

Microbial Diversity and Bioremediation of a Hydrocarbon-Contaminated Aquifer (Vega Baja, Puerto Rico)

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Abstract: Hydrocarbon contamination of groundwater resources has become a major environmental and human health concern in many parts of the world. Our objectives were to employ both culture and culture-independent techniques to characterize the dynamics of microbial community structure within a fluidized bed reactor used to bioremediate a diesel-contaminated groundwater in a tropical environment. Under normal operating conditions, 97 to 99% of total hydrocarbons were removed with only 14 min hydraulic retention time. Over 25 different cultures were isolated from the treatment unit (96% which utilized diesel constituents as sole carbon source). Approximately 20% of the isolates were also capable of complete denitrification to nitrogen gas. Sequence analysis of 16S rDNA demonstrated ample diversity with most belonging to the α , β and γ subdivision of the *Proteobacteria*, *Bacilli*, and *Actinobacteria* groups. Moreover, the genetic constitution of the microbial community was examined at multiple time points with a Functional Gene Array (FGA) containing over 12,000 probes for genes involved in organic degradation and major biogeochemical cycles. Total community DNA was extracted and amplified using an isothermal ϕ 29 polymerase-based technique, labeled with Cy5 dye, and hybridized to the arrays in 50% formimide overnight at 50°C. Cluster analysis revealed comparable profiles over the course of treatment suggesting the early selection of a very stable microbial community. 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 [*nirS*, *nirK*, and *nosZ*], dissimilatory sulfite reductases [*dsrAB*], potential metal reducing C-type cytochromes, and methane monooxygenase [*pmoA*]) were repeatedly detected. Genes for degradation of MTBE, nitroaromatics and chlorinated compounds were also present, indicating a broad catabolic potential of the treatment unit. FGA's demonstrated the early establishment of a diverse community with concurrent aerobic and anaerobic processes contributing to the bioremediation process.

Keywords: Bioremediation, functional gene microarray, biofilm, tropical environments.

Introduction

Spills and leaks of petroleum hydrocarbons from storage facilities and distribution systems have resulted in the contamination of soil and water systems worldwide. Because of the threat they represent to public health and the need for restoring renewable and non-renewable resources, multiple cleanup strategies for petroleum products have been developed [6, 8, 22]. Microbial

degradation of hydrocarbons, through either naturally occurring processes or engineered systems, has been successfully used to reduce concentrations of these pollutants to safer environmental levels. Pump and treat systems are one engineering approach that allows the design of a treatment units for optimum biological operation. When combined with fixed film microbial growth, such strategies have shown effectiveness in the processing of sewage and contaminated groundwater [5, 10, 18].

One of the major factors limiting the microbial degradation of hydrocarbons in fixed-film communities is the availability of oxygen as final electron acceptor. This limitation can be solved by enhancing microbial activity with alternative electron acceptors [2, 17, 21]. Many anaerobic bacteria are capable of utilizing complex hydrocarbon mixtures, such as crude oil, as carbon and energy sources [1, 9, 7, 15]. Therefore, under inadequate aeration conditions, the use of anaerobic systems with alternative electron acceptors such as nitrate and sulfate can be beneficial. Indeed, a practical advantage in integrating electron acceptors in bioremediation applications is that they are water soluble, inexpensive and favor a more diverse community.

Fluidized bed reactors (FBR) packed with activated carbon as the biomass carrier, have been used for the restoration of contaminated waters [3, 10, 11]. The surface adhesion of microbes, local growth and exopolymer production lead to the formation of robust biofilm communities [20, 26]. The careful study of biofilm communities in FBRs can provide important knowledge for better design and more cost effective operation of engineered restoration strategies. Cultured-based methods are useful for understanding the physiological potential of microorganisms but do not necessarily provide comprehensive information on the composition of 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*. In order to improve our understanding of biofilms, culture-dependent approaches should be complemented with culture-independent techniques.

