 5 minutes followed by 35 cycles of denature at 94°C, annealing at 56.9°C, extension at 72°C, and a final extension at 72°C for 10 minutes in a Perkin Elmer Gene Amp PCR System 2400. A total of 50 ng/µl of each PCR product was used to prepare the samples which wrere delivered for single-strand sequencing using the 519F-forward primer to MacroGen Company facilities in Korea (http://www.macrogen.com) following their specifications. The sequences were analyzed using BLAST (http://www.ncbi.nih.gov/BLAST/) to get a preliminary identification of the strains. The sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/) of the European Bioinformatics Institute (EMBL-EBI) and the BioEdit Sequence Alignment Editor software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The cluster analysis was performed using the PHYLIP 3.65 software package.

Total DNA extraction. Five grams of activated carbon will be used for total community DNA extraction for the following samples (30 days, 61 days, 153 days and 212 days of treatment operation) following a protocol for soils and sediments described previously by Zhou *et*. *al*. (1996).

Cloning of 16S rDNA PCR products from the BAC bacterial communities. 16S rDNA clone libraries of the microbial community samples for 61 and 212 days of the bioreactor treatment operation were constructed. A 16S rDNA fragment of 900 bp was amplified from the community DNA. The oligonucleotides used for the PCR reaction were the forward universal primer UNIV 519F (5'-CAGCMGCCGCGGTAATWC-3') and the reverse universal primer UNIV 1392R (5'-ACGGGCGGTGTGTRC-3'). A total of 50 µl of PCR reaction was prepared as followed: 5.0 µl of 10X *Taq* polymerase buffer B, 6.0 µl of 25mM $MgCl_2$, 1.0 µl of dNTP's mix [2.5mM each (1:1:1:1 proportion)], 0.75 µl of [20 mg/ml] BSA, 1.0 µl of each primer [50pM/µl], 0,5 µl of *Taq* polymerase enzyme (2.5U) (Promega®), the ddH_20 volume was adjusted by the amount of DNA template (100 ng) added. The PCR cycling parameters were: denaturation at 94°C for 5 minutes followed by 35 cycles of denature at 94°C, annealing at 56.9°C, extension at 72°C, and a final extension at 72°C for 10 minutes in a Perkin Elmer Gene Amp PCR System 2400. The amplified products were purified as described in the Wizard® SV Gel and PCR Clean-up system (Promega®) and stored at -20°C until cloning step.

The 900 bp 16S rDNA products were cloned following the manufacturer protocol manual of the pGEM® T-Vector System (Promega®). A total of 50 clones of each library were randomly selected for further analysis. Clones were grown in 3 ml of Luria broth

with [50 mg/ml] of Ampicilin and incubated at 37°C in a rotary shaker at 120 rpm overnight. Plasmid mini preps were performed using the Wizard® Plus SV Minipreps DNA purification system (Promega®) and stored at -20°C until sequencing. The DNA concentrations were estimated as described previously. Dilutions were done as specified in the sequencing preparation protocol of MacroGen Inc., Korea (www.macrogen.com). A total of 15 µl dilutions with concentrations of 100 ng/µl were done for each plasmid preparation. A forward UNIV-519F primer dilution was prepared to 10 µl with concentration of 5 pmole/µl for every 5 samples. Each clone sequences were analyzed using BLAST (http://www.ncbi.nih.gov/BLAST/) for preliminary identity results. The sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/) of the European Bioinformatics Institute (EMBL-EBI) and the BioEdit Sequence Alignment Editor software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Cluster analysis was performed using the PHYLIP 3.65 software package to compare the clone libraries.

Diversity indices and statistical analyses of the 16S clone libraries were done. The Shannon-Weiner index (H), Simpson's reciprocal index (1/D), richness (S), the number of phylotypes, evenness (EH), Jackard index, SChao, SAce were calculated using the following programs: DOTUR: Distance based OTU and richness determination software (www.plantpath.wisc.edu/fac/joh/dotur.html), and ASLO (Limnology and Oceanography: Methods/ http://www.aslo.org/lomethods/free/2004/0114.pdf). Web-LIBSHUFF was used to determine whether the clone libraries were significantly different from one another by comparing coverage curves of libraries to each first in an X/Y fasion and then the reverse Y/X (http://libshuff.mib.uga.edu).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) of the BAC communities.

A total of 100 ng of the bioreactor's community DNA samples were used as template for 16S rDNA PCR reaction as described in the improved protocol for T-RFLP by capillary electrophoresis (Grüntzig *et. al*., 2002) with some modifications.

The primers used for the amplification were: the labeled-forward primer 519F-IR700 (5'-CAGC(AC)GCCGCGGTAAT(AT)C-3') and the reverse primer 1392R (5'-ACGGGCGGTGTGTACA-3'). The PCR reactions was carried in a total volume of 50 µl using 5.0 µl of 10X *Taq* polymerase reaction buffer B, 6.0 µl of 25mM $MgCl_2$, 1.0 µl of dNTP's mix [2.5mM each in a 1:1:1:1 proportion], 1.0 µl of each 16S rDNA primer [50pM/µl], 0.5 µl of [10 mg/ml] BSA, 0.5 µl of *Taq* polymerase enzyme (2.5U) and 34 µl of ddH_2O adjusted by the amount of DNA template added. The cycling parameters were: denaturation at 94°C for 5 minute, followed by 35 cycles of melting at 94°C for 1 minute, annealing at 56.9°C for 1 minute, extension at 72°C for 3 minutes and a final extension at 72°C for 10 minutes using a Perkin Elmer Gene Amp PCR System 2400.

The PCR products were purified as described in the protocol of Wizard SV Gel and PCR clean-up system of Promega®. The purified samples were run in a 1.0% agarose gel (1X TAE) with 1 µg of Lambda *Hind*III molecular marker to estimate DNA concentrations prior to the restriction enzyme digestions.

A total of 200 ng of each labeled 16S rDNA products were used for restriction digestions separately with the following enzymes: *Hae*III, *Rsa*I, and *Msp*I (Promega®). Each digestion reaction consisted of 2.0 µl of 10X Reaction buffer, 5 units of each restriction enzyme and the ddH_2O volume was adjusted by the amount of PCR product

added for a total reaction volume of 20 µl. The digestions were incubated at 37°C in a water bath for 4 hours followed by 10 minutes at 65°C to inactive the enzymes. Samples were processed using the LI-COR Biosciences NEN®DNA Analyzer Model 4300 (LI-COR Inc.).

Aliquots of each digestion were prepared by mixing with an equal amount of IR2 stop solution (LI-COR Biosciences) in a 1:1 proportion. A 5.5% acrylamide gel (acrylamide gel matrix KB^{Plus}-LICOR) of 0.25 mm of thickness was prepared with 150 µl of 10% ammonium per-sulfate and 20 ml of 15% TEMED with a polymerization time of 1 hour and 30 minutes. The samples were denatured at 94ºC for 3 minutes and kept at 4ºC until loading the gel. A pre-run step for 20 minutes was performed using TBE 1X buffer (KB^{Plus}-LICOR) with the following parameters: voltage 1,500 (V), current 40 (mA), and a power of 40 (W). After the pre-run, one microliter of each denatured sample was loaded in the gel using the molecular sizing standard 50-700 bp (KB^{Plus}-LICOR). The samples were run for 3 hours and 30 minutes with the same pre-run parameters.

The T-RFLP fingerprints were collected in a TIF image and analyzed using the Gel Pro Analyzer 3.1 software. Changes in the microbial community of the bioreactor were analyzed using the number and proportion of peaks observed in the T-RFLP electropherograms for each sample with the different enzymes as the main comparative criteria. The similarity analysis between samples was performed using EstimateS Win7.5.0 software (http://purl.oclc.org/estimates).

Functional Gene Arrays of the biofilm communities. Functional gene microarrays (FGA's) were done in the facilities of the Environmental Sciences Division, in Oak Ridge National Laboratory, Tennessee under the supervision of Dr. Jizhong Zhou and Dr. Christopher W. Schadt. The array slides contained more than 21,000 experimental genes probes including genes for the main biogeochemical cycles, organic compound degradation and metal resistance as well as numerous replicated control probes including conserved 16s rDNA and several human genes that can be spiked in to the hybridizations.

Five grams of activated carbon were used for total community DNA extraction following a protocol for soils and sediments (Zhou *et. al*., 1996). DNA was cleaned following extraction using the Sephacel Purification of DNA from environmental samples protocol (Ogram *et. al.*, 1987). DNA samples were further purified as specified by Promega Wizard DNA clean-up Kit system using a Vacuum Manifold. DNA pellets were eluted in 50 µl of 10mM Tris-HCl pH 8.5 (pre-warmed at 65°C) and followed by a desalting procedure. The DNA concentrations and purity were measured using 1 µl of the samples in a Nanodrop 24.7 instrument.

Whole Community Genome Amplification (WCGA) by the Rolling Circle Amplification (RCA) was done in triplicate following the protocol described in the TempliPhi 500 Amplification Kit (Amersham Biosciences) with 10-30 ng/µl of template DNA as modified at ORNL (Wu *et. al*., unpublished). One microliter of each DNA was dispensed in 10 µl of sample buffer (containing random hexamers) and incubated at room temperature for 10 minutes. A master mix containing 10 µl of reaction buffer (contains salts and deoxynucleotides), 1 µl of enzyme mixture [1 U/µl] (contains *phi* 29 DNA polymerase and random primers in 50% glycerol), and additionally 1 µl of SSB [2.67

µg/µl] (single strand binding protein) and 1 µl of 1mM of spermidine were added for each reaction. A positive control DNA plasmid mixture SP5-10 and a negative control just ddH_2O were used. The reactions were incubated 3 hours at 30°C followed by an enzyme inactivation step at 65°C for 10 minutes on an ABI 9700 thermocycler (Applied Biosystems). One microliter of the amplification reactions was run in a 0.6% agarose gel (1X TAE) to visualize the results.

The amplified community DNA was then labeled with Cy5 dUTP by random priming. To each RCA product, 20 µl of 3mM random hexamers and 0.3 µl of 10mM spermidine were added. Samples were denatured at 99.9°C for 5 minutes and immediately chilled on ice. A total of 20 µl of the labeling master mix containing 2.5 µl of dNTP's mix [2.5mM dTTP, 5mM dACG-TP], 1.0 µl of Cy5-dUTP fluorochrome, 0.7 µl of 490 ng/µl of recA, 2.0 µl of Klenow enzyme and 13.8 µl of MilliQ ddH_2O were added to each reaction tube. The samples were incubated at 37°C for 6 hours in an ABI 9700 thermocycler (Applied Biosystem). The labeled-target DNA was purified using the a QIAquick PCR purification Kit (QIAGEN), dried for one hour in a Thermo Savant SPD 1010 SpeedVac System and stored at -20°C until hybridization.

The FGA's microarrays slides were cross-linked using a UV Stratalinker 1800 at 6,000 µjoules x 100 of energy and pre-hybridization and hybridization steps were performed as described in the manufacturer Ultra GAPS™ Coated Slides instruction manual (Corning Life Sciences) with some modifications. The slides were pre-hybridized for one hour at 50°C with 100 ml of the buffer (50% formamide, 5X SSC, 0.1% SDS, 0.1 mg/ml BSA [bovine serum albumine] and ddH_2O, final concentrations). The dried-labeled samples were resuspended in 40 µl of freshly prepared hybridization

solution (final concentrations: 50% formamide, 5X SSC, 0.10% SDS, 0.1 µg/µl of Salmon Sperm DNA [10 µg/µl], 1mM spermidine and ddH_2O). The probes solutions were incubated at 95°C for 5 minutes and kept at 60°C on a ABI 9700 thermocycler (Applied Biosystems) prior to hybridization. Hybridization was performed at 50°C overnight. The array slides were washed three times with buffer I (pre-warmed at 50°C/Final concentrations: 1X SSC, 0.1% SDS) for 5 minutes in continuous shaking. Two additional washes were done with buffer II (Final concentrations: 0.1X SSC, 0.1% SDS) at room temperature for 10 minutes followed by four washes with buffer III (0.1X SSC, final concentrations) by 1 minute. Finally the slides were dried by centrifugation and stored in dark until scanning.

The microarrays were scanned with a Scan Array Express Microarray Scanner (Perkin Elmer) at a resolution of 10µm. The laser power and photomultiplier tube (PMT) gain were 100% and 85% respectively. The 16-bit TIFF files were quantified using the ImaGene 6.0 Premium program (www.biodiscovery.com). Local background measurements were subtracted for each spot. The poor quality spots were flagged and removed from the data set and further analysis. The signal-noise ratio (SNR) was also computed for each spot to discriminate true signals from noise. The SNR was calculated with the following equation: SNR = Signal mean-Background mean/ Background standard deviation. A SNR ≥ 3 was used as an additional criterion for the minimum signal determination and spots with lower SNRs were removed from the data set. Statistical analysis was done using the Microsoft Excel 2003 program. Cluster analysis of the data was performed with the Gene Cluster and TreeView 1.60 programs (http://rana.stanford.edu/software). Similarities between BAC community samples were

performed using the statistical software EstimateS Win 7.5.0 (http://purl.oclc.org/estimates).

RESULTS

Fluidized bed bioreactor

A pump and treat remediation strategy was implemented for seven months to remediate the diesel contaminated plume (approx. 45,360 L). Through either attachment or agglomeration, a fixed-film developed on the porous activated carbon media resulting in a biofilm community. At a flow rate of 3.7 L/min, the biofilm was composed of over 10^7-10^8 total cultivable cells per gram of activated carbon. The TPH level varied considerably for each treated batch. However, removal efficiency of total petroleum hydrocarbon (TPH) achieved a sustainable 98% of the total applied organic load with a 15 to 20 min hydraulic retention time. When hydrocarbons levels at the inlet port surpassed 1,000 ppm, one additional recycle step was required to obtain concentrations below 50 ppm. In general, high removal of total hydrocarbons was achieved within the first ten days after the startup phase. Bioaugmentation of the treatment column with a diesel-degrading consortium grown from local soil samples could have contributed to the early colonization of the GAC and success of the remediation process.

A summary of the physicochemical parameters at the bioreactor's influent and effluent sampling ports during groundwater treatment are presented in Table 1. In general, the pH of the bioreactor maintained neutral levels throughout the treatment process. Stable pH levels (7.58 ± 0.22) and tropical temperatures ($26.8 \pm 1.8^{o}C$) perhaps provided advantageous conditions for microbial growth and hydrocarbon degradation. Concurrent with the biofilm development onto the BAC, a drop in aerobic plate counts was observed from 10^7 CFU/gGAC to 10^5 CFU/gGAC. In addition to oxygen, both nitrate and sulfate (alternative electron acceptors) were consumed, indicating that the

microbial community was capable of utilizing organic compounds without additional oxygen amendments. Low oxygen concentration in the effluent, a high electron acceptor demand and alternative electron acceptors consumption within the treatment phase suggest a strong link between microbial activity and anaerobic respiration. The addition of nutrients to an equalization tank prior to treatment provided the necessary nitrogen and phosphorous sources required for growth at the various treatment components as the groundwater appeared to be deficient for these elements. Both, phosphorus and nitrate uptake in the reactor were high while nitrite was not produced in the treatment unit. Excess or low consumption of ammonium is also indicative of dissimilatory nitrate utilization. Nitrate uptake increased progressively in the system with the highest observed value after 153 days of operation (net consumption of 12.1 mg/L). Sulfate uptake increased from 9.7 mg/L in 181 days to 16.5 mg/L by 212 days of operation.

Table 1. Summary of physical and chemical parameters observed during the operation of the treatment unit.

[1]**Parameter**	***Influent (days)***			***Effluent (days)***		
	61	***153***	***212***	***61***	***153***	***212***
Dissolved Oxygen (mg/L)	6.58	3.06	3.60	-	1.42	1.57
Turbidity (NTU)	66	446	150	15	999	21
$N\text{-}NH_3$ (mg/L)	1.35	4.80	0.13	1.56	6.60	0.27
$N\text{-}NO_3$ (mg/L)	6.7	19.9	0.8	4.8	7.8	0.6
$S\text{-}SO_4$ (mg/L)	-	16.7	18.2	-	7.0	1.7

[1]Average; n= 3; -, Not Available.

Characterization of BAC Microbial Communities

The composition, structure and stability of microbial populations in BAC biofilm communities were examined using both culture-dependent and molecular techniques. Characterization of the microbial populations indicates that the biofilm community was composed of at least 26 different coexisting bacterial groups (Appendix 2). Based on their morphology and Gram staining, 46% of the isolates were gram-negative rods, 34% gram-positive rods, 12% gram-negative cocci, and 8% gram-negative diplobacillus (Figure 2). Approximately 20% of the isolates were capable of complete denitrification to N_2 gas while 92% used diesel as their carbon source for growth.Robust growth was observed for the 46% of the positive diesel-degrading populations (strains DIESVBS1, DIESVBS7, DIESVBS8, DIESVBS9, DIESVBS11, DIESVBS16, DIESVBO1, DIESVBO3, DIESVBN1, DIESVBN2, and DIESVBN5). Other cultures were capable of growth in this media at a lower rate. The strain DIESVBO1 showed a very distinctive growth utilizing the diesel as a carbon source in a period time of two days and producing a “biosurfactant-like-substance” which turned the media to a white coloration. Biosurfactants are microbially produced surface-active compounds with both hydrophilic and hydrophobic regions which give them the property of aggregate with fluids such as water and hydrocarbons (Fietcher, 1992; Lin, 1996).

