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## **Bioremediation of Petroleum and Radiological Contaminated Soils at the Savannah River Site: Laboratory to Field Scale Applications**

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### **Introduction**

In the process of Savannah River Site (SRS) operations limited amounts of waste are generated containing petroleum, and radiological contaminated soils. Currently, this combination of radiological and petroleum contaminated waste does not have an immediate disposal route and is being stored in low activity vaults. SRS developed and implemented a successful plan for clean up of the petroleum portion of the soils *in situ* using simple, inexpensive, bioreactor technology. Treatment in a bioreactor removes the petroleum contamination from the soil without spreading radiological contamination to the environment. This bioreactor utilizes the bioventing process and bioaugmentation or the addition of the select hydrocarbon degrading bacteria. Oxygen is usually the initial rate-limiting factor in the biodegradation of petroleum hydrocarbons. Using the bioventing process allowed control of the supply of nutrients and moisture based on petroleum contamination concentrations and soil type. The results of this work have proven to be a safe and cost-effective means of cleaning up low level radiological and petroleum-contaminated soil.

Many of the other elements of the bioreactor design were developed or enhanced during the demonstration of a "biopile" to treat the soils beneath a Polish oil refinery's waste disposal lagoons (1). Aerobic microorganisms were isolated from the aged refinery's acidic sludge (pH 3) contaminated with polycyclic aromatic hydrocarbons (PAHs). Twelve hydrocarbon-degrading bacteria were isolated from the sludge. The predominant PAH degraders were tentatively identified as *Achromobacter*, *Pseudomonas Burkholderia*, and *Sphingomonas* spp. Several *Ralstonia* spp were also isolated that produce biosurfactants. Biosurfactants can enhance bioremediation by increasing the bioavailability of hydrophobic contaminants including hydrocarbons. The results indicated that the diversity of acid-tolerant PAH-degrading microorganisms in acidic oil wastes may be much greater than previously demonstrated and they have numerous applications to environmental restoration. Twelve of the isolates were subsequently added to the bioreactor to enhance bioremediation. In this study we showed that a bioreactor could be bioaugmented with select bacteria to enhance bioremediation of petroleum-contaminated soils under radiological conditions.

### **Materials and Methods**

*Microbial Characterization.* One gram samples (wet weight) of sludge or biopile material were suspended in 10 ml of 0.1% (w/v) sodium pyrophosphate buffer (pH 7), vortexed, and serial dilutions were made for the following microbial enumerations: acridine orange direct microscopic count (0.05% acridine orange), live and dead epifluorescent direct count with

BAClight (Molecular Probes, Eugene, OR), and colony forming units on 1% PTYG acidified agar (pH 4) exposed to naphthalene vapor for two weeks. Bacterial colonies with distinct morphotypes were picked and transferred to the same agar medium for purification and subsequent characterizations. The density of colony forming units on 1% PTYG agar at pH 7 and not exposed to naphthalene vapor was also determined after two weeks of incubation. The number of potential phenanthrene degraders was determined by spraying a saturated solution of phenanthrene in hexane directly to the colonies on the agar surface. After an additional week of incubation, colonies that cleared the phenanthrene crystals around their periphery were selected and characterized further.

After verification of purity of the microbial isolates verification of purity on nutrient agar, bacterial isolates were transferred to blood agar or R2A when blood agar did not support growth and incubated 3-4 d at 28°C. Bacterial cells were removed from the agar surface and suspended in 0.85% saline to achieve the appropriate Biolog OD. The cell suspensions were inoculated in triplicate to either GN or GP BIOLOG™ plates (Biolog™, Haywood, CA, USA) and incubated at 28°C. Identification and carbon utilization results were recorded after each incubation time. DNA for polymerase chain reaction (PCR) and partial sequencing of small subunit rRNA (SSU rRNA) genes was extracted from bacterial isolates. Samples of pure cultures were recovered from solid media and suspended in dH<sub>2</sub>O. The cells were centrifuged and the pellets were resuspended in 400 ul of bead beating solution (0.1 M NaCl, 0.5 M Tris·Cl [pH 8], 10% SDS). Glass beads (0.1 g, 0.1 mm diameter) were added to the samples which were treated with a Mini Bead Beater (Biospec Products, Bartlesville, Oklahoma) for 15 sec to 15 min. The nucleic acids were recovered by phenol:chloroform:isoamyl alcohol (25:24:1, Amresco, Solon, Ohio) and chloroform:isoamyl alcohol (24:1) extractions followed by precipitation with isopropanol (2). Each PCR reaction contained 1-2 ul of template DNA, 1ul each of forward and reverse primer, 1 Ready-To-Go PCR bead (Amersham Pharmacia, Piscataway, NJ). PCR products were visualized on a 1% agarose gel and purified using a PCR Clean-up Kit (Qiagen, Valencia, CA). Partial sequences of rRNA genes were obtained using an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA) with an ABI PRISM BigDye terminator sequencing Lab kit at the Genome Analysis Facility in the Botany Department at the University of Georgia, Athens. Isolates were tentatively identified by similarity to sequences in the GenBank data base using the FastA algorithm (3) of the GCG software package (Genetics Computer Group, Wisconsin). Phylogenetic trees were constructed using Jukes-Cantor and Neighbor-joining algorithms to determine evolutionary distance and branching order within the PHYLIP set of programs (4).