DNA microarrays have emerged as a leading technique for the analysis of gene expression in pure cultures and environmental samples. Recently, various formats of environmental microarrays have been proposed, developed and evaluated for species detection and microbial community analyses in complex environments [16, 24]. These studies demonstrated the great potential of this approach due to their high specificity, sensitivity and quantitative nature of the data [16]. Microarrays have thus become an excellent tool for microbial detection, identification and characterization in their natural environment. Functional gene arrays (FGAs) are designed for the detection of key genes involved in biological processes such as carbon, nitrogen and sulfur cycles and provide information about the microbial populations controlling these processes. These gene probes on such arrays can be used as signatures for monitoring the potential structure, activities and physiological status of microbial populations and communities that drive these processes in the environment [16].

We used growth-dependent isolation and characterization methods, and community DNA fingerprinting by FGAs, to study the structure of biofilm communities in a FBR fed diesel-contaminated

groundwater supplemented with oxygen, and nitrate/sulfate compounds as supplementary electron acceptors in a tropical environment. We found early selection of a highly diverse natural groundwater-colonized community on the activated carbon. The level of functional diversity observed in these communities could explain the stability and success of the treatment process.

Material and Methods

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 50-liter working volume polyvinyl chloride column reactor (15 cm diameter x 275 cm long) was used to treat the contaminated plume. Groundwater was pumped simultaneously from multiple extraction wells to a 3,000 L equalization tank. After collection, the contaminated groundwater was passively supplemented with an oxygen release compound (ORC[®]) and 0.1 g/L of a nutrient solution (NH₄Cl and KH₂PO₄) 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 (less than 50 ppm of total petroleum hydrocarbons [TPH] by EPA Method 8015), the treated water containing traces of nutrients and microorganisms was reinjected into the aquifer to accelerate *in situ* remediation. To enhance the startup phase of the bioreactor, a mixed-culture from soil samples was grown in minimum culture media (Bushnell-Hass) amended with free diesel product as the sole carbon source. Both soil and free product were collected from the diesel-impacted area. Indigenous populations capable of diesel degradation under aerobic and denitrifying conditions were selected and inoculated into the bioreactor (10⁸-10⁹ CFU/ml, 2.5-L total). 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 on site chemical analysis including dissolved oxygen, pH, temperature, electrical conductivity, turbidity (HORIBA U-10 Water Quality Checker/HACH Portable turbidimeter Model 2100P) and TPH using a UVF-3100 (Site Lab, CO).

Isolation and Characterization of GAC Bacteria

Biofilm samples were collected aseptically from the bottom 30% of the column sampling port on a monthly basis. Cells were removed and homogenized as previously described [11]. Viable bacterial numbers from GAC samples were determined by using R2A medium (Difco, Detroit, Mich.), which was designed for improved recovery of environmental heterotrophs. To isolate numerically dominant bacteria from GAC biofilm communities, dispersed biofilm bacteria from the reactors were diluted and then plated on R2A solid medium. Isolates picked from the terminal dilutions were subcultured three times to ensure purity and screened by traditional microbiological techniques including cell morphology, gram staining and the nitrate reduction test. To determine diesel utilization potential, each isolate was 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 [10ml/L]). The inoculated glass tubes were sealed and incubated in a rotary shaker for 5 days. Positive activity was measured daily by optical density using the HACK spectrophotometer (DR/4000U model) at 660nm. A range for absorbance and a growth scale were assigned to the isolates relative to the non-diesel control tube.

Isolates representing dominant populations were further characterized by partial 16S rRNA gene sequence analyses. DNA was extracted from biomass material collected by centrifugation. Lyses were performed using 25% sucrose TE buffer, lysozyme [5mg/ml], 0.25M EDTA, 10% sodium dodecyl sulfate (SDS), and Proteinase K [10mg/ml]. The DNA was precipitated using two salt solutions at high concentrations: 5M sodium chloride and 8M potassium acetate with 95% ethanol. Finally, the DNA was recovered and purified using 70% ethanol and resuspended in 50 μ L of TE buffer, pH 8.0. DNA concentrations were estimated with spectrophotometric measurements at 260nm and 280nm.