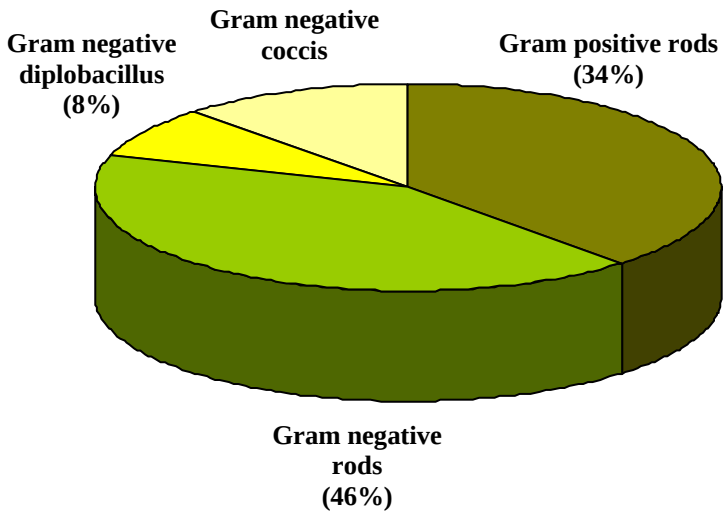
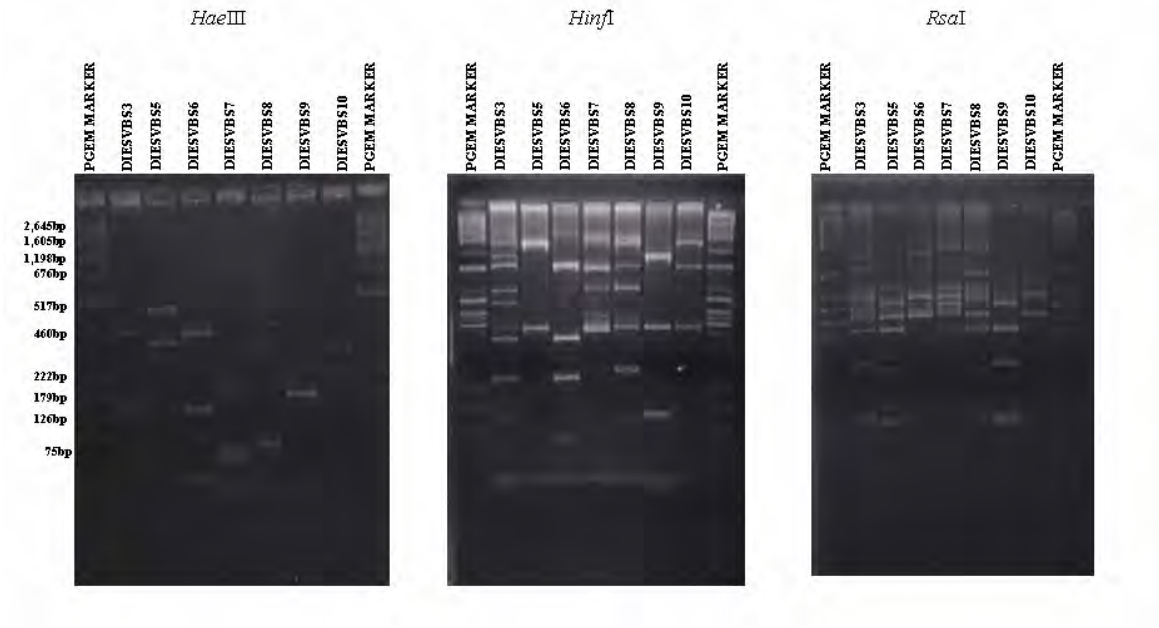


Figure 2. Distribution of BAC microbial isolates strains based on their morphology and Gram staining reaction.

The genotypic diversity of bacterial populations was analyzed by means of ARDRA. A 1,400bp fragment was amplified using the universal primers 8F and 1392R which is consistent with the pre-established size of other bacterial 16S rRNA genes (Woese, 1987; Gürtler and Stanisich, 1996). In general, enzyme digestions of the 16S rDNA products resulted in restriction patterns constituted by 2 to 7 bands with molecular size ranging between 75 to 1,100 bp for enzymes *Hae*III, *Hinf*I, and *Rsa*I. Figure 3 shows distinct profiles among the isolates.



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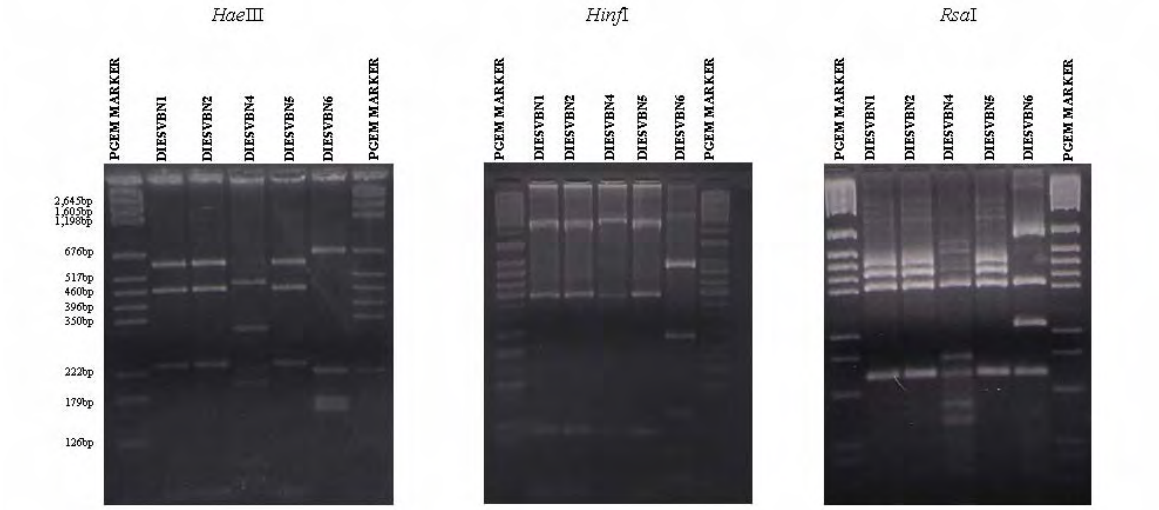

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Figure 3. Genotypic ARDRA profiles of isolated strains. Differences between isolates can be observed for the enzymes *Hae*III, *Hinf* I, and *Rsa*I. (A) Strains from the BAC unit after 30 days of operation and, (B) Strains isolated after 90 days of treatment.

Cluster analysis by means of ARDRA profiles revealed a great level of diversity when the isolates were compared with well-characterized hydrocarbon degraders isolated from the same treatment unit previously used for the restoration of a gasoline-contaminated aquifer (Figure 4). Only a few strains were similar to the gasoline-degraders. Furthermore, the isolated strains result in at least 19 genetically different groups as showed by their ARDRA restriction profiles. This observation demonstrated the degree of genetic heterogeneity of the biofilm community.

Based on ARDRA profiles, a total of sixteen (16) bacterial populations were further characterized by their partial 16S rDNA sequences (which constituted 77% of the BAC isolated strains). A 900 bp fragment was amplified using the universal primers 519F and 1392R. This analysis generated sequences with a molecular size between 768 to 830bp representing the 85-92% of the amplified product length. Similarity coefficients values higher than 0.7 were obtained for the 75% of the bacterial isolates. The sequence identity analysis was performed using the RDP database with NCBI related type strains as showed in Table 2. The phylogenetic analysis was done using the PHYLIP 3.65 software (Figure 5).

In general, the strains were phylogenetically related to three bacterial divisions: *Bacilli*, *Actinobacteria* and *Proteobacteria*. The *Proteobacteria* was represented by 11 bacterial strains distributed within the *alpha*, *beta* and *gamma* subdivisions. The *Proteobacteria* is composed by a representative strains which had been well-described as petroleum hydrocarbon-degrading species (Dojka *et. al.*, 1998; Rölling *et. al.*, 2001; Watanabe *et. al.*, 2000).

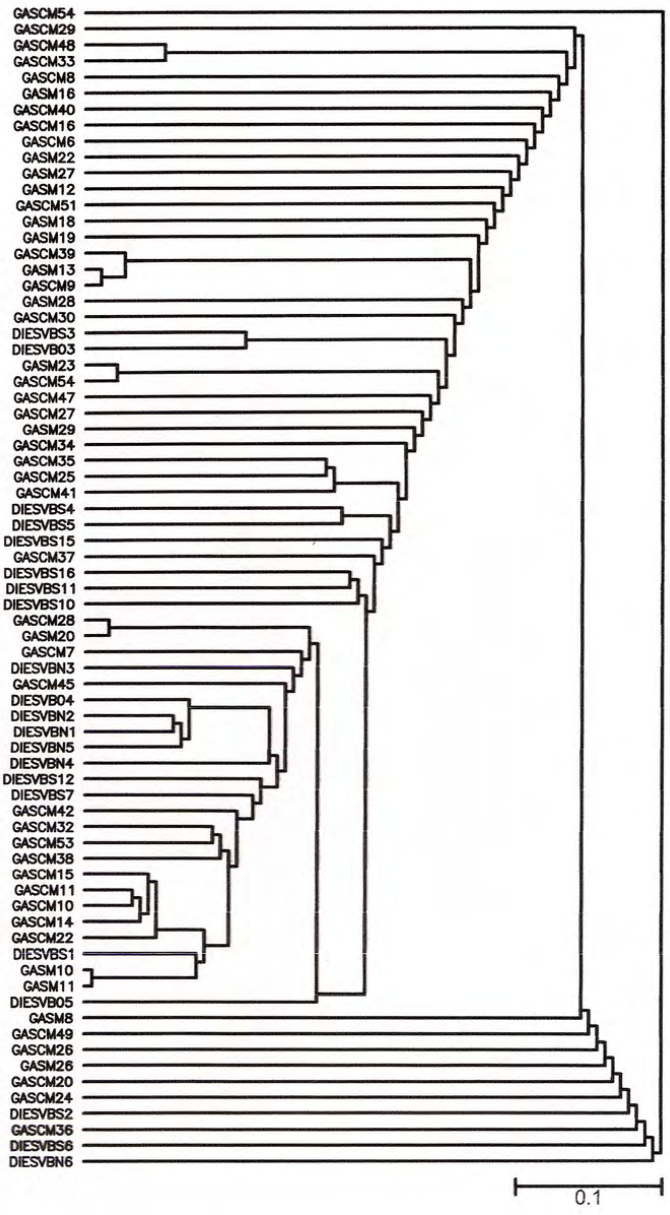


Figure 4. Cluster analysis of bacterial cultures by means of ARDRA. DIESVB series correspond to isolates obtained from the BAC unit in this study while GAS series correspond to cultures obtained in 2002 from the same treatment unit at a gasoline impacted site (Mayagüez, PR).

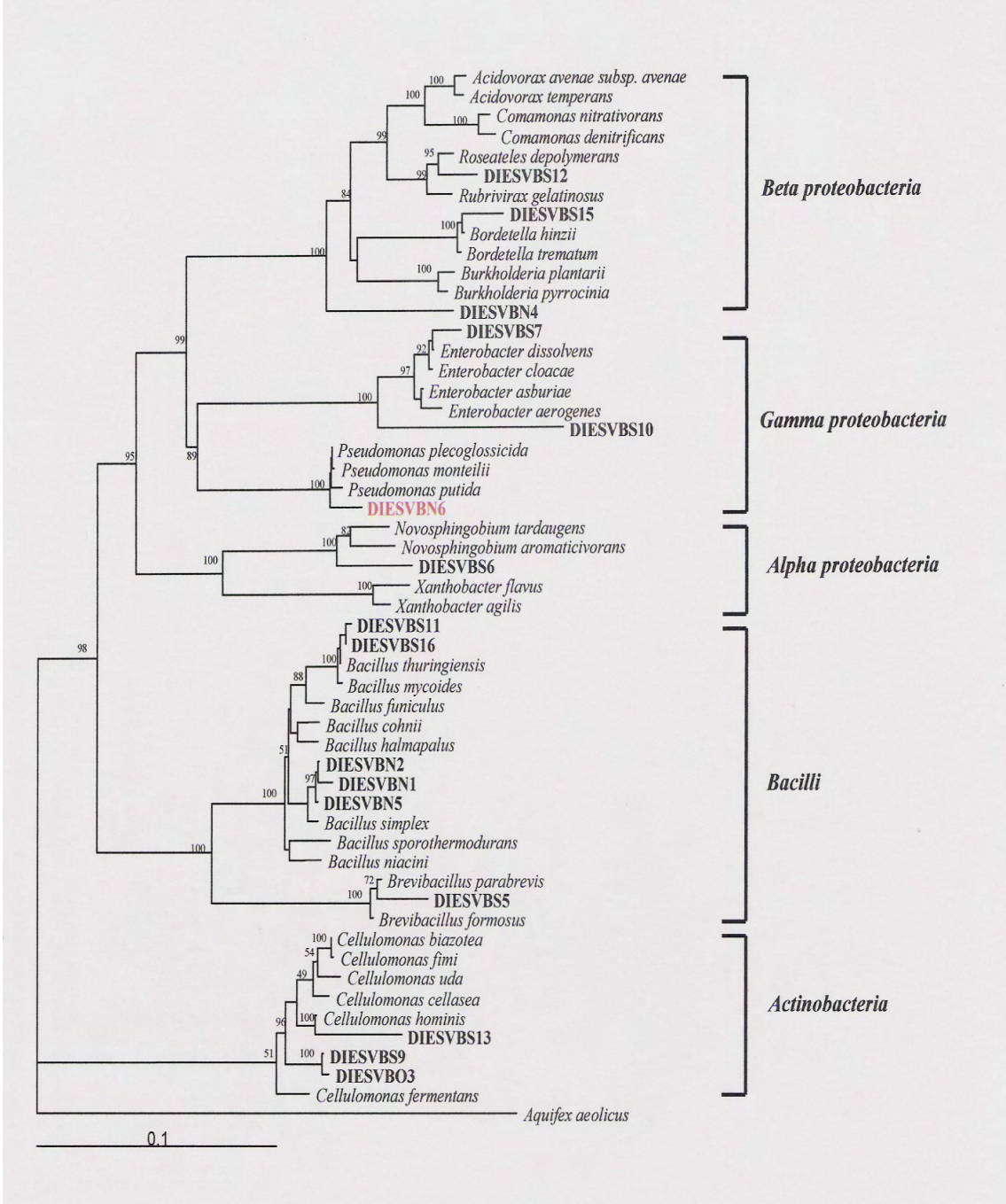


Figure 5. Phylogeny analysis of BAC strains based on partial 16S rDNA sequence analysis (Bootstraps values ≥ 45 are shown/ *Aquifex aeolicus*-Outgroup). .Strain DIESVBN6 (red color) can not grow on diesel as sole added carbon source.

Table 2. Analysis of partial 16S rDNA sequences for BAC isolated strains with diesel growth potential as a sole carbon source.

Strain ID	***Fragment length (bp)***	***[1]NCBI related type strain***	***[2]Sab***	***Phylogenetic Division***
DIESVBS5	815	*Brevibacillus parabrevis*	0.842	*Bacilli*
DIESVBS6	817	*Novosphingobium tardaugens*	0.810	α-*Proteobacteria*
DIESVBS7	810	*Enterobacter dissolvens*	0.937	γ-*Proteobacteria*
DIESVBS9	808	*Cellulomonas uda*	0.832	*Actinobacteria*
DIESVBS10	809	*Enterobacter asburiae*	0.616	γ-*Proteobacteria*
DIESVBS11	831	*Bacillus thuringiensis*	0.958	*Bacilli*
DIESVBS12	812	*Roseateles depolymerans*	0.894	ß-*Proteobacteria*
DIESVBS13	792	*Cellulomonas hominis*	0.785	*Actinobacteria*
DIESVBS15	821	*Bortedella hinzii*	0.901	ß-*Proteobacteria*
DIESVBS16	819	*Bacillus mycoides*	0.946	*Bacilli*
DIESVBO3	806	*Cellulomonas fermentans*	0.850	*Actinobacteria*
DIESVBN1	819	*Bacillus simplex*	0.943	*Bacilli*
DIESVBN2	830	*Bacillus simplex*	0.929	*Bacilli*
DIESVBN4	818	*Dechlorosoma sp.* PCC	0.949	ß-*Proteobacteria*
DIESVBN5	811	*Bacillus simplex*	0.947	*Bacilli*
DIESVBN6	800	*Pseudomonas plecoglossicida*	0.970	γ-*Proteobacteria*

[1] NCBI, National Center of Biotechnological Information
[2] Sab, Similarity Coefficient

Clone libraries of 16S rDNAs from BAC bacterial samples

In order to better understand the composition of the dominant microbial community present in the treatment unit, two 16S rDNA clone libraries were constructed for BAC-61 and BAC-212 samples. A 900bp gene product was amplified using the primers 519F and 1392R and a total of 50 clones were isolated for each library. Twenty four (24) clones of the gene library corresponding to sample BAC-61 days were partially sequenced. For the second library, a total of 43 clones were sequenced. In order to obtain a preliminary identification of each clone, the sequences were analyzed using the NCBI BLAST tool (Appendix 4 and 5) while phylogenetic analysis were performed using the software package PHYLIP 3.65 (Figures 6 and 7).

Five distinctive clusters resulted from the sample BAC-61. Cluster I was constituted by 17 clones (CO2, CO3, CO4, CO7, CO8, CO9, CO10, CO11, CO17, CO19, CO20, CO23, CO28, CO32, CO39, CO40, CO58) being the broader and most frequent member of the community. Cluster II was represented by CO31, cluster III (CO25 and CO55), cluster IV (CO60, CO33, CO22), and cluster V by CO21. Typically, clones were phylogenetically related to uncultured bacteriums with different degradation and metabolic potentials. The related-uncultured sequences were obtained from different hydrocarbon-contaminated sites such as lakes, aquifers and soils. The metabolic capacities of the related bacterium were diverse including polycyclic aromatic hydrocarbon degrading-bacteria, nitrogen-fixation, multiple metal resistance, ammonia and nitrite-oxidixing bacteria, as well as purpur-sulfur bacteria.

The second clone library BAC-212 days resulted in five main clusters as well. Cluster I was constituted by 18 clones (CM10, CM11, CM12, CM13, CM17, CM23,

CM24, CM31, CM35, CM36, CM41, CM55, CM68, CM69, CM72, CM94, CM95) as the dominant population of the community; cluster II (CM76); cluster III (CM91); cluster IV (CM4, CM25, CM32, CM33, CM40, CM42, CM48, CM49, CM83, CM93), and cluster V by CM34. A phylogenetic analysis revealed similarities to a metabolically diverse group of uncultured bacterium. The related-bacteria were isolated from hydrocarbon-contaminated sites as observed in the BAC-61 days community. The metabolic activities of these related bacterium includes: degradation of polycyclic aromatic hydrocarbon, methane-oxidation, manganese oxidixing bacteria, purple sulfur bacteria, denitrifier and falcultative anaerobic bacteria.

In order to evaluate the taxonomic diversity of the gene libraries, all the clone sequences were analyzed using the RDP database sequence match tool comparing with the NCBI type strains (Figures 6 and 7). The results showed that the BAC-61 days community was composed of 75% ß-proteobacteria representatives, 17% γ-proteobacteria and 8% α-proteobacteria. In contrast, the dominant community observed in BAC-212 days was composed of 77% γ-proteobacteria, 23% of ß-proteobacteria while α-proteobacteria was undetected.