***Catabolic potential of selected microbial isolates.*** Naphthalene, anthracene, phenanthrene, pyrene, acenaphthene, fluorene, fluoranthene, catechol, indole, 2,3- hydroxynaphthoic acid, 1,2 dihydroxynaphthalene, naphthenol, 9-fluorenone, 9-hydroxyfluorene, acenaphthol, 2,3- and 3,4 dihydroxybenzoic acid (98-99% purity as determined by HPLC), and bis(trimethyl-silyl)-trifluoroacetamide were purchased from Aldrich (St. Louis, MO). PAHs and standards were dissolved in acetone and filter sterilized using autoclaved 0.2 um nylon acrodiscs (Fisher Scientific, Fairlawn, NJ). After verification of clearance of phenanthrene crystals by isolates on agar plates, the purity of the isolates was confirmed. Purified isolates demonstrating clearance of phenanthrene crystals were then tested with the following compounds as potential degradation substrates; catechol, indole, anthracene, fluorene, acenaphthene, fluoranthene, and pyrene (98% purity, obtained from either Sigma or Aldrich, St. Louis, MO). Catechol 2, 3 dioxygenase

activity was determined by spraying a 0.1% catechol solution onto isolated colonies from cultures grown on a glucose (1%) minimal agar medium after incubation of 24 - 48 h. A positive reaction was observed as the appearance of a yellow color surrounding the colonies with 5 min indicating *meta* ring cleavage of catechol (5). Dihydroxylating dioxygenase activity was determined with pure cultures growing on R2A medium containing 1mM indole. A positive reaction was observed as the appearance of blue colonies indicating indigo production. The PAH substrate range was tested by adding a streak of the acetone dissolved PAH to one edge of an R2A agar plate using a sterile cotton swab and adjacent to a confluent streak of the microbial isolate. Degradation of the PAH was indicated by the disappearance of PAH crystals and/or appearance of chromogenic degradation products diffusing into the agar medium after 1 - 2 weeks of incubation. Microorganisms that demonstrated naphthalene degradation were grown to exponential phase in 50% TSB overnight. Bacterial cells were then pelleted by centrifugation and inoculated to 50 ml of minimal salts medium (MSM) (2) exposed to naphthalene vapor and incubated for one week at room temperature prior to extraction and analysis of PAH degradation products.

*Bioreactor Design and Operation.* A continuous airflow, packed bed reactor was used to remediate petroleum and radiological contaminated soil. Because of low temperature and pressure requirements, inexpensive materials were used in the construction of in-vessel bioreactors (6). The bioreactor was constructed from a modified skid pan. Modification of the skid-pan provided the following advantages: skid pans are readily available and relatively low cost, approximately \$1200 US for a 6 yd<sup>3</sup> pan; skid pans have built in design components that facilitate waste handling and movement, there are lifting lugs attached to the units that work with existing waste handling equipment; and the structural design of skid-pans are adequate to account for the loads that would be involved with the treatment of contaminated soils. The skid pan shell was modified by adding access ports for the addition of moisture, oxygen, and nutrients and monitoring of temperature, moisture and pressure. The skip-pan was equipped with a lid, a nuclear-grade High Efficiency Particulate Air (HEPA) filter system to contain radiological material and prevent release to the environment. A false floor, constructed of galvanized carbon steel grating material, was added to the system. This created air space below the soil bed that allowed for air flow upwards through the solid matrix. To facilitate access to the system a port was constructed on the lid of the unit. A liquid feed system was included in the design so that moisture and/or nutrients could be added to the system.