A 900 bp 16S rDNA gene product was obtained from each culture using the primers UNIV 519F (5'-CAGCMGCCGCGGTAATWC-3') and the reverse universal primer UNIV 1392R (5'-ACGGGCGGTGTGTRC-3'). A total of 50 μ L of PCR reaction was prepared as followed: 5.0 μ L of 10X *Taq* polymerase buffer B, 6.0 μ L of 25mM MgCl₂, 1.0 μ L of dNTP's mix [2.5mM each (1:1:1:1 proportion)], 0.75 μ L of [20 mg/ml] BSA, 1.0 μ L of each primer [50pM/ μ L], 0.5 μ L of *Taq* polymerase enzyme (2.5U) (Promega®), the ddH₂O 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/ μ L of each PCR product was used to prepare the samples which were delivered for single-strand sequencing using the 519F-forward primer to MacroGen Company 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.

Functional Gene Array Analysis of the Biofilm Communities

Five grams of activated carbon were used for total community DNA extraction following a protocol for soils and sediments [26]. DNA was cleaned following the Sephadex Purification of DNA from environmental samples protocol [12]. 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 μ L of 10mM Tris-HCl pH 8.5 (pre-warmed at 65°C) and followed by a desalting procedure. Whole Community Genome Amplification (WCGA) by the Rolling Circle Amplification (RCA) was done in triplicate with 10-30 ng/ μ L of template DNA following the protocol described previously [23]. Briefly, one microliter of each DNA was dispensed in 10 μ L of sample buffer (containing random hexamers) and incubated at room temperature for 10 minutes. A master mix containing 10 μ L of reaction buffer (contains salts and deoxynucleotides), 1 μ L of enzyme mixture [1 U/ μ L] (contains *phi* 29 DNA polymerase and random primers in 50% glycerol), and additionally 1 μ L of single strand binding protein [2.67 μ g/ μ L] and 1 μ L of 1mM of spermidine were added for each reaction. 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).

The amplified community DNA was labeled with Cy5 by random priming. To each labeling reaction, 20 μ L of 3mM random hexamers and 0.3 μ L of 10mM spermidine were added. Samples were denatured at 99.9°C for 5 minutes and immediately chilled on ice. A total of 20 μ L of the labeling master mix containing 2.5 μ L of dNTP's mix [2.5mM dTTP, 5mM dACG-TP], 1.0 μ L of Cy5 fluorochrome, 0.7 μ L of 490ng/ μ L of recA, 2.0 μ L of Klenow enzyme and 13.8 μ L of MilliQ ddH₂O 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 slides were cross-linked using a UV Stratlinker 1800 at 6,000 μ Joules 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. Briefly, the slides were pre-hybridized for one hour at 50°C with 100 mL of the buffer (final concentrations: 50% formamide, 5X sodium dodecyl sulfate

(SSC), 0.1% SDS, 0.1 mg/mL BSA [bovine serum albumine] and ddH₂O). 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 ddH₂O). 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 washings with buffer III (final concentration: 0.1X SSC) by 1 minute. Finally the slides were dried by centrifugation and stored in dark until scan.

The microarrays were scanned with a Scan Array Express Microarray Scanner (Perkin Elmer Precisely) 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: $SNR = (\text{Signal Mean} - \text{Background Mean}) / \text{Background Standard Deviation}$. A $SNR \geq 3$ is an accepted criterion for the minimum signal. Spots with lower SNRs were also removed from the data set. Cluster analysis of the data was performed with the Gene Cluster and TreeView 1.60 programs (<http://rana.stanford.edu/software>).

Results and Discussion

Chemical and Physical Analysis of Reactor Performance

A pump and treat remediation strategy was implemented for seven months to remediate the diesel contaminated plume. Through either attachment or agglomeration, a fixed-film developed on the porous media forming a biofilm community. At a flow rate of 3.8 L/min, the biofilm was composed of over 10^7 - 10^8 total cultivable cells per gram of activated carbon. The total petroleum hydrocarbon (TPH) level at the reactor's inlet port varied considerably from each treated batch. However, removal efficiency of TPH achieved a sustained 97-99% of the total applied organic load with a 14 min hydraulic retention time (Table 1). When hydrocarbons levels at the inlet port surpassed 1,000 ppm, one additional recycle step was required. In general, high removal of total hydrocarbons was achieved less than 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 success of the remediation process. A summary of the physical and chemical parameters of the groundwater at the bioreactor's influent and effluent sampling ports is presented in table 1.