The phylogenetic analysis for both communities showed similarities in the degrading potential of the established biofilm. As mentioned before, in the initial stages of treatment operation the number of aerobic plate counts was high, but decreased thus indicating a shift of the microbial community toward a more anaerobic-dominated structure. Changes in the community structure were observed by a shift of abundant aerobic degrader clones in the BAC-61 days to the presence of a high number of anaerobic and facultative-degrader clones in the BAC-212 days. Although a shift in the

microbial structure occurred, gene libraries revealed the community maintained efficient hydrocarbon degradation potential through 7 months of restoration process.

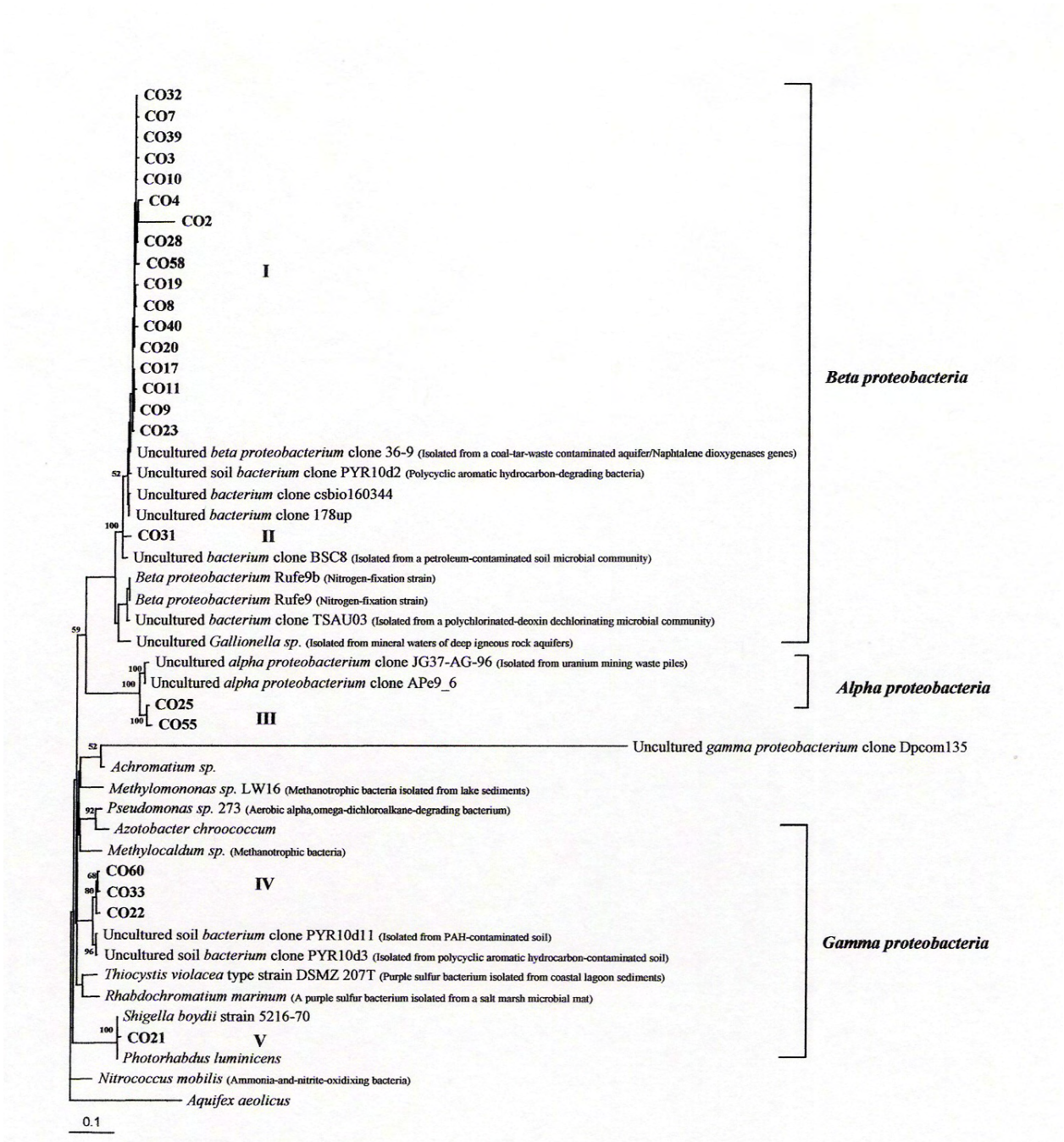


Figure 6. Phylogenetic analysis of the 16S rDNA clone library representing the microbial community composition after 61 days of treatment (Bootstraps values ≥ 45 are shown/ *Aquifex aeolicus*-Outgroup).

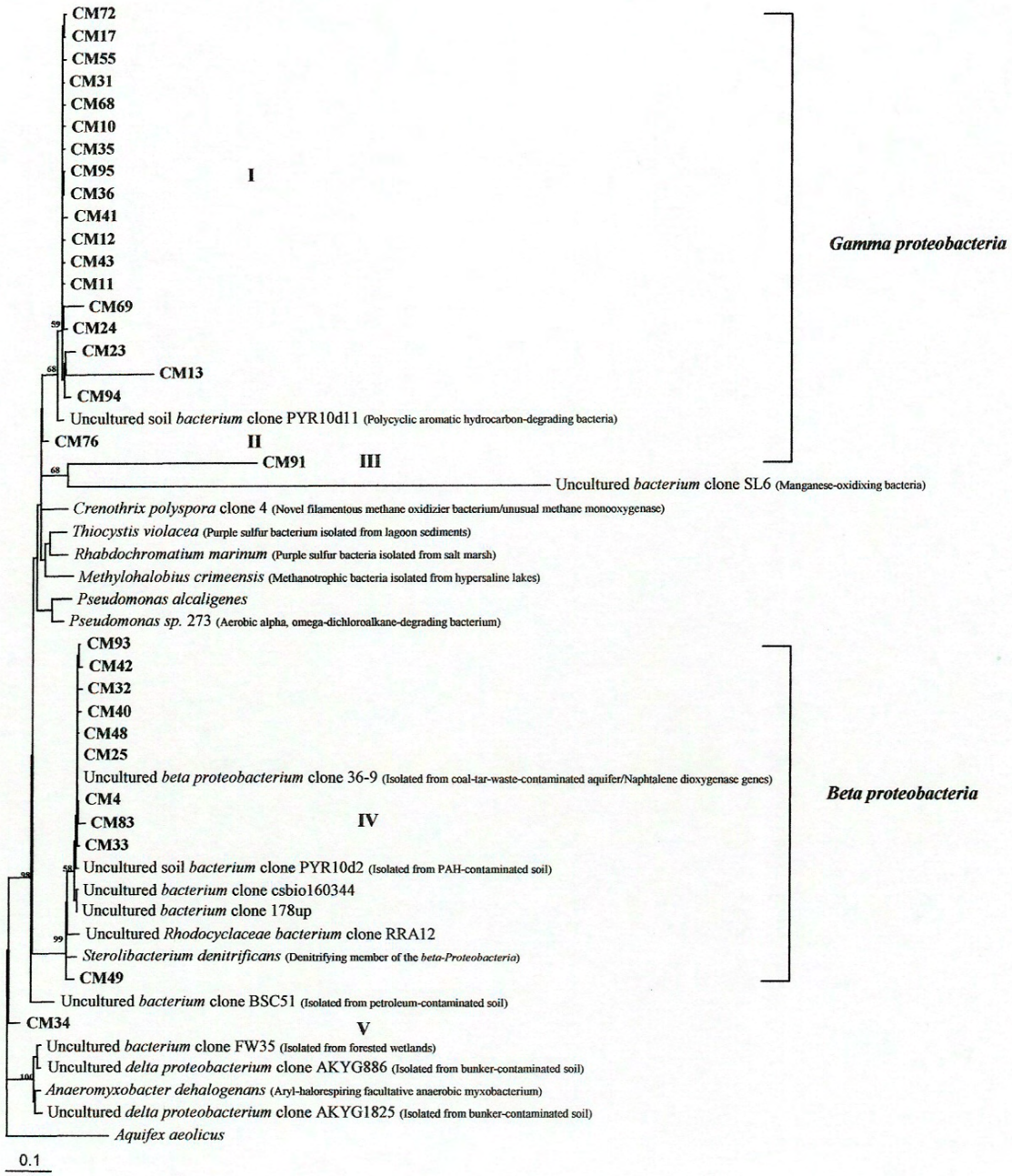


Figure 7. Phylogenetic analysis of the 16S rDNA clone library representing the microbial community composition after 212 days of treatment (Bootstraps values ≥ 45 are shown/ *Aquifex aeolicus*- Outgroup).

Statistical indices for both gene libraries are showed in Table 4. The unique distance to define an OTU for both libraries was 0.03 or 97% of similarity (Dunbar, J., 2004; Singleton *et. al*., 2004; Stout and Nusslein, 2005) between sampled clones. Richness analysis showed greater diversity for community BAC-212 days with 16 different operational taxonomic units (OTU's) while the BAC-61 days community had only 6 different OTU's. The Shannon and Simpson's diversity indices were calculated. Both Shannon and Simpson's indexes showed that clone library BAC-212 had higher diversity than BAC-61. The Jaccard, SAce, and SChao richness index values were calculated in order to corroborate richness between samples. All indexes indicate that library BAC-212 had the highest level of richness when compared with clone library BAC-61.

To measure how well the sample represents the larger environment, the Good Coverage Index was calculated (Table 3 and Figure 8) using the program ASLO (www.aslo.org/methods/free/2004/0114a.html). For clone library BAC-61, the coverage was 76% and 63% for library BAC-212. At 61 days, the biofilm appears to be composed by a less diverse community with a high level of dominance of a few representative clones. An increase in diversity was observed in clone library BAC-212 with more phylotypes represented at lower frequencies. In order to exhaustively sample and fully cover the community BAC-212 days, a larger library (>100 clones) will be necessary.

Rarefraction curves were done using the program DOTUR (http://www.plantpath.wisc.edu/fac/joh/dotur.html). This is a method used to compare observed richness among environments that have been unequally sampled (Hughes and Bohannan, 2004). After 100 repeated randomizations of the samples, the results

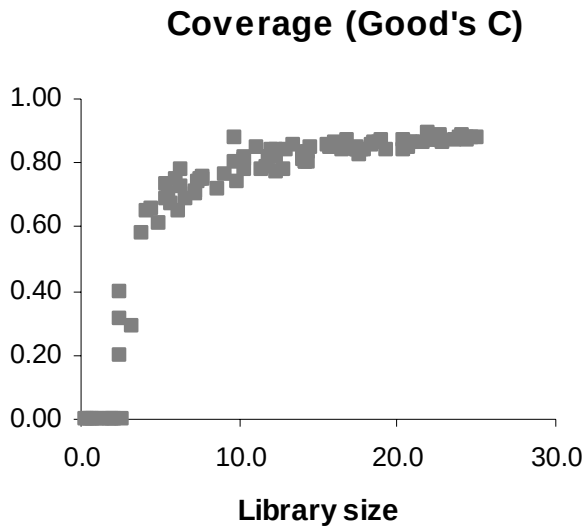
demonstrated an increase in richness for clone library BAC-212 (Figure 9). The graphic curves revealed that if clone library BAC-61 has sampled more clones, the distribution will keep the same graphic pattern. In contrast, for clone library BAC-212, additional clone samples will be necessary to reach a continuous tendency in the graphic pattern correspondent to coverage data.

In order to evaluate a level of differences among clone libraries, a p-value was calculated using Web-LIBSHUFF program (http://libshuff.mib.uga.edu). The following formula was used to calculate the standard p-value of two libraries: $p=1-(1-a)^{k\ (k-1)}$. A confidence percent had been established at p=0.05 with k being the number of clone libraries to be studied. The standard p-value for two clone libraries was 0.0253. The Web-LIBSHUFF results revealed a p-value minor of the minimum expected p-value of 0.001 indicating significance differences between the two 16S rDNA clone libraries.

Table 3. Statistical indexes for 16S rDNA clone libraries.

Clone Library	***Shannon (H)***	***Simpson's (1/D)***	***[1]Richness Observed***	***Jaccard***	***SAce***	***SChao***	***Coverage %***
BAC-61	1.2	0.42	6 (25)	9.0	11	7.5	76
BAC-212	2.1	0.19	16 (43)	83	91	94	63

[1]Numbers in parentheses indicate the number of 16S rDNA clones used in the analyses. Richness is the number of phylotypes observed. Each phylotype consisted of either unique clone or a group of clones that had sequence similarities of over 97%.



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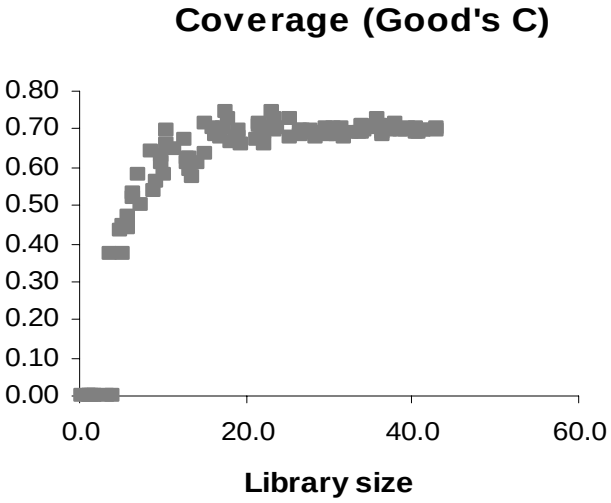

B

Figure 8. Good coverage curves for 16S rRNA clone libraries. (A) BAC-61 days, and (B) BAC-212 days.

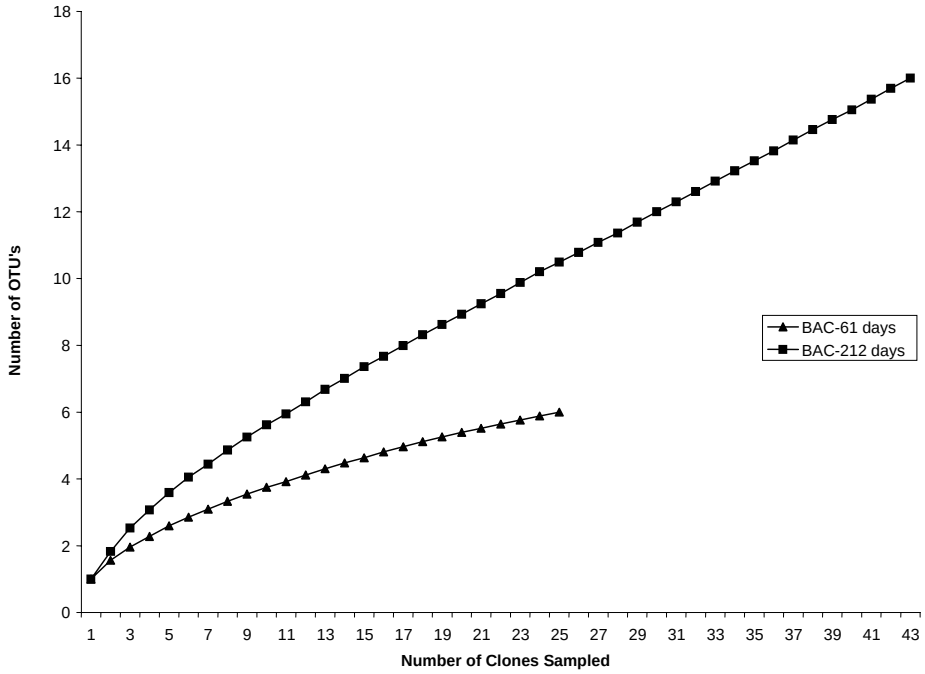

Figure 9. Rarefraction curves obtained for both clone libraries (BAC-61 days, and BAC-212 days).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) of the BAC communities

In order to have an overview of the possible changes in the biofilm structure established at the treatment unit, 16S rDNA T-RFLP was performed for samples BAC-30, BAC-61, BAC-153 and BAC-212 days. Total community 16S rDNA was amplified using the primer pair labeled 519F-IR700 and 1392R. PCR amplicons were digested separately with three restriction enzymes: *Hae*III, *Rsa*I and *Msp*I and the community T-RF patterns was analyzed in a gel based DNA sequencer.

T-RF's profiles revealed a wide range of 16S rDNA fragments ranging from 45 to 695 bp representing different bacterial populations within the BAC community (Figure 10). The restriction analysis for the enzyme *Hae*III showed similarities among the

community samples. Bacterial populations represented by T-RF's of 695, 439, 387, 204, 100, 96, and 45 bp were common for all the samples. Although similarities were observed, a unique population was represented by a T-RF of 487 bp for BAC-212 community. Changes in community structure were also assess with some populations (T-RF's of 270 and 108 bp) presented in early stages and absent in later stages of treatment.

The restriction profile for the enzyme *Rsa*I revealed shared bacterial populations among all communities. Common populations were represented by 387, 337, 245, 230, 145, 139 and 45 bp T-RF's. A unique 140 bp fragment was observed in BAC-30 and BAC-212 communities. Finally with the restriction enzyme *Msp*I, a conserve fingerprint profile was observed for BAC communities with T-RF's of 650, 360, 337, 225, 216 and 45 bp. Unique fragments were also observed for BAC-153 and BAC-212, while community structure changes were represented by a 204 bp T-RF not detected in BAC-212 community.

Furthermore, similarity analysis of the T-RFLP's showed strong relations among communities with some variations which supports the T-RF's profiles as described previously (Table 4). Changes in bacterial populations within the biofilm at the early and last stage of operation were observed as demonstrated by 16S rDNA clone libraries.

Table 4. Similarity value (%) of BAC T-RFLP community profiles.