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

¹Average; n= 3 to 6; -, not available. TPH, total petroleum

¹ Parameter	Influent FBR unit			Effluent FBR unit		
	Day 61	Day 153	Day 212	Day 61	Day 153	Day 212
TPH (mg/L)	554.3	1064.9	999.8	9.4	32.0	6.9
DO (mg/L)	6.58	3.06	3.60	-	1.42	1.57
Turbidity (NTU)	66	446	150	15	999	21
N-NH ₃ (mg/L)	1.35	4.80	0.13	1.56	6.60	0.27
N-NO ₃ (mg/L)	6.7	19.9	0.8	4.8	7.8	0.6
S-SO ₄ (mg/L)	-	16.7	18.2	-	7.0	1.7

hydrocarbons; DO, dissolved oxygen.

The pH of the bioreactor as measured at the influent and effluent was neutral throughout the treatment process. Stable pH levels (7.58 ± 0.22) and tropical temperatures ($26.8 \pm 1.8^\circ\text{C}$) perhaps provided advantageous conditions for the sustainable growth of microbes and degradation of hydrocarbons. In addition to oxygen, both nitrate and sulfate were consumed (alternative electron acceptors), indicating the microbial community was able to utilize 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 of the microbial activity to anaerobic respiration. The addition of nutrients to an equalization tank prior to treatment provided excess resources required for the development of microbial assemblages in the various treatment components. Excess of ammonium and nitrate consumption is indicative of dissimilatory nitrate reduction. Nitrate uptake increased progressively during the operation of the system with the highest observed value at Day 153 (net consumption of 12.1 mg/L). Sulfate uptake increased from 9.7 mg/L in Day 153 to 16.5 mg/L by Day 212.

Characterization of GAC Microbial Community

The composition, structure and stability of microbial populations in FBR biofilm community was examined using both culture-dependent and independent methods. Our

culture-based results demonstrated a great diversity of aerobic and facultative diesel degrading bacteria within the biofilm community. Gram positive and negative rods were regularly isolated from the treatment unit. Characterization of the microbial populations indicates that the biofilm community was composed of at least 25 different coexisting bacterial groups. Approximately 20% of the isolates were also capable of complete denitrification to N₂ gas. Abundant ammonium levels as growth nitrogen source, isolation of denitrifying bacteria and nitrate uptake within the treatment unit suggest that nitrate respiration might have contributed to the success of the biodegradation process. Phylogenetic analysis of 16S rRNA partial sequences revealed a great level of diversity related to three bacterial divisions: *Bacilli*, *Actinobacteria* and *Proteobacteria* (Figure 1). The *Proteobacteria* was represented by 11 bacterial strains distributed within the *alpha*, *beta* and *gamma* subdivisions. The members of the *Proteobacteria* group included representative strains of well-described petroleum hydrocarbon-degrading species [4, 14, 21].

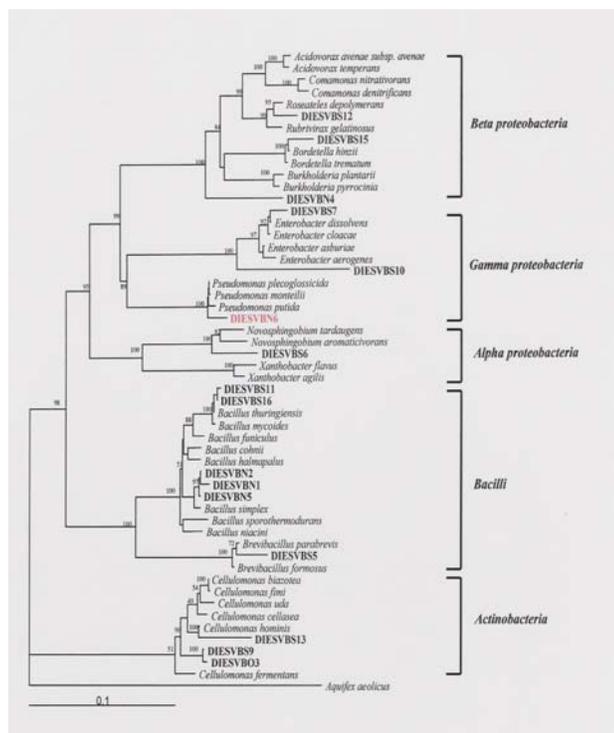


Figure 1: Phylogenetic tree of GAC strains based on partial 16S rDNA sequence analysis (Bootstraps values ≥ 45 are shown) with *Aquifex aeolicus* used as the outgroup. The bar indicates the difference of 10 nucleotides per 100.

Functional Gene Array of GAC Microbial Community Samples

The FGA's of GAC microbial communities were used as a generic gene profiling and comparative tool. Application of a 50-mer oligonucleotide array to environmental samples was successfully used by Rhee *et al.* and Tiquia *et al.* [13, 19]. 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.



Figure 2: 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) GAC-30 days; (B) GAC-61 days; (C) GAC-153 days; (D) GAC-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 2: Pairwise similarity value (%) of GAC communities

Gene arrays	% Similarity		
	Day 30	Day 61	Day 153
Day 61	47.5	-	-
Day 153	49.6	74.4	-
Day 212	61.4	69.8	73.8

Table 3: Summary of total hybridization results.

Probe Category	Total Gene Probe Number	Total Hybridized Probes	Gene ID Category
Metabolic Genes (C, N, S cycles)	5,769	333	<i>Geobacter sp.</i> cytochrome family, <i>nirS</i> , <i>nirK</i> , <i>nifH</i> , <i>nosZ</i> , <i>amoA</i> , <i>pmo</i> , <i>pmoA</i> , <i>dsrA</i> , <i>dsrB</i>
Organic Degradation	4,014	270	Nitrobenzene, naphthalene, biphenyl, 2,4-D, MTBE, toluene, nitrobenzene
Metal Resistance	2,402	172	Mercury, copper, arsenic, nickel, cobalt, cadmium
Total Genes	12,185	775	

Table 4: Hybridization results obtained for representative genes involved in diesel degradation and detected in all samples from the GAC community.

Gene Name	Gene Description / Source / Gene ID	¹ Signal Noise Ratio			
		Day 30	Day 61	Day 153	Day 212
Phthalate	Putative phthalate ester hydrolase / <i>Arthrobacter keyseri</i> / 13242052_108	13.28 (7.73)	3.38 (1.31)	7.65 (0.61)	12.41 (12.91)
Phthalate	Phthalate dioxygenase large subunit / <i>Arthrobacter keyseri</i> / 13242054_353	4.83 (2.23)	4.31 (1.34)	9.57 (1.56)	7.60 (4.66)
Phthalate	3,4-dihydroxyphthalate 2-decarboxylase / <i>Arthrobacter keyseri</i> / 13242058_587	5.33 (1.53)	3.90 (1.41)	12.01 (0.59)	6.85 (2.51)
MTBE	Alkane 1-monooxygenase / <i>Pseudomonas fluorescens</i> / 13445194_108	3.44 (1.38)	3.69 (1.45)	9.76 (0.39)	4.34 (1.73)
Benzoate/ anaerobic	Thiolase (acetyl-CoA acetyltransferase) / <i>Bacillus halodurans</i> C-125 / 15614592_1076	4.71 (2.59)	4.39 (2.03)	10.71 (1.14)	4.83 (1.49)
Thiocyanate	Carbon monoxide dehydrogenase / <i>Sulfolobus solfataricus</i> P2 / 15898062_686	4.29 (1.56)	4.64 (1.86)	9.08 (0.91)	5.47 (2.64)
Phthalate	phthalate permease / <i>Sulfolobus tokodaii</i> str. 7 / 15922956_410	9.37 (5.65)	2.82 (1.14)	4.03 (0.60)	19.55 (12.12)
Protocatechuate	Protocatechuate 3,4-dioxygenase, alpha subunit / <i>Caulobacter crescentus</i> / 16126648_384	2.94 (1.01)	6.59 (3.99)	20.30 (2.24)	13.90 (11.30)
Protocatechuate	Putative protocatechuate 3,4-dioxygenase / <i>Sinorhizobium meliloti</i> 1021 / 16265236_532	3.37 (1.32)	11.62 (6.23)	31.43 (1.86)	12.51 (7.28)
Biphenyl	Biphenyl dioxygenase / <i>Ralstonia eutropha</i> / 1890342_658	5.46 (2.27)	2.31 (0.67)	3.65 (0.60)	7.22 (4.97)
Aniline	Aniline dioxygenase beta-subunit / <i>Acinetobacter sp.</i> YAA / 2627148_399	3.14 (1.57)	3.57 (1.39)	5.33 (0.71)	4.23 (1.41)
Protocatechuate	3,4-dioxygenase beta chain / <i>Bradyrhizobium japonicum</i> USDA 110 / 27:31794411_472	5.11 (1.33)	4.04 (1.52)	9.51 (1.06)	6.13 (2.88)
Cyclohexanol	Cyclohexanone monooxygenase / <i>Bradyrhizobium japonicum</i> USDA 110 / 27382095_773	4.44 (1.76)	4.24 (1.73)	9.32 (0.82)	6.40 (2.90)
Phthalate	3,4-dihydroxy-3,4-dihydrophthalate dehydrogenase / <i>Terrabacter sp.</i> / 27531096_403	4.44 (2.33)	6.35 (3.73)	6.63 (0.82)	4.25 (1.59)