T-RFLP	***% Similarity***		
	BAC-30	**BAC-61**	**BAC-153**
BAC-61	56.9	-	-
BAC-153	44.0	52.2	-
BAC-212	43.9	48.9	41.5

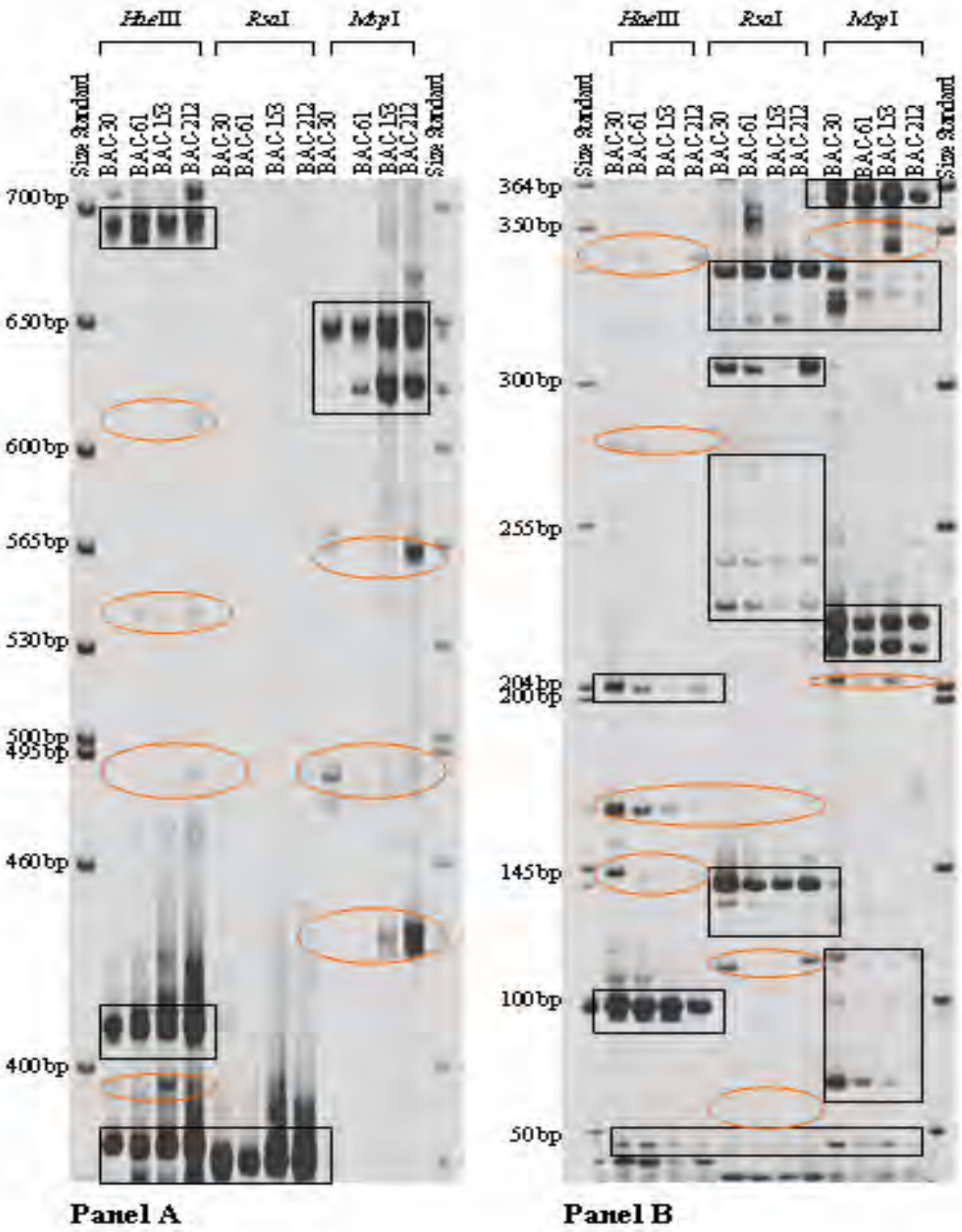

Figure 10. T-RFLP fingerprints of the BAC microbial communities. (Black rectangles identified T-RF's presents in all samples: Orange ovals identified unique T-RF's or changes in bacterial abundance and distribution: Panel B is a continuance of Panel A representing the same gel).

Functional Gene Array of Microbial Community Samples

To evaluate the metabolic potential within the biofilm community, functional gene microarrays consisting of more than 21,000 genes probes was performed in triplicate. The array was constituted by three main gene categories: biogeochemical cycles, metal resistance, and organic degradation genes (Table 5).

A total of 270 genes for organic degradation (including naphthalene, toluene [aerobic and anaerobic], octane, biphenyl, pyrene, xylene, phenanthrene, and benzene); and 333 genes involved in metabolic activities (some nitrogenases [*nir*S, *nir*K, and *nos*Z], dissimilatory sulfite reductases [dsr*AB*], cytochrome c family of *Geobacter* sp., and methane monooxygenase [*pmo*A]) were detected (Table 6, Appendix 7). Furthermore, genes for MTBE, explosive degradation, and chlorinated compounds were also present, thus indicating the broad catabolic potential of the microbial community.

Table 5. Summary of FGA total hybridization results and representative gene categories for diesel BAC microbial communities.

Probe Category	***Total Gene Probe Number***	***Total Hybridized Probes***	***Gene ID Category***
Metabolic Genes (C, N, S cycles)	5,769	333	*Geobacter sp.* cytochrome family, *nir*S, *nir*K. *nif*H, *nos*Z, *amo*A, *pmo*, *pmo*A, *dsr*A, *dsr*B
Organic Degradation	4,014	270	Nitrobenzene, naphthalene, biphenyl, 2,4-D, MTBE, toluene, nitroluene, acetylene, benzoate, cyclohexanol, phthalate, thiocyanate
Metal Resistance	2,402	172	Mercury, copper, arsenic, nickel, cobalt, cadmium
Total Genes	**12,185**	**775**	

Table 6. Pairwise similarity value (%) for FGA's of BAC microbial communities.

FGA's	*% Similarity*		
	BAC-30	**BAC-61**	**BAC-153**
BAC-61	47.5	-	-
BAC-153	49.6	74.4	-
BAC-212	61.4	69.8	73.8

Similar to T-RFLP data, FGA cluster analysis revealed strong similarities among the samples (Figure 11, Table 6). Probe hybridization patterns indicated an early selection of a core microbial community although the Shannon diversity index increased progressively from 5.99 (BAC-30 days) to 6.38 (BAC-212 days) during the seven months of operation (Table 7). The highest Shannon and Simpson's diversity indices were observed in BAC-212 community. These observations are similar to those obtained with clone libraries. The Chao and Jaccard richness indices showed that gene diversity in the community was increasing with treatment time reaching the highest value at the end of operation.

A list of genes involved in organic degradation and consistently present in the microbial community is presented in Table 8. Some organic degradation pathways were present in all BAC community samples such as genes involved in the degradation of: anaerobic benzoate, biphenyl, thiocyanate, protocatechuate, toluene anaerobic, acetylene, phthalate and MTBE. These results supported the idea that of a stable community core with an expanding potential for organic and hydrocarbon degradation. Differences in the presence of organic degradation genes were observed between samples including unique genes present exclusively during the first stage of operation while others genes were only detected at later times (Appendix 7).

Table 7. Statistical indexes estimated from FGA's data.

Community Samples	*Chao 1 Mean (SD analytical)*	*Jacc 1 Mean*	*Shannon Mean (SD runs)*	*Simpson Mean (SD runs)*
BAC-30	451.70 ± 1.54	451.70	5.99 ± 0.12	407.04 ± 58.35
BAC-61	625.84 ± 0.15	791.78	6.25 ± 0.07	473.01 ± 51.28
BAC-153	722.80 ± 0.00	911.07	6.33 ± 0.04	481.15 ± 33.84
BAC-212	791.00 ± 0.00	991.25	6.38 ± 0.00	493.84 ± 0.00

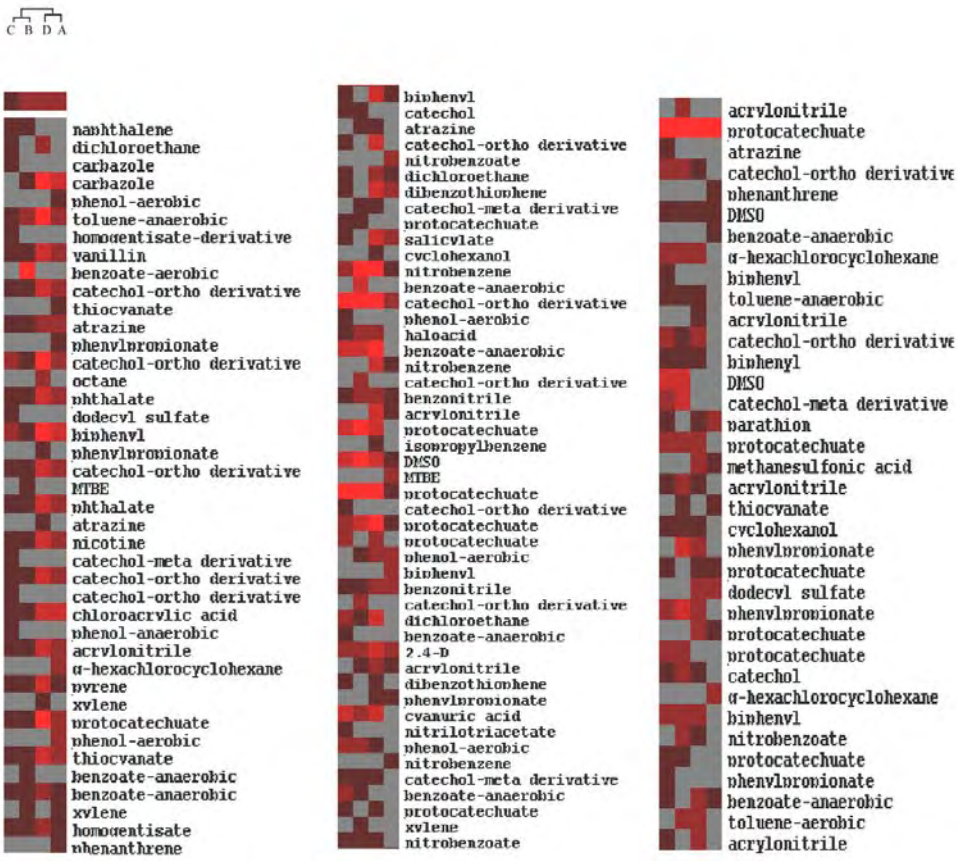

Figure 11. Hierarchical cluster analysis of bioreactor community samples relationships based on Functional Gene Arrays. The figure was generated using hierarchical cluster analysis (CLUSTER) and visualized with TREEVIEW. Biofilm community samples were represented as: (A) BAC-30 days; (B) BAC-61 days; (C) BAC-153 days; (D) BAC-212 days. Each row represents the hybridization pattern for the organic degradation genes detected in the samples. Gray color indicates no signal; increase in intensity levels represents higher hybridization signal level.

Table 8. Hybridization results for genes detected in all BAC-samples with potential involvement in diesel transformation.

		[1]Signal Noise Ratio			
Gene Name	***Gene Description / Source / Gene ID***	***BAC-30***	***BAC-61***	***BAC-153***	***BAC-212***
Phthalate	Putative phthalate ester hydrolase /	13.28	3.38	7.65	12.41
	Arthrobacter keyseri / 13242052_108	(7.73)	(1.31)	(0.61)	(12.91)
Phthalate	Phthalate dioxygenase large subunit /	4.83	4.31	9.57	7.60
	Arthrobacter keyseri / 13242054_353	(2.23)	(1.34)	(1.56)	(4.66)
Phthalate	3,4-dihydroxyphthalate 2-decarboxylase / *Arthrobacter keyseri*	5.33	3.90	12.01	6.85
	/ 13242058_587	(1.53)	(1.41)	(0.59)	(2.51)
MTBE	Alkane 1-monooxygenase /	3.44	3.69	9.76	4.34
	Pseudomonas fluorescens / 13445194_108	(1.38)	(1.45)	(0.39)	(1.73)
Benzoate/	Thiolase (acetyl-CoA acetyltransferase) /	4.71	4.39	10.71	4.83
anaerobic	*Bacillus halodurans* C-125 / 15614592_1076	(2.59)	(2.03)	(1.14)	(1.49)
Thiocyanate	Carbon monoxide dehydrogenase /	4.29	4.64	9.08	5.47
	Sulfolobus solfataricus P2 / 15898062_686	(1.56)	(1.86)	(0.91)	(2.64)
Phthalate	phthalate permease /	9.37	2.82	4.03	19.55
	Sulfolobus tokodaii str. 7 / 15922956_410	(5.65)	(1.14)	(0.60)	(12.12)
Protocatechuate	Protocatechuate 3,4-dioxygenase, alpha subunit /	2.94	6.59	20.30	13.90
	Caulobacter crescentus / 16126648_384	(1.01)	(3.99)	(2.24)	(11.30)
Protocatechuate	Putative protocatechuate 3,4-dioxygenase /	3.37	11.62	31.43	12.51
	Sinorhizobium meliloti 1021 / 16265236_532	(1.32)	(6.23)	(1.86)	(7.28)
Biphenyl	Biphenyl dioxygenase /	5.46	2.31	3.65	7.22
	Ralstonia eutropha / 1890342_658	(2.27)	(0.67)	(0.60)	(4.97)
Aniline	Aniline dioxygenase beta-subunit /	3.14	3.57	5.33	4.23
	Acinetobacter sp. YAA / 2627148_399	(1.57)	(1.39)	(0.71)	(1.41)
Protocatechuate	3,4-dioxygenase beta chain / *Bradyrhizobium japonicum*	5.11	4.04	9.51	6.13
	USDA 110 / 27:31794411_472	(1.33)	(1.52)	(1.06)	(2.88)
Cyclohexanol	Cyclohexanone monooxygenase / *Bradyrhizobium japonicum*	4.44	4.24	9.32	6.40
	USDA 110 / 27382095_773	(1.76)	(1.73)	(0.82)	(2.90)
Phthalate	3,4-dihydroxy-3,4-dihydrophthalate dehydrogenase /	4.44	6.35	6.63	4.25
	Terrabacter sp. / 27531096_403	(2.33)	(3.73)	(0.82)	(1.59)
Toluene/	Benzylsuccinate synthase gamma subunit /	6.26	5.40	15.41	7.98
anaerobic	*Thauera aromatica* / 3184130_28	(1.97)	(2.11)	(0.96)	(2.23)
Acetylene	Probable ephA protein /	4.39	6.57	11.40	6.48
	Pirellula sp. 1 / 32473431_370	(1.97)	(4.21)	(1.18)	(2.06)
Biphenyl	Biphenyl dihydrodiol dehydrogenase /	6.86	4.22	16.08	8.15
	Bacillus sp. JF8 / 32562914_541	(2.19)	(2.41)	(1.26)	(2.56)
Acetylene	Acetylene hydratase Ahy /	5.68	4.27	11.74	6.42
	Pelobacter acetylenicus / 33325847_969	(2.28)	(2.19)	(0.91)	(3.39)
Protocatechuate	Putative protocatechuate 3,4 dioxygenase / marine α	22.03	11.30	38.79	8.89
	proteobacterium SE45 / 38490070_560	(14.05)	(12.20)	(13.74)	(4.81)
Thiocyanate	ACDS complex carbon monoxide dehydrogenase /	8.40	3.94	5.02	7.48
	Methanopyrus kandleri / 38503097_1862	(3.54)	(1.47)	(0.44)	(5.86)
Biphenyl	Receptor-like histidine kinase /	12.42	13.16	12.20	6.23
	Rhodococcus erythropolis / 3868875_3209	(6.00)	(13.32)	(2.18)	(4.32)
Benzoate/	Ferredoxin, 2Fe-2S /	3.43	3.18	6.46	4.70
anaerobic	uncultured bacterium 580 / 40063438_226	(0.79)	(1.31)	(1.06)	(2.71)
Benzoate/	Ferredoxin / *Desulfotomaculum*	3.26	2.93	4.54	5.98
anaerobic	*thermocisternum* / 4028019_136	(0.70)	(1.07)	(0.59)	(2.73)
Thiocyanate	Carbon monoxide dehydrogenase / *Thermoproteus tenax* /	4.47	3.65	8.93	6.01
	41033719_176	(1.47)	(1.36)	(0.86)	(2.91)

[1]Average (Standard Deviation); n=6.

Functional gene arrays revealed information about how signal intensity varies within genes present in the biofilm community (Wu *et. al*., 2001; Rhee *et. al*., 2004) during the treatment process for a given biogeochemical or organic degradation pathway of interest (Figure 12). The results showed that some genes maintain a constant abundance during the 7 months of treatment as seen for the MTBE, naphthalene, biphenyl, and aerobic phenol degradation pathways. In contrast, the anaerobic benzoate gene had a low signal during treatment until the last stage at 212 days with a significant signal increase. This observation is consistent with various lines of evidence suggesting a shifted to anaerobic bacterial dominance at later stages of operation.

Genes encoding for relevant metabolic activities as nitrogen fixation, nitrogen reduction and sulfur dissimilatory pathways were also present in all community samples with variations in their signal intensity (Figure 13). Genes as *nar*G, *nir*S and *nir*K involves in dissimilatory nitrate and nitrite reduction increased in their hybridization signal intensity toward the end of operation. Again, the detection of such genes could be indicative of more anaerobic contributions after the removal of hydrocarbons. Genes involved in sulfur dissimilatory pathways as *dsr*A and *dsr*B increased its hybridization signal as well by the middle stage of treatment with a maximum signal level at the end of the operation. In general, FGA's demonstrated great dynamics in the genetic potential of the community over time and the establishment of a highly diverse microbial community with concurrent aerobic and anaerobic processes contributing to the restoration process.