¹Average (Standard Deviation); n = 6.

Table 2: cont'd

Gene Name	Gene Description / Source / Gene ID	¹ Signal Noise Ratio			
		Day 30	Day 61	Day 153	Day 212
Toluene/ anaerobic	Benzylsuccinate synthase gamma subunit / <i>Thauera aromatica</i> / 3184130_28	6.26 (1.97)	5.40 (2.11)	15.41 (0.96)	7.98 (2.23)
Acetylene	Probable ephA protein / <i>Pirellula sp.</i> 1 / 32473431_370	4.39 (1.97)	6.57 (4.21)	11.40 (1.18)	6.48 (2.06)
Biphenyl	Biphenyl dihydrodiol dehydrogenase / <i>Bacillus sp.</i> JF8 / 32562914_541	6.86 (2.19)	4.22 (2.41)	16.08 (1.26)	8.15 (2.56)
Acetylene	Acetylene hydratase Ahy / <i>Pelobacter acetylenicus</i> / 33325847_969	5.68 (2.28)	4.27 (2.19)	11.74 (0.91)	6.42 (3.39)
Protocatechuate	Putative protocatechuate 3,4 dioxygenase / marine α proteobacterium SE45 / 38490070_560	22.03 (14.05)	11.30 (12.20)	38.79 (13.74)	8.89 (4.81)
Thiocyanate	ACDS complex carbon monoxide dehydrogenase / <i>Methanopyrus kandleri</i> / 38503097_1862	8.40 (3.54)	3.94 (1.47)	5.02 (0.44)	7.48 (5.86)
Biphenyl	Receptor-like histidine kinase / <i>Rhodococcus erythropolis</i> / 3868875_3209	12.42 (6.00)	13.16 (13.32)	12.20 (2.18)	6.23 (4.32)
Benzoate/ anaerobic	Ferredoxin, 2Fe-2S / uncultured bacterium 580 / 40063438_226	3.43 (0.79)	3.18 (1.31)	6.46 (1.06)	4.70 (2.71)
Benzoate/ anaerobic	Ferredoxin / <i>Desulfotomaculum</i> <i>thermocisternum</i> / 4028019_136	3.26 (0.70)	2.93 (1.07)	4.54 (0.59)	5.98 (2.73)
Thiocyanate	Carbon monoxide dehydrogenase / <i>Thermoproteus</i> <i>tenax</i> / 41033719_176	4.47 (1.47)	3.65 (1.36)	8.93 (0.86)	6.01 (2.91)

¹Average (Standard Deviation); n = 6.