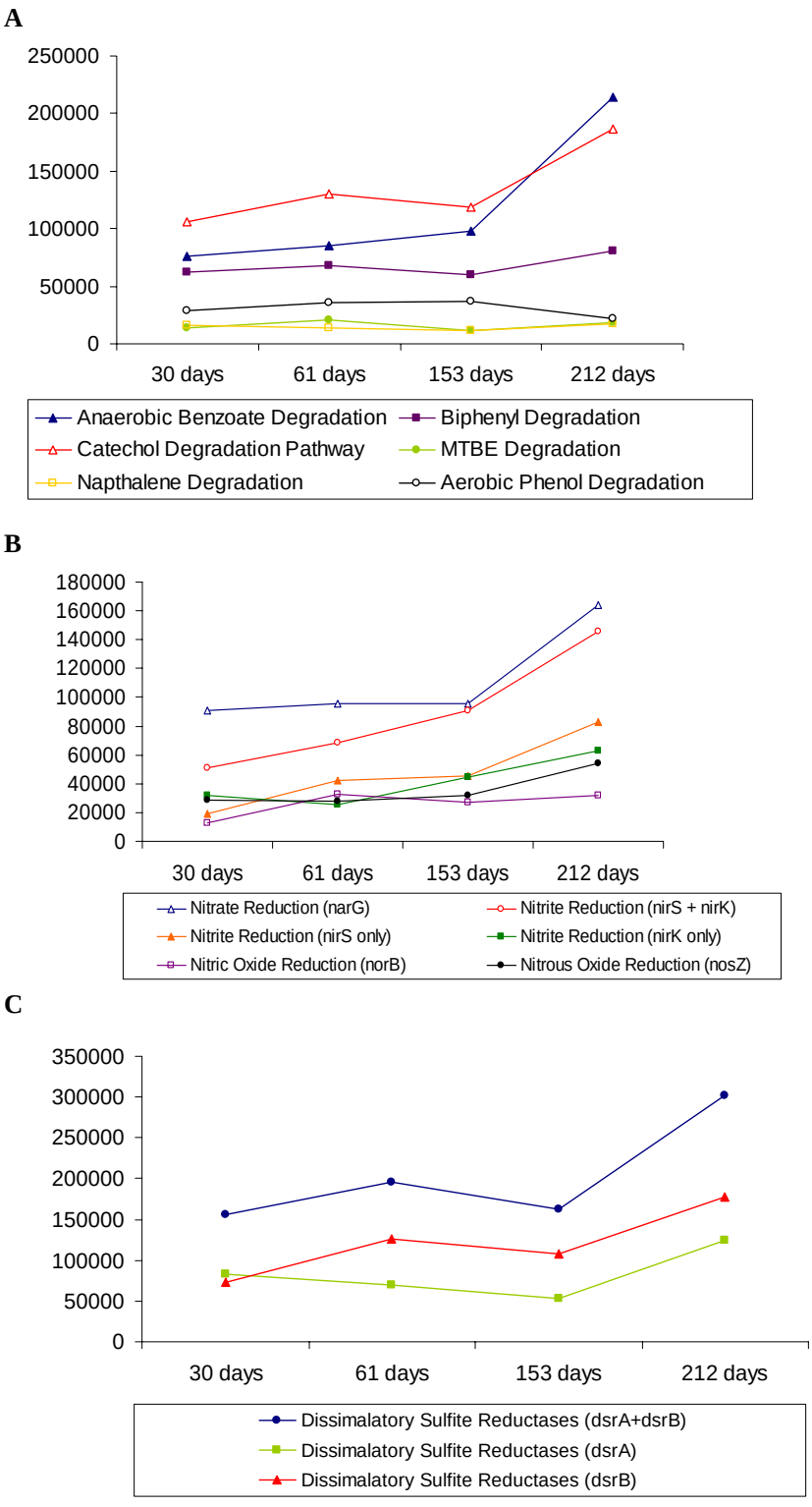

Figure 12. Cumulative Signal intensity patterns of some metabolic and organic degradation pathways of the BAC community samples as showed by FGA's. (A) Organic degradation; (B) denitrification processes and, (C) Sulfur dissimilatory pathways.

DISCUSSION

Bioremediation process

Fixed-film biological systems developed on porous media have been employed for bioremediation. In this study, granular activated carbon (GAC) was used to pack a fluidized bed bioreactor as the carrier matrix for microbial growth. Hydrocarbon-contaminated groundwater was pumped upwards through a bed of GAC, fluidizing media. This technique combined the absorptive and high surface-area-per-unit-volume properties of activated carbon with biological treatment (Massol-Deyá *et. al*., 1995, 1997).

In the process, the contaminants are transformed to harmless end products such as carbon dioxide, water and new biomass. Diesel constituents can be reduced to levels significantly below discharge limits with treatment times of minutes. Furthermore, recycling in the reactor can be effectively employed for treatment of high organic loads. Before treatment, the water was passively amended with oxygen using Oxygen Release Compound (3-7 mg/L) while nutrients such as nitrogen and phosphate (NH_4Cl and KH_2PO_4) were adjusted to at a ratio of 30:5:1 (carbon: nitrogen: phosphorous).

The use of the GAC-FBR treatment for restoration of the diesel-contaminated groundwater had significant advantages such as: (i) low operation and maintenance cost; (ii) low energy requirements (small mobile treatment unit); (iii) adsorbent granular activated carbon carrier provides high surface area for microbial growth; (iv) rugged and reliable treatment; (v) on-site degradation of contaminants; (vi) treatment times of minutes; and (vii) removal efficiencies greater than 98% without off-gases.

The FBR was operated as a discontinuous batch one-pass-flow system without recycle at a flow rate of 3.8 L/min. The biofilm, composed of over 10^7-10^8 total aerobic cultivable cells/g GAC, reached removal efficiency for total petroleum hydrocarbon (TPH) of 98% with only 15 to 20 min hydraulic retention time. Stable pH levels and temperatures were beneficial for the sustainable growth of microbes and degradation of hydrocarbons (Table 1).

Dissolved oxygen concentrations ranged between 3-7 mg/L at influent and below 2 mg/L in the effluent during operation. These concentrations were similar for the same treatment unit used for the restoration of a gasoline-contaminated aquifer at the University of Puerto Rico, Mayagüez Campus (Ara-Rojas, 2004). Based on the dissolved oxygen content, the remediation system is classified as micro-aerophilic. Micro-aerophilic systems are bioremediation processes conducted with dissolved oxygen concentrations above 2 mg/L, but below 21 mg/L (Mikesell *et. al.*, 1993). Low oxygen concentration (Table 1), high electron acceptor demand, and consumption of alternative electron acceptors within the treatment phase suggest concurrent aerobic/anaerobic activity in a heterogeneous system. Increase in nitrate removal with sulfate uptake increase is also indicative of anaerobic respiration (Table 1). Abundant ammonium levels, isolation of denitrifying bacteria, and nitrate uptake within the treatment unit suggest that dissimilatory nitrate respiration was key to the biodegradation process. Based on the physical and nutrients parameters it can be concluded that the biofilm established in the treatment unit was composed by bacterial populations able to grow under fluctuations of physical and nutrient conditions probably selecting for diverse and metabolically flexible hydrocarbon-degrading community.

BAC microbial community

There has been continued interest in understanding the biodiversity and structure of microbial communities inhabiting in both natural and artificially managed environments. Based on culture and culture-independent analysis of the hydrocarbon-degrading biofilm community a great morphological, physiological and genetic diversity were observed.

One mechanism to promote the availability of hydrocarbons through the microbial biofilm is by production of biosurfactants. These secondary metabolites useful in biotechnological bioremediation processes, enhance nutrient transport across membranes, act in host-microbe interactions, and provide biocidal and fungicidal protection to the producing organism (Jennings and Tanner, 2000).

Strain DIESVBO1 exhibited robust growth on minimum media with diesel as the only carbon source and produced a white color substance. There is a possibility that strain DIESVBO1 can produce a biosurfactant which enhance its diesel degradation potential helping the microbial biofilm as well during the bioremediation process.

Characterization of the 16S rDNA genes had been well-established as a standard method for the identification of species, genera and families of bacteria (Woese 1987; Gürtler and Stanisich, 1996). These genes are similar in length (approx. 1.5kb) throughout the bacterial kingdom and contain highly conserved regions as well as others that vary according to species and family. The ARDRA analysis is based in band patterns generated by restriction enzymes. The number and molecular size of the restriction bands are influenced by the presence, frequency and absence of the enzyme recognition site. A cluster analysis of restriction patterns generated using ARDRA

showed a high genotypic diversity within cultivable members of the biofilm community (Figure 3). At least 19 different groups could be identified when compared with isolated strains for the same treatment unit in a gasoline-contaminated aquifer (Figure 4). Only strain DIESVBS1 revealed some genetic similarity with the gasoline-degrader strains GASM10 and GASM11, demonstrating selection of a different and unique community using the same treatment unit.

In general, bacterial populations were taxonomically related to three bacterial divisions: *Bacilli, Actinobacteria* and *Proteobacteria*. A total of 44% of cultures partially sequenced belonged to *alpha, beta,* and *gamma* subdivisions of the *Proteobacteria*. The culture distribution within the *Proteobacteria* division is as follow: α-*proteobacteria* (DIESVBS6); ß-*proteobacteria* (DIESVBS12, DIESVBS15, DIESVBN4); and γ-*proteobacteria* (DIESVBS7, DIESVBS10, and DIESVBN6). The *Proteobacteria* division is well known by the diversity of groups involve in petroleum hydrocarbon degradation (Dojka *et. al*., 1998; Macnaughton *et. al*., 1999; Rölling *et. al*., 2001, and Watanabe *et. al.,* 2000). The *Bacilli* division was represented by 37% of the strains and 19% of the isolates were closely related to the *Actinobacteria* division.

Strain DIESVBS6 was closely related to *Novosphingobium tardaugens* (Sab 0.810). This genus belongs to diverse *Sphingomonas* group known by its remarkable metabolic capability to degrade a wide range of organic pollutants such as polycyclic aromatic hydrocarbons (PAH's). Tiirola *et. al*. (2002) investigated microbial diversity in a fluidized bed reactor treating polycholorophenol-contaminated groundwater. Their results revealed *Novosphingobium* strain MT1 as a dominant member responsible for the

potentially degradation of main contaminants present in the groundwater (2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, and pentachlorophenol).

DIESVBS5 was related with *Brevibacillus parabrevis*. Although some species of *Brevibacillus* had been reported as bacterial contaminants in the food industry such as *B. agri*, and *B. borstelensis* (De Clerk *et. al*., 2004), some *Brevibacillus sp.* had environmental relevance. *Brevibacillus laterosporus* is an aerobic spore-forming bacterium that is characterize by its ability to produce crystalline inclusions and its potential used for biological control (de Oliveira *et. al.*, 2004). Petrie *et. al.* (2003) characterized iron(III)-reducing microbial communities from acidic sediments contaminated with uranium(VI). Their T-RFLP's findings revealed fragments closely related to *Paenibacillus* and *Brevibacillus* genus.

Isolates DIESVBS11, DIESVBS16, DIESVBN1, DIESVBN2, DIESVBN3 and DIESVBN5 were related to different *Bacillus sp*. (*B. thuringiensis*, *B. mycoides*, and *B. simplex*, respectively). *B. thuringiensis* has pathogenic properties and it is commonly used as an organic bio-pesticide against insect pests (Hill *et. al*., 2004; Priest *et. al*., 2004). Recent discoveries showed the ability of some *Bacillus sp*. to produce biosurfactants under aerobic and anaerobic conditions (Youseff *et. al*., 2005). This finding can be a promising alternative to enhance bioremediation processes when bioaccessibility of hydrocarbons becomes a rate-limiting factor.

Strain DIESVBS12 was phylogenetically related to *Roseateles depolymerans*, an aerobic-phototrophic bacteria related to the ß-proteobacteria (Suyama *et. al*., 2002). DIESVBN4 was related to *Dechlorosoma sp.* with a Sab of 0.949. *Dechlorosoma sp*. is a member of a metabolically diverse group of organisms capable of anaerobic growth in

perchlorate. The ubiquity of *Dechlorosoma* species in different environments including petroleum-contaminated soil, river sediments, pristine soils and aquifer sediments had been demonstrated (Achenbach *et. al.,* 2001; Logan *et. al.*, 2001; Lack *et. al*., 2002; Zhang *et. al*., 2005). Physiological studies of *Dechlorosoma suillum sp. nov*., and *Dechlorosoma agitata sp. nov*. demonstrated their Fe(II) oxidizing potential when nitrate or chlorate served as the electron acceptor under strictly anaerobic conditions (Achenbach *et. al.*, 2001; Lack *et. al*., 2002).

Pseudomonas sp. and *Burkholderia sp*. were routinely found within the BAC community. *Pseudomonas sp.* has been investigated by their wide range of catabolic abilities for toluene oxidation by different pathways (McClay *et. al.* 1995; Esteve-Nuñez *et. al*., 2001). *Burkholderia sp*. are an important component of the soil microbial community able to fix nitrogen and capable to utilize a wide range of organic compounds as carbon sources. This metabolic versatility makes *Burkholderia* an excellent tool for biodegradation of environmental pollutants (Falcão Salles *et. al.*, 2002). Due to their hydrocarbon degrading properties, *Pseudomonas sp*. and *Burkholderia sp*. are commonly found in contaminated sites and in restoration processes.

A genotypic characterization of BAC cultures showed a high degree of heterogeneity in the microbial community. As mentioned before, few similarities were found when compared with the ARDRA profiles. The gasoline-degraders strains were phylogenetically represented by the bacterial divisions: *Cythophaga-Flexibacter-Bacteroides*, *Proteobacteria* and *Gram-positive*-bacteria. The 16S rDNA partially sequences showed gasoline-degraders strains related to species such as *Delftia sp*., *Pseudomonas sp*., *Bosea sp*., *Gordonia sp*., *Rhodococcus sp*., *Flavobacterium sp*.,

Hydrogenophaga sp. (Ara-Rojas, 2004) with similarity coefficients greater than those obtained for diesel-degrading isolates (Table 2). It is clearly demonstrated that BAC community supported a wide range of microbial populations within the multi-species consortia perhaps enhancing the hydrocarbon degradation efficiency.

Characterization of the BAC community by culture-independent techniques

Analysis of microbial communities involved in *in-situ* hydrocarbon biodegradation activities has been a challenge to microbiologists. Culture-independent techniques such as 16S rDNA clone libraries, terminal restriction fragment length polymorphism (T-RFLP) and DNA microarrays can provide important insights about microbial community characteristics such as viable biomass, community structure (microbial population genotypic profiles), nutritional and physiological status and metabolic activities.

In order to characterize the BAC community, clone libraries based on 16S rDNA gene were constructed for two samples representing early and late stages of treatment (BAC-61 days and BAC-212 days). The results showed great diversity with multiple clones being strongly related (97-99% of similarity) with uncultured *proteobacterium* of hydrocarbon degradation abilities (Appendix 4 and 5). Dominance of clones related to *Proteobacteria* correlates with the phylogenetic analysis the BAC cultures as well (Table 2).

Clone library BAC-61 was composed by 75% of *beta*, 17% *gamma*, and 8% *alpha-proteobacteria*. Phylogenetic analysis resulted in a main cluster constituted by 17 clones closely-related with uncultured *beta proteobacterium* clone 36-9 and clone PYR10d2

(Figure 6). The uncultured *beta proteobacterium* clone 36-9 was previously described at a coal-tar-waste-contaminated aquifer with aerobic naphthalene degradation activity (Baker and Madsen, 2002). The uncultured soil bacterium clone PYR10d2 was obtained from a bioreactor treating contaminated soil with PAH's (Singleton *et. al*., 2006). The *gamma* proteobacterium clones were related to uncultured soil bacterium PYR10d11 and the purple-sulfur bacteria strains *Thiocystis violacea* and *Rhabdochromatium marinum*, from lagoon sediments and salt marsh microbial mats, respectively (Guyoneaud *et. al*., 1997; Dilling *et. al*., 1995). The *alpha* bacterial division was represented by a few clones related to uncultured proteobacteria clone Ape9_6 and clone JG37-AG-96. The clone JG37-AG-96 was obtained from uranium waste piles and mill tailings (Geissler *et. al*., unpublished).

Clone library BAC-212 days showed a significant shift in the biofilm community distribution. The *gamma* proteobacteia was composed by 75% of the sampled clones, 23% *beta*-proteobacteria and alpha subdivision were not detected (Figure 7). The main cluster of *gamma* proteobacteria clones was related (90-98%) with soil bacterium PYR10d11 (isolated from a bioreactor treating PAH-contaminated soil), and the bacterial strains *Thiocystis violacea* and *Rhabdochromatium marinum*. An increase in the abundance of bacterial populations related to purple sulfur bacteria was observed although hydrocarbon degradation capacity was sustained. The beta *proteobacteria* sampled clones were related (89-98%) to clone 36-9 and clone PYR10d2 previously described. A lower percent of similarity was found between the clone libraries demonstrating a community change to more diverse in a later operation phase (Appendix 4 and 5).

Changes in the community structure were observed between the clone libraries in the abundance and diversity of sampled clones as well as the metabolic pathways. A shift from aerobic-degrading bacterium to more anaerobic scenery was observed based on the identity and phylogenetic analysis. Statistical analysis of the clone libraries correlated in the fact that bacterial populations at the early stage of treatment were less diverse (6 different OTU's), than the older community dominated by 16 different OTU's (Table 4). Diversity between the clone libraries was assessed using the Shannon and Simpson's indices. Both indices consistently indicated higher diversity levels in the BAC-212 community. The Shannon index is influenced by the richness rather than the Simpson's index which is heavily influenced by the abundances of the most common OTU's (Hughes and Bohannan, 2004). In terms of richness (Jaccard, SAce and SChao), estimated values were significantly higher for BAC-212 community as well.

The coverage percent for clone libraries was 76% for BAC-61 and 63% for BAC-212 community (Table 3). Coverage and rarefraction curves (Figures 8 and 9) suggest that sampling of additional clones were necessary to better cover the bacterial diversity present in BAC-212 sample. Finally, to evaluate the degree of similarity among libraries, the p-value was calculated by using the Web-LIBSHIFF program. With a standard p-value of 0.0253 for two libraries, the estimated p-value was lower than the minimum (0.001) established by the program. Definitively, the p-value supported the conclusion that the community in the bioreactor changed with time to a more mature and diverse structure.

Terminal restriction fragment length polymorphism was performed in order to determine the presence of common or unique bacterial populations presented in the BAC

community samples. A wide range of bacterial populations represented by 16S rDNA terminal fragments were observed among samples (Figure 10). Bacterial populations presented in all community samples showed terminal fragments ranging from 695 bp to 45 bp. This result clearly indicates the existence of a core microbial community structure since the initial stages and during the 7 months of operation.

Unique T-RF's were observed demonstrating the presence of bacterial populations only in some operation stages. For example, with the enzyme *Hae*III an approximately 395 bp 16S r-DNA terminal fragment was observed only for BAC-153 community and a 145 bp fragment for BAC-30 indicative of some structure changes in the bacteria distribution within the biofilm. The *Msp*I profiles showed unique fragments such as 430 bp for BAC-153 and BAC-212 (older operation stages) and 345 bp only for BAC-153.

Changes in bacterial abundance were also observed with significant variations in T-RF's intensities. For example, with *Rsa*I enzyme differences in the intensities of a 230 bp T-RF were observed among samples, as well in *Hae*III for a 204 bp terminal fragment. Intensities changes in a conserved bacterial population respresented by a T-RF of 45 bp were also observed for all the enzymes among the community samples. It was demonstrated the utility of this molecular approach giving information about the microbial community composition and the heterogeneity represented by diverse 16S rDNA terminal fragments.

T-RFLP revealed that changes in the community structure was occurring by the presence of unique T-RF's among samples, the absence of some 16S rDNA fragments and changes in the T-RF's intensities demonstrating the possibility of changes in the

abundance of some bacterial populations at a given stage. These changes could be associated with a change from an aerobic dominated community to a more anaerobic microbial scenery as supported by the clone library data.

In addition to estimating species richness or diversity T-RFLP pattern of a community could be viewed as a community fingerprint and used to assess the similarity of different communities (Liu *et. al*., 1997). Similarity analysis of BAC communities by means of T-RFLP showed values that correlate with the clone libraries with a difference between BAC-61 and BAC-212 with only 48.6% of similarity between T-RF's profiles (Table 4). The initial stages of operation were represented by BAC-30 and BAC-61 communities with a 56.9% of similarity, while the last stages (BAC-153 and BAC-212) resulted in a lower similarity percent of 41.5%. These results demonstrated a change to a high diverse community because of the observed diminution in similarity between samples at the last months of operation.

Taxonomic and identity bacterial information for the BAC community T-RFLP data was so difficult to elucidate because there was inconsistency between the T-RF's and the data generated for the *in-silico* restriction analysis (Genscript-Restriction Enzyme Map Analysis/ http://www.genscript.com/cgi-bin/tools/enzyme_cuttingtool) of the isolated strains and the sampled clones (Appendix 6). No similarities were found between the expected molecular sizes generated *in-silico* with the terminal fragments observed for the community samples (Figure 10, Appendix 6).

In addition to PCR factors, the composition of T-RFLP profiles can be influenced by factors related to the restriction digestion, such as partially digested PCR products observed in T-RFLP's profiles of pure cultures and environmental samples (Egert and

Friedrich, 2003). Incompletely digested PCR products from a complex microbial community may result in additional T-RF's and, overestimation of diversity data, and inconsistency making comparisons with *in-silico* restriction analysis as observed in this study.

Egert and Friedrich (2003) found the presence of non-terminal restriction fragments in T-RFLP profiles when a complex microbial community was examined. They designated these fragments as pseudo-terminal restriction fragments. Clones for two bacterial and two archaeal clone libraries were analyzed with the enzymes *Msp*I and *Alu*I, respectively. Each clone was expected to display a single T-RF, moreover additional RF's amplicons were observed being false T-RF's. Restriction endonucleases require double-stranded DNA at the restriction site, based on that the presence of single-stranded amplicons was checked using mung bean nuclease, which degrades single-stranded DNA. After mung bean nuclease digestion, pseudo-T-RF's were not detected in environmental, clone and pure culture-derived T-RFLP's. This data indicates clearly that pseudo terminal fragments are formed by the presence of single-stranded DNA. It is highly recommended to limit the number of PCR cycles to a minimum because pseudo terminal formation increases linearly with the cycle number.

The transformation of environmental contaminants is a complex process that is influenced by the nature and amount of the contaminant present, the structure and dynamics of the microbial community, and the interplay of geochemical and biological factors (Rhee *et. al*., 2004). Microarray is a powerful technology that is widely used to study biological processes.

Functional gene array analysis were also performed to study the genetic profile of the BAC community in order to assess possible gene rearrangements in ecological and environmental processes such as nitrification, denitrification, sulfate reduction and organic contaminat degradation. A cluster analysis of the array data (Figure 11, Table 6) revealed strong similarities among the samples which correlates with the early selection of a core microbial community structure as observed by T-RFLP profiles (Figure 10). Statistical analysis showed higher gene diversity in the BAC-212 community (Table 7). Richness was evaluated by the SChao and Jackard indeces resulting in progressive gene diversity increase during the 7 months of treatment. This observation is consistent with the presence of more OTU's at older stages of operation.

The FGA's for the BAC microbial communities were used in this study as a generic gene profiling and comparative tool. Application of a 50-mer oligonucleotide FGA's to environmental samples was successfully used by Rhee *et. al.* (2004) and Tiquia *et. al*. (2004). Five micrograms of microbial DNA community samples were labeled with Cy5 and hybridized with array slides in triplicates. Good hybridization signals were observed for different genes involve in the organic degradation of naphthalene, benzoate and alkenes correlating with the sample type thus demonstrating the significant utility of this approach.

A total of 775 genes were detected by FGA's in the BAC community samples: 270 genes for organic degradation, 333 genes involved in metabolic activities (nitrification, denitrification and sulfur reduction), and 172 metal resistance genes (Table 5 and 8). Furthermore genes for MTBE, explosive degradation and chlorinated compounds were also found. These results indicate the great biofilm potential for

hydrocarbon degradation as revealed by the bioremediation efficiency at the site. The BAC community was composed by bacterial populations with a broad catabolic capacity as showed by the phylogenetic analysis of the isolated strains and clone libraries. Differences in the presence of genes were also found among samples with unique genes present only during specific treatment stages (Appendix 7).

DNA microarray can be used to monitor bacterial gene abundane and rearrangements that occurred within a biofilm among different community samples. Signal intensity equals to gene abundance, based on that fact it was observed that some genes were constant during the treatment process such as naphthalene, biphenyl, and aerobic phenol pathways (Figure 13). Some anaerobic genes had a low signal in the early stage with a significant increase by the last months as the case for the anaerobic benzoate gene. These results corroborate the increase in clone representatives related to anaerobic bacteria as observed in the clone library for the 212 days community sample (Appendix 5).

Functional gene arrays revealed information about how gene abundance varies within the biofilm community during the treatment process for a given biogeochemical or organic degradation pathway of interest (Figure 12). Genes involves in nitrate and nitrite reduction as *nar*G, *nir*S, and *nir*K showed an increase in their intensity, being more abundant by the later treatment stage which operated under more oxygen deficiencies. Genes representing the sulfur dissimilatory pathways (*dsr*A and *dsr*B) had a maximum intensity level by the end of treatment.

Detection of biodegradation genes in the bioreactor and profiling the differences in microbial community structure was successfully accomplished by FGA's. This analysis was suitable for (i) diversity analyses of the biofilm community involved in the

restoration phase of the diesel-contaminated site; (ii) to screen samples and obtain data on the presence of an extensive array of metabolic processes as well as (iii) to characterize temporal changes within the system. These observations were also consistent with culture-based data and field observations of robust and stable hydrocarbon removal efficiency. Furthermore, our results suggest that tropical environments may harbor unique and complex microbial assemblages, which are yet poorly understood. Understanding the microbial structure of this unique tropical system may help improve treatment design, operations and maintenance, as well as to identify additional applications for the restoration of other sites.

CONCLUSIONS

Several observations suggest that the sustainable diesel degradation was associated with microbial colonization of the BAC media. (i) Cell density determined by microscopical observations and cell counts increased coincident with nutrient uptake, indicating that growth was occurring. (ii) Almost 92% of all isolated cultures were capable of utilizing diesel compounds as sole carbon and energy source. (iii) Turbidity in the effluent was higher than measurements in the influent indicating microbial growth in the treatment column. (iv) Uptake of oxygen, nitrate and sulfate were indicative of both aerobic and anaerobic respiration activity. (v) Probe hybridization patterns indicated that aerobic, denitrifying, and sulfate- and iron-reducing bacteria were present within the biofilm community. (vi) A highly diverse with a broad catabolic potential community was selected in the treatment unit as determined by 16S rDNA clone libraries, T-RFLP's and FGA's.

The integration of cultured-dependent and culture-independent molecular approaches allowed a comprehensive characterization of microbial communities in a bioreactor unit treating diesel-contaminated groundwater. Understanding these traits will help to improve and develop new methods in bioremediation taking advantage of the microbial diversity associated to tropical environments.

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APPENDIX

Appendix 1. Petroleum hydrocarbon composition of diesel fuel.

Compound	Number of Carbons	EC	Weight Percent	Reference
Straight Chain Alkanes	-	-	-	-
n-Octane	8	8	0.1	BP, 1996
n-Nonane	9	9	0.19-0.49	BP, 1996
n-Decane	10	10	0.28-1.2	BP, 1996
n-Undecane	11	11	0.57-2.3	BP, 1996
n-Dodecane	12	12	1.0-2.5	BP, 1996
n-Tridecane	13	13	1.5-2.8	BP, 1996
n-Tetradecane	14	14	0.61-2.7	BP, 1996
n-Pentadecane	15	15	1.9-3.1	BP, 1996
n-Hexadecane	16	16	1.5-2.8	BP, 1996
n-Heptadecane	17	17	1.4-2.9	BP, 1996
n-Octadecane	18	18	1.2-2.0	BP, 1996
n-Nonadecane	19	19	0.7-1.5	BP, 1996
n-Eicosane	20	20	0.4-1.0	BP, 1996
n-Heneicosane	21	21	0.26-0.83	BP, 1996
n-Docosane	22	22	0.14-0.44	BP, 1996
n-Tetracosane	24	24	0.35	BP, 1996
Branched Chain Alkanes	-	-	-	-
3-Methylundecane	12	-	0.09-0.28	BP, 1996
2-Methyldodecane	13	-	0.15-0.52	BP, 1996
3-Methyltridecane	14	-	0.13-030	BP, 1996
2-Methyltetradecane	15	-	0.34-0.63	BP, 1996
Alkyl Benzenes	-	-	-	-
Benzene	6	6.5	0.003-0.10	BP, 1996
Toluene	7	7.58	0.007-0.70	BP, 1996
Ethylbenzene	8	8.5	0.007-0.20	BP, 1996
o-Xylene	8	8.81	0.001-0.085	BP, 1996
m-Xylene	8	8.6	0.018-0.512	BP, 1996
p-Xylene	8	8.61	0.018-0.512	BP, 1996

Appendix 1 (continuation)

Compound	Number of Carbons	EC	Weight Percent	Reference
Styrene	9	8.83	<0.002	BP, 1996
1-Methyl-4-isopropylbenzene	10	10.13	0.003-0.026	BP, 1996
1,3,5-Trimethylbenzene	9	9.62	0.09-0.24	BP, 1996
n-Propylbenzene	9	9.47	0.03-0.048	BP, 1996
Isopropylbenzene	9	9.13	<0.01	BP, 1996
n-Butylbenzene	10	10.5	0.031-0.046	BP, 1996
Biphenyl	12	-	0.01-0.12	BP, 1996
Naphtheno-Benzenes	-	-	-	-
Fluorene	13	16.55	0.034-0.15	BP, 1996
Fluoranthene	16	21.85	0.0000007-0.02	BP, 1996
Benz(b)fluoranthene	20	30.14	0.0000003-0.000194	BP, 1996
Benz(k)fluoranthene	20	30.14	0.0000003-0.000195	BP, 1996
Indeno (1,2,3-cd) pyrene	22	35.01	0.000001-0.000097	BP-1996
Alkyl Naphthalenes	-	-	-	-
Naphthalene	10	11.69	0.01-0.80	BP, 1996
1-Methylnaphthalene	11	12.99	0.001-0.81	BP, 1996
2-Methylnaphthalene	11	12.84	0.001-1.49	BP, 1996
1,3-Dimethylnaphthalene	12	14.77	0.55-1.28	BP, 1996
1,4-Dimethylnaphthalene	12	14.6	0.110-0.23	BP, 1996
1,5-Dimethylnaphthalene	12	13.87	0.16-0.36	BP, 1996
Polynuclear Aromatics	-	-	-	-
Anthracene	14	19.43	0.000003-0.02	BP, 1996
2-Methyl anthracene	15	20.73	0.000015-0.018	BP, 1996
Phenanthrene	14	19.36	0.000027-0.30	BP, 1996
1-Methylphenanthrene	15	20.73	0.000011-0.024	BP, 1996

Appendix 1 (continuation)

Compound	*Number of Carbons*	*EC*	*Weight Percent*	*Reference*
2-Methylphenanthrene	15	-	0.014-0.18	
Polynuclear Aromatics	-	-	-	-
3-Methylphenanthrene	15	-	0.000013-0.011	BP, 1996
4&9-Methylphenanthrene	15	-	0.00001-0.034	BP, 1996
Pyrene	16	20.8	0.000018-0.015	BP, 1996
1-Methylpyrene	17	-	0.0000024-0.00137	BP, 1996
2-Methylpyrene	17	-	0.0000037-0.00106	BP, 1996
Benz(a)anthracene	18	26.37	0.0000021-0.00067	BP, 1996
Chrysene	18	27.41	0.000045	BP, 1996
Triphenylene	18	26.61	0.00033	BP, 1996
Cyclopenta(cd)pyrene	18	-	0.000002-0.0000365	BP, 1996
1-Methyl-7-ispropylphenanthrene	18	-	0.0000015-0.00399	BP, 1996
3-Methylchrysene	19	-	<0.001	BP, 1996
6-Methylchrysene	19	-	<0.0005	BP, 1996
Benz(a)pyrene	20	31.34	0.000005-0.00084	BP, 1996
Benz(e) pyrene	20	31.17	0.0000054-0.000240	BP, 1996
Perylene	20	31.34	<0.0001	BP, 1996
Benz(ghi)perylene	22	34.01	0.0000009-0.00004	BP, 1996
Picene	22	-	0.0000004-0.000083	BP, 1996

* BP (1996). Summary tables of laboratory analysis for diesel and fuel oil #2, personal communication from B. Alberston, Friedman and Bruya, Inc., Seattle, WA, developed for Bristish Petroleum.

Appendix 2: Morphological and biochemical characterization of 26 isolated strains from the BAC unit treating a diesel-contaminated aquifer in Vega Baja, P.R.

Strain ID	***Cell Type***	***Colony Morphology***	***Nitrate Reduction Test*** **(NO_3-NO_2)**	***Nitrogen Reduction Test*** **(NO_3-N_2)**	***Diesel Growth Test***
DIESVBS1	Gram negative rod	Circular, white and entire margin	Negative	Negative	Robust
DIESVBS2	Gram positive rod	Irregular and white	Negative	Negative	Progressive
DIESVBS3	Gram negative short rod	Circular, white and flat	Positive/Gas production	-	Moderate
DIESVBS4	Gram positive rod	Undulate margin and white	Positive/Gas production	-	Moderate
DIESVBS5	Gram negative rod	Flat and white	Negative	Negative	Moderate
DIESVBS6	Gram negative short rod	Flat and yellow	Negative	Negative	Progressive
DIESVBS7	Gram negative diplobacillus	Flat and ivory	Positive	-	Robust
DIESVBS8	Gram positive rod	Circular, and white	Positive	-	Robust
DIESVBS9	Gram negative short rod	Circular and yellow	Positive/Gas production	-	Robust
DIESVBS10	Gram negative short rod	Irregular margin and white	Positive	-	Moderate
DIESVBS11	Gram negative rod	Irregular margin and white	Positive	-	Robust
DIESVBS12	Gram positive rod	Flat, circular and ivory	-	Positive	Progressive
DIESVBS13	Gram positive rod	Circular, entire margin and white	Negative	Negative	Progressive
DIESVBS14	Gram negative short rod	Irregular margin and white	-	Positive	Progressive

Appendix 2 (continuation)

Strain ID	***Cell Type***	***Colony Morphology***	***Nitrate Reduction Test*** **(NO_3-NO_2)**	***Nitrogen Reduction Test*** **(NO_3-N_2)**	***Diesel Growth Test***
DIESVBS15	Gram negative short rod	Irregular , undulate margin and white	Positive	-	Progressive
DIESVBS16	Gram negative rod	Rhizoid form and white	Positive	-	Robust
DIESVBO1	Gram negative rod	Flat and pink	-	Positive	Robust/Biosurfactant like substance
DIESVBO2	Gram positive short rod	Flat , entire margins and yellow	-	Positive	Moderate
DIESVBO3	Gram negative cocci	Flat and ivory	Positive	-	Robust
DIESVBO4	Gram negative diplobacillus	Flat and orange	Positive	-	Negative
DIESVBN1	Gram positive rod	Irregular and light pink	-	Positive	Robust
DIESVBN2	Gram positive rod	Irregular, filamentous margins and white	Negative	Negative	Robust
DIESVBN3	Gram negative cocci	Flat and yellow	Negative	Negative	Moderate
DIESVBN4	Gram negative cocci	Filamentous margins and dark pink	Positive	-	Moderate
DIESVBN5	Gram positive rod	Irregular, filamentous margins and ivory	Positive	-	Robust
DIESVBN6	Gram negative rod	Irregular, filamentous margins and white	Negative	Negative	Negative

Appendix 3. Identification of BAC isolated strains using the BLAST sequence match tool based on partial 16S rDNA sequences.