To evaluate the total genotypic diversity within the biofilm community, samples representing various times in the operation history were examined. A cluster analysis of the microarray data revealed strong similarities among the samples (Figure 2; Table 2). Probe hybridization patterns indicated early selection of a core microbial community although the Shannon Diversity Index increased progressively from 5.99 to 6.38 during the seven months of operation. 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 [*nirS*, *nirK*, and *nosZ*], dissimilatory sulfite reductases [*dsrAB*], cytochrome c family of *Geobacter sp.*, and methane monooxygenase [*pmoA*]) were detected (Table 3 and 4). Furthermore, genes for the degradation of MTBE, nitroaromatics and chlorinated compounds were also present, thus indicating the broad catabolic potential of a microbial community selected under diesel as the main carbon and energy source. The results showed genes with steady abundance during the 7 months of treatment as seen for the MTBE, naphthalene, biphenyl, and aerobic phenol degradation pathways (Table 4).

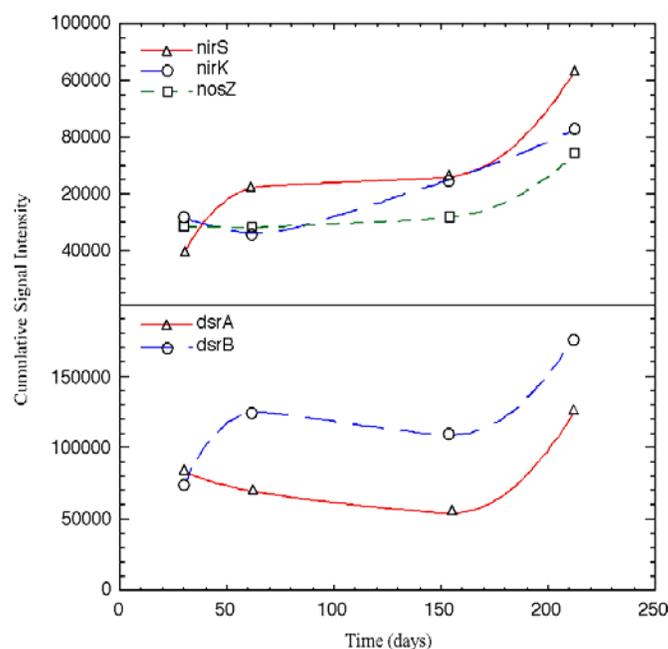


Figure 3: Cumulative signal intensity patterns of dissimilatory nitrate (*nirS*, *nirK*, *nosZ*) and sulfate reduction (*dsrAB*) pathways.

In contrast, the anaerobic benzoate gene had initial low signals but increased with time. 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 reduction and sulfur dissimilatory pathways were also present in all community samples with variations in their signal intensity (Table 4, Figure 3). Genes as *nirS* and *nirK* involves in dissimilatory nitrate and nitrite reduction increased in their hybridization signal intensity toward the end of operation. Again, detection of such genes could be indicative of the contribution of anaerobic processes in the removal of hydrocarbons. Genes involved in sulfur dissimilatory pathways as *dsrA* and *dsrB* increased its hybridization signal with a maximum signal level at the end of the operation (Figure 3). In general, FGA's demonstrated the establishment of a highly diverse community with concurrent aerobic and anaerobic processes contributing to the restoration process.

Several observations suggest that the sustainable diesel degradation was associated with early microbial colonization of the GAC media.

- (i) Cell density determined by microscopical observations and indirect cell counts increased coincident with nutrient uptake, indicating that growth was occurring.
- (ii) Almost 96% of all isolated cultures were capable of utilizing diesel compounds as sole carbon and energy source.
- (iii) Uptake of oxygen, nitrate and sulfate were indicative of both aerobic and anaerobic respiration activity.
- (iv) Probe hybridization patterns indicated that aerobic, denitrifying, and sulfate- and iron-reducing bacteria were present within the biofilm community.
- (v) A highly diverse with a broad catabolic potential and stable community was selected early within the treatment unit as determined by FGAs.

In conclusion, in our preliminary analysis the functional gene oligonucleotide microarray was suitable for diversity analyses of the biofilm community involved in the restoration phase of the diesel-contaminated site. The microarray was useful to screen samples and for obtaining data on the presence of an extensive array of metabolic processes as well as to characterize temporal changes within the system. These observations were 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 operations and maintenance, as well as to identify additional application strategies for the restoration of other sites.

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