Strain ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
DIESVBS5	*Brevibacillus parabrevis* strain:IFO 12334T	Genomic DNA	98
	Brevibacillus parabrevis strain M3	Genomic DNA	98
	Brevibacillus agri strain R-20067	Bacterial contaminants in semi-final gelatine extracts	98
DIESVBS6	*Sphingomonas sp*. clone FI012	Human oral cavity	99
	Sphingomonas sp. strain B28161	From paper processing machines	98
	Novosphingobium pentaromativorans strain US6-1	High-molecular-mass polycyclic aromatic hydrocarbon-degrading bacterium isolated from estuarine sediment	96
DIESVBS7	Uncultured bacterium clone: Sc-EB04.	Suiyo Seamount hydrothermal vent water	99
	Enterobacter dissolvens LMG 2683	Phytopathogens within the Enterobacteriaceae	98
	Enterobacter sp. D1	A toxaphene degrading bacterium isolated from aged contaminated soil in Picacho, Chinandega, Nicaragua	98
DIESVBS8	Uncultured bacterium clone 1700b-13	Colonization and Succession of Microbes in extreme environments volcanic deposit from 1700	94
	Uncultured bacterium clone B18	Polycyclic aromatic hydrocarbon-degrading microbial communities	91
	Bacterium PE03-7G26	Freshwater sediment	93
DIESVBS9	*Isoptericola variabilis* strain:c95	Phenol-degrading *Variovorax* strains responsible for efficient trichloroethylene degradation in a chemostat enrichment culture	99
	Cellulomonas variformis strain MX5	Symbiotic bacterium isolated from the hindgut of the *Mastotermes darwiniensis*	99
	Actinomycetaceae isolate SR 272	Genomic DNA	99

Appendix 3 (continuation)

Strain ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
DIESVBS10	*Enterobacter cloacae* isolate CR1	Rhizobacteria (PGPR) from corn and its effects on two corn varieties	94
	Enterobacter hormaechei subsp. steigerwaltii strain EN-562T	Surgical skin wound of a 49 year old patient with tonsillar carcinoma	94
	Enterobacter hormaechei subsp. oharae strain EN-314T	Mouth swab 2 year old infant	94
DIESVBS11	*Bacillus cereus* strain CICC10185	Genomic DNA	100
	Bacillus sp. P07	Genomic DNA	100
	Bacillus anthracis strain JH18	Genomic DNA	100
DIESVBS12	*Beta proteobacterium* MBIC3293	Isolated from the river water of Shichigasyuku	99
	Roseateles depolymerans strain 61B2 (DSM11814)	Bacterial isolates degrading aliphatic polycarbonates	98
	Roseateles sp. MC12	Soil bacteria that degrade aliphatic polyesters available commercially as biodegradable plastics	98
DIESVBS13	Bacterium WS01_1416	Cr(VI) reducing *Cellulomonas spp*. from subsurface soils	96
	Cellulomonas parahominis strain W7385	Genomic DNA	96
	Uncultured *actinobacterium* clone BPC1_H06	Acid-impacted and pristine subalpine stream sediments	95
DIESVBS15	*Alcaligenes sp*. O-1	Degradation of 2-aminobenzenesulfonate	99
	Alcaligenes sp. mp-2	Microbial Diversity Hotspot Lake Waiau water	99
	Unidentified *bacterium* clone W1B-B04	Genomic DNA	99

Appendix 3 (continuation)

Strain ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	*Similarity %*
DIESVBS16	*Bacillus cereus* strain CICC10185	Genomic DNA	100
	Bacillus sp. P07	Genomic DNA	100
	Bacillus anthracis strain JH18	Genomic DNA	100
DIESVBO1	*Xanthobacter agilis*	Genomic DNA	87
	Unidentified *eubacterium* clone vadinBA44	Fluidized bed anaerobic digestor fed by vinasses (waste from a wine distillery)	86
	Xanthobacter viscosus sp. nov.	Genomic DNA	86
DIESVBO2	*Diaphorobacter sp*. R-25011	Denitrification using defined growth media	86
	Uncultured *beta proteobacterium* clone:OS1L-9	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading denitrifiers in activated sludge anaerobic degradation of 3,4-dihydroxybenzoate	86
	Acidovorax sp. 3DHB1	Genomic DNA	86
DIESVBO3	*Isoptericola variabilis* strain:c95	Phenol-degrading *Variovorax* strains responsible for efficient trichloroethylene degradation in a chemostat enrichment culture	99
	Cellulomonas variformis strain MX5	Symbiotic bacterium isolated from the hindgut of the *Mastotermes darwiniensis*	99 99
	Actinomycetaceae isolate SR 272	Genomic DNA	
DIESVBN1	*Bacillus sp*. Bt176 OTU1	Rhizospheric and soil eubacterial communities PCB-degrading	99
	Bacillus sp. OUCZ63	Bacteria associated with plant roots at a contaminated site	99
	Bacillus megaterium isolate CECRIbio 04	Naphthalene storage tank biofilm	99

Appendix 3 (continuation)

Strain ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
DIESVBN2	Low G+C Gram-positive *bacterium* D-N(1)-1B	Antibiotic Resistant Bacteria in the Sediment of Southern Basin of Lake Biwa	99
	Bacillus sp. Bt176 OTU1	Rhizospheric and soil eubacterial communities PCB-degrading	99
	Bacillus sp. OUCZ63	Bacteria associated with plant roots at a contaminated site	99
DIESVBN3	Uncultured beta *proteobacterium* clone S-04	Phenol-degrading bacteria	92
	Uncultured *Comamonas sp.* clone 6H	Isolated in activated sludge (atrazine-degrading bacterial consortia isolated from bulk- and maize rhizosphere soil)	91
	Uncultured *bacterium* clone E65	Deep sea sediment core from the tropic western Pacific warm pool	91
DIESVBN4	*Azospira sp.* R-25019	Denitrification using defined growth media	99
	Uncultured *beta proteobacterium* clone ccslm36	Microbial populations of TCE-contaminated site before and after in situ bioremediation treatment	99
	Dechlorosoma sp. PCC 16S	Perchlorate-reducing, hydrogen-oxidizing heterotroph activated sludge	99
DIESVBN5	Low G+C Gram-positive *bacterium* D-N(1)-1B	Antibiotic Resistant Bacteria in the Sediment of Southern Basin of Lake Biwa	99
	Bacillus sp. Bt176 OTU1	Rhizospheric and soil eubacterial communities PCB-degrading	99
	Bacillus sp. OUCZ63	Bacteria associated with plant roots at a contaminated site	99
DIESVBN6	*Pseudomonas putida* isolate BCNU106	Toluene-tolerant bacterium	99
	Pseudomonas putida strain NA-1	Nicotinic acid hydroxylation transformation	99
	Pseudomonas sp. XQ-3	Contaminated Soil (Genomic DNA)	99

Appendix 4. Identity clone analysis for the 16S rDNA gene library of the microbial community for 61 days of bioremediation treatment using the BLAST sequence match tool.

Clone ID	*Top Three BLAST Hits Sequence Match*	*BLAST Sequence Match Source Information*	**Similarity %**
CO2	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	98
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone 178up	Equine fecal contamination	97
CO3	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO4	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	98
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone 178up	Equine fecal contamination	97
CO7	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO8	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98

Appendix 4 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CO9	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	99
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO10	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	99
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO11	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO17	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	99
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO19	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98

Appendix 4 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CO20	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	99
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO21	*Shigella boydii* strain 5216-70	Genomic DNA	99
	Uncultured *gamma proteobacterium* clone Dpcom135	Raw liquid sewage	99
	Uncultured *bacterium* clone C436	From human stool sample	99
CO22	Uncultured soil *bacterium* clone PYR10d11	PAH-contaminated soil	97
	Thiocystis violacea type strain DSMZ 207T	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Rhabdochromatium marinum	A purple sulfur bacterium from a salt marsh microbial mat	89
CO23	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	99
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO25	Uncultured alpha *proteobacterium* clone:APe9_6	Microorganisms in aposymbiotic pea aphids, *Acyrthosiphon pisum*	97
	Uncultured *bacterium* clone 267ds10	Equine fecal contamination	97
	Uncultured alpha *proteobacterium* clone JG37-AG-96	Bacterial communities found in uranium mining waste piles and mill tailings	96

Appendix 4 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CO28	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO31	*Beta proteobacterium* Rufe9b	Nitrogen-fixation strains isolated from wild rice	95
	Uncultured bacterium clone:TSAU03	Polychlorinated-dioxin dechlorinating microbial community	95
	Beta proteobacterium Rufe9	Nitrogen-fixation strains isolated from wild rice	95
CO32	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	99
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO33	Uncultured soil *bacterium* clone PYR10d11	PAH-contaminated soil	97
	Thiocystis violacea type strain DSMZ 207T	Purple sulfur bacterium isolated from coastal lagoon sediments	91
	Rhabdochromatium marinum	A purple sulfur bacterium from a salt marsh microbial mat	90
CO39	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98

Appendix 4 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CO40	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	99
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO55	Uncultured alpha *proteobacterium* clone:APe9_6	Microorganisms in aposymbiotic pea aphids, *Acyrthosiphon pisum*	97
	Uncultured *bacterium* clone 267ds10	Equine fecal contamination	96
	Uncultured alpha *proteobacterium* clone JG37-AG-96	Bacterial communities found in uranium mining waste piles and mill tailings	96
CO58	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d2	Degradation of polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CO60	Uncultured soil *bacterium* clone PYR10d11	PAH-contaminated soil	97
	Thiocystis violacea type strain DSMZ 207T	Purple sulfur bacterium isolated from coastal lagoon sediments	91
	Rhabdochromatium marinum	A purple sulfur bacterium from a salt marsh microbial mat	90

Appendix 5. Identity clone analysis for the 16S rDNAgene library of the microbial community for 212 days of bioremediation treatment using the BLAST sequence match tool.

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	*Similarity %*
CM1	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	89
CM3	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum-contaminated soil	90
CM4	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CM6	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *gamma proteobacterium* clone HPDOMI2D01	Microbial communities from stromatolites of Hamelin Pool in Shark Bay, Western Australia	90
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM10	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum-contaminated soil	90
CM11	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
CM12	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
CM13	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	92

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM16	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	89
CM17	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum-contaminated soil	90
CM20	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *gamma proteobacterium* clone HPDOMI2D01	Microbial communities from stromatolites of Hamelin Pool in Shark Bay, Western Australia	90
	Uncultured *bacterium* clone RB146	Microbial community structure in rhizosphere of Phragmites	89
CM22	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	88

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM23	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Uncultured bacterium clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	89
CM24	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured bacterium clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	89
CM25	Uncultured beta proteobacterium clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured bacterium clone 178up	Equine fecal contamination	98
CM30	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	95
	Uncultured bacterium clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	89

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM31	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	91
	Uncultured bacterium clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
CM32	Uncultured beta proteobacterium clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured bacterium clone 178up	Equine fecal contamination	98
CM33	Uncultured beta proteobacterium clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured bacterium clone 178up	Equine fecal contamination	98
CM34	Uncultured delta proteobacterium clone AKYG1825	Farm soil adjacent to a silage storage bunker	90
	Uncultured bacterium clone FW35	Forested wetland	90
	Uncultured delta proteobacterium clone AKYG886	Farm soil adjacent to a silage storage bunker	90
CM35	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured bacterium clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
	Pseudomonas sp. Pss 14	Novel species from farm soils in Korea	89

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM36	Uncultured soil bacterium clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	91
	Uncultured bacterium clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
CM40	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CM41	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Rhabdochromatium marinum	A purple sulfur bacterium from a salt marsh microbial mat	89
CM42	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	98
	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone 178up	Equine fecal contamination	97

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM43	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	90
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
CM45	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
CM48	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CM49	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	96
	Uncultured *bacterium* clone 178up	Equine fecal contamination	96
	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM55	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	90
CM66	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	90
CM68	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	90
CM69	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	93
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	93
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	86
CM72	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	89

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM75	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
CM76	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	94
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	89
CM79	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	89
CM82	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	96
	Pseudomonas sp. 273	An aerobic alpha,omega-dichloroalkane degrading bacterium	89

Appendix 5 (continuation)

Clone ID	Top Three BLAST Hits Sequence Match	BLAST Sequence Match Source Information	Similarity %
CM83	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	98
	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Uncultured *bacterium* clone 178up	Equine fecal contamination	97
CM91	Uncultured *bacterium* clone B182	Deep sea sediment	89
	Uncultured *gamma proteobacterium* clone 92	100 meter deep seawater	89
	Uncultured *gamma proteobacterium* clone 9	100 meter deep seawater	89
CM93	Uncultured *beta proteobacterium* clone 36-9	Naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters	99
	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	98
	Uncultured *bacterium* clone 178up	Equine fecal contamination	98
CM94	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	96
	Uncultured *bacterium* clone SL6	Manganese-oxidizing microorganisms and manganese deposition during biofilm formation on stainless steel in a brackish surface water	97
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	90
CM95	Uncultured soil *bacterium* clone PYR10d11	Polycyclic aromatic hydrocarbons in a bioreactor treating contaminated soil	97
	Thiocystis violacea	Purple sulfur bacterium isolated from coastal lagoon sediments	90
	Uncultured *bacterium* clone:BSC51	Petroleum contaminated soil	90

Appendix 6: *In silico* analysis of terminal restriction fragments for the BAC isolated strains and sampled clones

Strain ID/Clone ID	***Hae*III Terminal restriction fragment (bp)**	***Rsa*I Terminal restriction fragment (bp)**	***Msp*I Terminal restriction fragment (bp)**
DIESVBS5	384	346	68
DIESVBS6	382	344	66
DIESVBS7	189	346	70
DIESVBS8	347	11	336
DIESVBS9	3	99	14
DIESVBS10	186	11	67
DIESVBS11	351	347	617
DIESVBS12	196	13	68
DIESVBS13	199	96	598
DIESVB15	290	342	99
DIESVBS16	352	348	6
DIESVBO1	653	NON-CUT	608
DIESVBO2	549	312	70
DIESVBO3	196	94	457
DIESVBN1	384	346	618
DIESVBN2	385	347	619
DIESVBN3	189	742	67
DIESVBN4	348	9	66
DIESVBN5	387	349	621
DIESVBN6	336	98	55
CO2	332	308	67
CO3	333	309	68
CO4	333	309	68
CO7	333	309	68
CO8	332	10	67
CO9	332	308	67
CO10	333	309	68
CO11	331	307	66
CO17	330	306	65
CO19	334	12	69
CO20	334	310	69
CO21	189	346	69
CO22	349	345	595

Appendix 6 (continuation)

Strain ID/Clone ID	*Hae*III Terminal restriction fragment (bp)	*Rsa*I Terminal restriction fragment (bp)	*Msp*I Terminal restriction fragment (bp)
CO23	330	8	65
CO25	378	340	63
CO28	333	309	68
CO31	334	310	69
CO32	334	310	69
CO33	347	10	132
CO39	331	307	68
CO40	323	299	58
CO55	377	339	62
CO58	333	309	68
CO60	348	344	594
CM1	349	345	595
CM3	349	345	67
CM4	331	307	68
CM6	349	701	595
CM10	349	345	595
CM11	348	115	594
CM12	349	345	595
CM13	429	425	697
CM16	347	343	593
CM17	347	343	593
CM20	349	11	595
CM22	346	342	592
CM23	346	10	592
CM24	347	9	593
CM25	332	308	67
CM30	343	7	610
CM31	347	9	593
CM32	333	11	68
CM33	333	309	68
CM34	187	110	113
CM35	349	345	616

Appemdix 6 (continuation)

Strain ID/Clone ID	*Hae*III Terminal restriction fragment (bp)	*Rsa*I Terminal restriction fragment (bp)	*Msp*I Terminal restriction fragment (bp)
CM35	349	345	616
CM36	349	11	595
CM40	331	307	66
CM41	345	341	591
CM42	341	317	6
CM43	349	11	595
CM45	349	11	595
CM48	331	307	66
CM49	331	307	68
CM55	355	351	601
CM66	349	345	67
CM68	349	345	595
CM69	175	7	49
CM72	347	343	593
CM75	348	344	594
CM76	348	10	67
CM79	349	345	6
CM82	350	346	617
CM83	333	309	68
CM91	185	342	64
CM93	334	11	68
CM94	347	343	4
CM95	350	346	595

Appendix 7. Organic degradation shared genes results by Functional gene microarrays of the BAC community samples

		30 days	*61 days*	*153 days*	*212 days*
GeneName	*Description*	*SNR Average*	*SNR Average*	*SNR Average*	*SNR Average*
MTBE	putative alkane 1-monooxygenase [Burkholderia cepacia].	ND	3.97 (1.89)	ND	3.96 (1.99)
biphenyl	dihydroxy naphthalene/biphenyl dioxygenase [Novosphingobium aromaticivorans].	ND	15.12 (16.88)	33.65 (7.99)	8.64 (5.45)
cresol	flavoprotein subunit p-cresol methylhydroxylase [Novosphingobium aromaticivorans].	ND	15.51 (0.91)	11.45 (2.84)	4.74 (3.18)
benzoate-anaerobic	4-hydroxybenzoyl-CoA reductase HbaD subunit (Rhodopseudomonas palustris CGA009)	6.19 (3.07)	ND	ND	13.57 (8.01)
phthalate	transporter permease 2 [Arthrobacter keyseri].	ND	ND	3.74(0.36)	ND
phthalate	putative phthalate ester hydrolase [Arthrobacter keyseri].	13.28 (7.73)	3.38 (1.31)	7.65 (0.61)	12.41 (12.91)
phthalate	phthalate dioxygenase large subunit [Arthrobacter keyseri].	4.83 (2.23)	4.31 (1.34)	9.57 (1.56)	7.60 (4.66)
phthalate	3,4-dihydroxyphthalate 2-decarboxylase [Arthrobacter keyseri].	5.33 (1.53)	3.90 (1.41)	12.01 (0.59)	6.85 (2.51)
MTBE	alkane 1-monooxygenase [Pseudomonas fluorescens].	3.44 (1.38)	3.69 (1.45)	9.76 (0.39)	4.34 (1.73)
acetylene	epoxide hydrolase [Mesorhizobium loti MAFF303099].	ND	3.56 (1.51)	ND	5.25 (2.85)
benzoate-anaerobic	Acetyl-CoA acetyltransferase (Acetoacetyl-CoA thiolase) (Wautersia eutropha)	ND	7.83 (5.52)	29.21 (4.87)	9.97 (6.89)
MTBE	alkane 1-monooxygenase [Prauserella rugosa].	ND	3.73 (1.66)	9.67 (2.23)	3.89 (1.29)
toluene-aerobic	benzyl alcohol dehydrogenase [Acinetobacter calcoaceticus].	3.52 (1.88)	ND	ND	5.72 (2.85)
phenol-aerobic	phenol hydroxylase large subunit [uncultured microorganism PCRTD02].	ND	ND	16.06 (6.52)	ND
octane	alkane-1-monooxygenase [Rhodococcus fascians].	ND	ND	ND	3.88 (1.20)
Protocatechuate	protocatechuate 3,4-dioxygenase beta subunit [Silicibacter sp. DSS-3].	ND	5.79 (2.93)	8.18 (1.63)	ND
protocatechuate	protocatechuate 3,4-dioxygenase beta subunit [Sulfitobacter sp. GAI-37].	4.16 (1.79)	ND	ND	4.05 (1.78)
MTBE	alkane-1-monooxygenase [Rhodococcus sp. Q15].	ND	2.44 (0.99)	ND	ND
benzoate-anaerobic	thiolase (acetyl-CoA acetyltransferase) [Bacillus halodurans C-125].	4.71 (2.59)	4.39 (2.03)	10.71 (1.14)	4.83 (1.49)
benzoate-anaerobic	Acetyl-CoA c-acetyltransferase (acetoacetyl-CoA thiolase) (acaB-1) [Sulfolobus solfataricus P2].	7.63 (3.20)	ND	ND	13.56 (7.67)
thiocyanate	Carbon monoxide dehydrogenase, large chain (cutA-1) [Sulfolobus solfataricus P2].	4.29 (1.56)	4.64 (1.86)	9.08 (0.91)	5.47 (2.64)
toluene-aerobic	Toluene-4-monooxygenase system protein A. carboxy end fragment (tmoA) [Sulfolobus solfataricus P2].	4.67 (2.07)	ND	ND	ND
thiocyanate	Carbon monoxide dehydrogenase, medium chain. (cutB-2) [Sulfolobus solfataricus P2].	4.13 (1.20)	3.08 (1.06)	ND	5.56 (2.59)
phthalate	445aa long hypothetical 4-methyl-o-phthalate/phthalate permease [Sulfolobus tokodaii str. 7].	9.37 (5.65)	2.82 (1.14)	4.03 (0.60)	19.55 (12.12)

Appendix 7 (continuation)

		30 days	*61 days*	*153 days*	*212 days*
GeneName	Description	SNR Average	SNR Average	SNR Average	SNR Average
octane	putative alkane-1-monooxygenase 4 [Rhodococcus erythropolis].	ND	5.36 (2.99)	ND	ND
protocatechuate	protocatechuate 3,4-dioxygenase, alpha subunit [Caulobacter crescentus CB15].	2.94 (1.01)	6.59 (3.99)	20.30 (2.24)	13.90 (11.30)
protocatechuate	putative protocatechuate 3,4-dioxygenase alpha chain protein [Sinorhizobium meliloti 1021].	3.37 (1.32)	11.62 (6.23)	31.43 (1.86)	12.51 (7.28)
naphthalene	PUTATIVE 2-HYDROXYCHROMENE-2-CARBOXYLATE ISOMERASE PROTEIN [Ralstonia solanacearum GMI1000].	ND	3.78 (1.61)	9.55 (2.61)	ND
protocatechuate	PROBABLE PROTOCATECHUATE 3,4-DIOXYGENASE (BETA CHAIN) OXIDOREDUCTASE PROTEIN [Ralstonia solanacearum GMI1000].	ND	4.81 (3.05)	10.39 (2.09)	7.04 (3.64)
benzoate-anaerobic	PROBABLE ACETOACETYL-COA REDUCTASE OXIDOREDUCTASE PROTEIN [Ralstonia solanacearum GMI1000].	ND	4.13 (2.59)	8.39 (1.37)	4.20 (0.85)
phenol-aerobic	phenol hydroxylase subunit PhkA [Burkholderia kururiensis].	4.88 (2.25)	2.37 (0.82)	ND	4.35 (1.43)
phthalate	PHTHALATE TRANSPORTER. [Escherichia coli].	ND	10.94 (1.42)	11.87 (3.84)	3.93 (1.98)
MTBE	putative alkane-1-monooxygenase [Rhodococcus sp. Q15].	5.27 (2.74)	ND	ND	ND
benzoate-anaerobic	benzoyl CoA reductase subunit [Azoarcus evansii].	ND	ND	5.90 (2.10)	ND
benzoate-anaerobic	benzoyl CoA reductase subunit [Azoarcus evansii].	ND	ND	7.41 (2.54)	ND
cyclohexanol	cyclohexanol dehydrogenase [Xanthobacter flavus].	ND	4.50 (2.68)	7.73 (1.66)	3.49 (2.01)
biphenyl	biphenyl dioxygenase [Wautersia eutropha].	5.46 (2.27)	2.31 (0.67)	3.65 (0.60)	7.22 (4.97)
protocatechuate	protocatechuate 3,4-dioxygenase beta subunit [Corynebacterium glutamicum ATCC 13032].	ND	ND	7.42 (2.28)	7.07 (3.57)
benzoate-anaerobic	oxidoreductase [Corynebacterium glutamicum ATCC 13032].	3.46 (1.58)	ND	ND	4.16 (2.83)
thiocyanate	Cyanate hydratase (Cyanase) (Cyanate lyase) (Cyanate hydrolase)(Aquifex aeolicus)	3.49 (1.48)	3.63 (1.77)	ND	ND
thiocyanate	Cyanate hydratase (Cyanase) (Cyanate lyase) (Cyanate hydrolase) (Pseudomonas aeruginosa)	ND	9.17 (5.00)	14.61 (4.47)	ND
benzene	putative dioxygenase ferredoxin subunit [Streptomyces coelicolor A3(2)].	ND	ND	5.87 (0.55)	ND
thiocyanate	CO dehydrogenase/acetyl-COA synthase beta subunit [Methanosarcina mazei Go1].	3.20 (1.50)	ND	ND	ND
benzoate-anaerobic	aldehyde dehydrogenase [Xanthomonas campestris pv. campestris str. ATCC 33913].	ND	4.11 (2.11)	7.69 (2.14)	3.19 (1.25)
protocatechuate	protocatechuate 3,4-dioxygenase alpha chain [Xanthomonas axonopodis pv. citri str. 306].	ND	5.35 (2.67)	16.71 (2.01)	4.97 (2.63)
protocatechuate	protocatechuate 3,4-dioxygenase beta chain [Xanthomonas axonopodis pv. citri str. 306].	ND	4.13 (2.07)	6.55 (1.38)	ND

Appendix 7 (continuation)

		30 days	*61 days*	*153 days*	*212 days*
GeneName	Description	SNR Average	SNR Average	SNR Average	SNR Average
phenol-aerobic	phenol hydroxylase [Xanthomonas axonopodis pv. citri str. 306].	ND	5.65 (2.79)	ND	ND
protocatechuate	protocatechuate 3,4-dioxygenase beta subunit [Acinetobacter lwoffii].	ND	ND	7.21 (2.55)	ND
phenol-aerobic	phenol hydroxylase oxygenase component [Acinetobacter radioresistens].	ND	ND	ND	3.91 (1.33)
xylene	XylL [Pseudomonas putida].	2.71 (1.11)	2.46 (1.21)	ND	ND
benzoate-anaerobic	glutaryl-CoA dehydrogenase [Bordetella bronchiseptica RB50].	ND	ND	7.36 (1.19)	3.76 (2.03)
protocatechuate	COG3485: Protocatechuate 3,4-dioxygenase beta subunit [Pseudomonas fluorescens PfO-1]	ND	5.66 (2.61)	ND	ND
benzoate-anaerobic	COG1775: Benzoyl-CoA reductase/2-hydroxyglutaryl-CoA dehydratase subunit, BcrC/BadD/HgdB [Geobacter metallireducens].	3.69 (0.88)	ND	6.21 (0.68)	5.10 (1.19)
naphthalene	COG3917: 2-hydroxychromene-2-carboxylate isomerase [Rhodospirillum rubrum].	2.90 (1.44)	ND	8.00 (1.78)	3.99 (2.01)
naphthalene	COG3917: 2-hydroxychromene-2-carboxylate isomerase [Rhodospirillum rubrum].	ND	ND	7.57 (2.75)	3.33 (2.06)
naphthalene	COG3917: 2-hydroxychromene-2-carboxylate isomerase [Rhodospirillum rubrum].	3.16 (1.12)	2.79 (0.96)	ND	5.23 (1.90)
benzoate-anaerobic	COG1775: Benzoyl-CoA reductase/2-hydroxyglutaryl-CoA dehydratase subunit, BcrC/BadD/HgdB [Desulfitobacterium hafniense].	ND	ND	20.12 (4.15)	6.99 (4.01)
naphthalene	COG3917: 2-hydroxychromene-2-carboxylate isomerase [Nostoc punctiforme].	6.76 (3.12)	ND	ND	ND
protocatechuate	COG3485: Protocatechuate 3,4-dioxygenase beta subunit [Pseudomonas syringae pv. syringae B728a].	ND	11.95 (1.51)	33.00 (11.71)	7.18 (6.19)
biphenyl	ferredoxin reductase BPH [Rhodococcus globerulus].	3.60	ND	3.14 (0.31)	ND
acetylene	epoxide hydrolase [Streptomyces globisporus].	ND	ND	ND	3.80 (1.75)
thiocyanate	Thiocyanate hydrolase beta subunit (Thiobacillus thioparus)	ND	5.68 (3.57)	14.67 (4.38)	3.96 (2.10)
biphenyl	2,3-dihydroxybiphenyl 1,2-dioxygenase [Bacillus sp. JF8].	5.86 (3.11)	ND	ND	ND
benzoate-anaerobic	acetoacetyl-CoA reductase [Bordetella parapertussis 12822].	ND	ND	5.12 (0.77)	4.01 (1.48)
aniline	aniline dioxygenase beta-subunit [Acinetobacter sp. YAA].	3.14 (1.57)	3.57 (1.39)	5.33 (0.71)	4.23 (1.41)
aniline	HYPOTHETICAL OXIDOREDUCTASE [Mycobacterium bovis AF2122/97].	ND	5.06 (2.79)	14.55 (3.46)	6.00 (3.77)
protocatechuate	protocatechuate 3,4-dioxygenase beta chain [Bradyrhizobium japonicum USDA 110].	5.11 (1.33)	4.04 (1.52)	9.51 (1.06)	6.13 (2.88)
cyclohexanol	cyclohexanone monooxygenase [Bradyrhizobium japonicum USDA 110].	4.44 (1.76)	4.24 (1.73)	9.32 (0.82)	6.40 (2.90)
phthalate	3,4-dihydroxy-3,4-dihydrophthalate dehydrogenase [Terrabacter sp. DBF63].	4.44 (2.33)	6.35 (3.73)	6.63 (0.82)	4.25 (1.59)
benzoate-anaerobic	ferredoxin [Tropheryma whipplei str. Twist].	ND	3.41 (1.34)	8.20 (1.00)	3.05 (1.34)
phenol-aerobic	phenol hydroxylase component phL [Pseudomonas stutzeri].	3.45 (1.65)	ND	ND	ND
xylene	putative oxygenase component of xylene monooxygenase [Sphingomonas sp. P2].	ND	ND	ND	3.57 (1.65)
benzoate-anaerobic	putative glutaryl-CoA dehydrogenase [Streptomyces avermitilis MA-4680].	ND	ND	ND	5.61 (2.44)

Appendix 7 (continuation)

		30 days	*61 days*	*153 days*	*212 days*
GeneName	Description	SNR Average	SNR Average	SNR Average	SNR Average
phenol-aerobic	phenol hydroxylase [uncultured soil bacterium].	ND	ND	11.20 (2.07)	ND
biphenyl	dioxygenase (Pseudomonas pseudoalcaligenes)	2.63 (1.23)	ND	ND	ND
benzoate-anaerobic	ferredoxin [Halobacterium salinarum].	ND	3.41 (1.22)	13.27 (1.30)	9.45 (6.83)
biphenyl	2,3-dihydroxybiphenyl 1,2-dioxygenase [Comamonas testosteroni].	ND	4.99 (2.24)	14.88 (2.87)	5.40 (2.51)
toluene-anaerobic	benzylsuccinate synthase gamma subunit [Thauera aromatica].	6.26 (1.97)	5.40 (2.11)	15.41 (0.96)	7.98 (2.23)
toluene-anaerobic	benzylsuccinate synthase beta subunit [Thauera aromatica].	ND	3.88 (1.15)	12.23 (0.94)	4.32 (2.07)
acetylene	probable ephA protein-Mycobacterium tuberculosis (strain H37RV) [Pirellula sp. 1].	4.39 (1.97)	6.57 (4.21)	11.40 (1.18)	6.48 (2.06)
biphenyl	biphenyl dihydrodiol dehydrogenase [Bacillus sp. JF8].	6.86 (2.19)	4.22 (2.41)	16.08 (1.26)	8.15 (2.56)
xylene	XylL [Pseudomonas sp. SV15].	ND	4.71 (2.27)	ND	ND
biphenyl	dioxygenase (Pseudomonas pseudoalcaligenes)	3.77 (0.80)	ND	4.26 (0.46)	5.63 (2.74)
acetylene	acetylene hydratase Ahy [Pelobacter acetylenicus].	5.68 (2.28)	4.27 (2.19)	11.74 (0.91)	6.42 (3.39)
benzoate-anaerobic	probable short chain dehyrogenase; putative acetoacetyl-CoA reductase [Bordetella bronchiseptica RB50].	ND	6.29 (4.20)	21.19 (4.14)	8.19 (5.01)
phenol-aerobic	phenol hydroxylase component; PoxA [Ralstonia sp. E2].	ND	6.43 (2.74)	12.16 (2.71)	ND
phenol-aerobic	phenol hydroxylase component; PoxF [Ralstonia sp. E2].	2.89 (1.19)	ND	ND	ND
biphenyl	probable biphenyl-2,3-diol 1,2-dioxygenase III [Chromobacterium violaceum ATCC 12472].	ND	ND	4.30 (0.91)	ND
protocatechuate	Protocatechuate 3,4-Dioxygenase beta chain [Rhizobium sp. NGR234].	ND	8.87 (1.03)	23.93 (7.88)	8.28 (5.64)
benzoate-anaerobic	D-subunit of benzoyl-CoA reductase [Thauera aromatica].	ND	10.45 (5.87)	ND	ND
naphthalene	ferredoxin [Pseudomonas putida].	ND	ND	ND	3.97 (1.94)
protocatechuate	putative protocatechuate 3,4 dioxygenase alpha subunit [marine alpha proteobacterium SE45].	22.03 (14.05)	11.30 (2.20)	38.79 (13.74)	8.89 (4.81)
protocatechuate	putative protocatechuate 3,4 dioxygenase alpha subunit [Roseovarius nubinhibens].	ND	8.11 (1.93)	15.87 (4.44)	4.33 (2.26)
protocatechuate	putative protocatechuate 3,4 dioxygenase beta subunit [marine alpha proteobacterium Y4I].	ND	ND	7.21 (1.08)	2.73 (1.44)
thiocyanate	ACDS complex carbon monoxide dehydrogenase 1(Methanopyrus kandleri)	8.40 (3.54)	3.94 (1.47)	5.02 (0.44)	7.48 (5.86)
naphthalene	salicylaldehyde dehydrogenase [Pseudomonas sp. ND6].	4.29 (1.76)	ND	4.91 (0.53)	5.31 (2.83)
biphenyl	receptor-like histidine kinase [Rhodococcus erythropolis].	12.42 (6.00)	13.16 (1.32)	12.20 (2.18)	6.23 (4.32)
benzoate-anaerobic	putative alcohol dehydrogenase [Rhodopseudomonas palustris CGA009].	ND	4.97 (2.55)	11.08 (2.95)	ND
phthalate	possible phthalate dioxygenase [Rhodopseudomonas palustris CGA009].	ND	3.38 (1.22)	ND	3.77 (1.94)
thiocyanate	putative cyanate lyase [Rhodopseudomonas palustris CGA009].	ND	8.29 (6.55)	23.75 (4.20)	9.15 (5.36)
thiocyanate	carbon monoxide dehydrogenase medium subunit [Rhodopseudomonas palustris CGA009].	ND	3.88 (1.71)	7.13 (1.33)	4.20 (2.09)
thiocyanate	carbon monoxide dehydrogenase subunit [Geobacter sulfurreducens PCA].	3.69 (1.14)	ND	4.95 (0.97)	3.84 (1.33)

Appendix 7 (continuation)

		30 days	*61 days*	*153 days*	*212 days*
GeneName	Description	SNR Average	SNR Average	SNR Average	SNR Average
benzoate-anaerobic	ferredoxin, 2Fe-2S [uncultured bacterium 580].	3.43 (0.79)	3.18 (1.31)	6.46 (1.06)	4.70 (2.71)
benzoate-anaerobic	ferredoxin [Desulfotomaculum thermocisternum].	3.26 (0.70)	2.93 (1.07)	4.54 (0.59)	5.98 (2.73)
thiocyanate	carbon monoxide dehydrogenase middle subunit CoxM/CutM homologues [Thermoproteus tenax].	4.47 (1.47)	3.65 (1.36)	8.93 (0.86)	6.01 (2.91)
benzoate-anaerobic	ferredoxin [Campylobacter jejuni].	9.16 (4.18)	ND	ND	13.19 (11.22)
toluene-aerobic	xylB (Pseudomonas putida)	3.34 (1.78)	ND	ND	ND
benzoate-anaerobic	atoB; B1549_C1_166 [Mycobacterium leprae].	ND	4.00 (1.99)	ND	ND
protocatechuate	protocatechuate 3,4-dioxygenase, beta subunit [Pseudomonas putida KT2440].	ND	ND	ND	5.46 (3.10)
toluene-aerobic	AreB [Acinetobacter sp. ADP1].	ND	6.28 (3.15)	ND	5.88 (3.58)
protocatechuate	protocatechuate-3,4-dioxygenase beta subunit (Bradyrhizobium japonicum)	ND	2.61 (0.97)	6.52 (1.33)	ND
phenol-anaerobic	4-hydroxybenzoate decarboxylase [Clostridium hydroxybenzoicum].	ND	ND	8.00 (3.14)	ND
phenol-aerobic	phenol hydroxylase component [Ralstonia sp. KN1].	ND	5.32 (2.65)	12.24 (3.37)	4.90 (2.36)
phenol-aerobic	phenol hydroxylase component [Ralstonia sp. KN1].	ND	5.59 (2.62)	7.24 (1.74)	ND
phenol-aerobic	phenol hydroxylase (Geobacillus stearothermophilus)	5.47 (2.54)	ND	ND	ND
biphenyl	2,3-dihydroxybiphenyl 1,2-dioxygenase [Rhodococcus sp. RHA1].	ND	2.66 (0.71)	10.12 (0.87)	3.72 (1.75)
biphenyl	2,3-dihydroxybiphenyl 1,2-dioxygenase [Rhodococcus sp. RHA1].	ND	4.73 (3.07)	9.21 (1.58)	4.36 (1.38)
protocatechuate	a-subunit of protocatechuate 3,4-dioxygenase [Pseudomonas putida].	ND	4.27 (1.85)	6.19 (0.74)	2.72 (1.38)
benzoate-anaerobic	probable acetyl-CoA acetyltransferase [Salmonella enterica subsp. enterica serovar Typhi str. CT18].	2.93 (0.91)	ND	ND	ND
cyclohexanol	cyclohexanone monooxygenase 1 [Brevibacterium sp. HCU].	ND	ND	ND	2.94 (1.44)