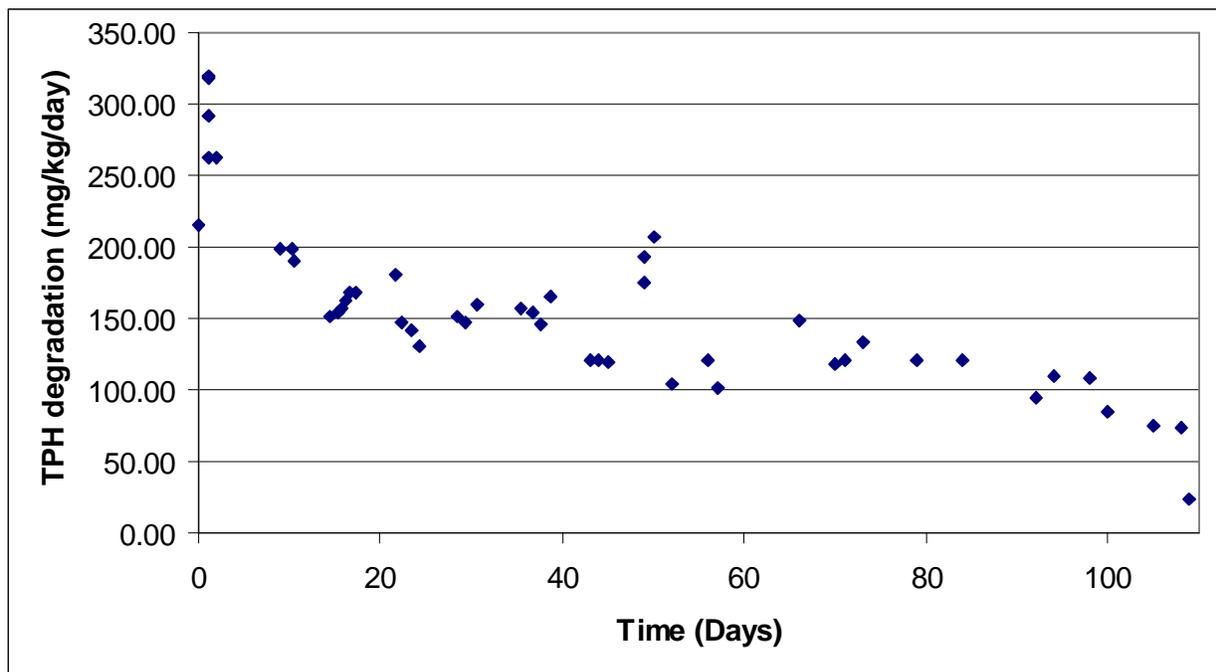
Two B-12 boxes, containing 7340 lb. of soil contaminated with approximately 30 g/kg petroleum and 60 disintegrations per minute of beta/gamma activity were loaded into the bioreactor. While loading, the soil was amended with compost, ammonium nitrate, and 10-10-10 fertilizer. An even distribution of water and bacterial inoculum (2 L of each culture in log phase) was added to the top of the soil layer and allowed to sink into the soil bed. The bioreactor was then capped with the bioreactor lid and connected to the air and liquid distribution systems. Temperatures, pressures and carbon dioxide levels were measured at least weekly while the bioreactor was operating. Temperature and pressure levels were taken using fixed gauges attached to the reactor. A carbon dioxide/methane analyzer (LFG 10 CEA Instruments, Emerson NJ) was used to detect gasses coming from the HEPA filter outlet line and the pressure relief valve. Air flow through the system was varied between 35 and 100 SCFH. Liquid, soil, and gas samples were taken from the reactor periodically. The liquid and soil samples were taken directly from the reactor access

port. These samples were used to monitor the nutrient, moisture, pH, redox, and hydrocarbon levels in the system. However, sampling soil did require additional radiological monitoring and transportation support. Gas samples were pulled after the HEPA filter. These samples were used to monitoring volatile organic compounds, oxygen, and carbon dioxide concentration over time.

## Results and Discussion

A total of 45 bacteria spp. were isolated from the sludge on an acidic minimal agar medium exposed to naphthalene vapor or phenanthrene crystals. Of the 45 bacterial isolates, four grew in nutrient broth acidified to pH 4. A subset of the microbial isolates was characterized by classical taxonomic criteria, BIOLOG®, and analysis of 16S rRNA genes. Analysis of the 16S RNA gene revealed at least 12 groups of related bacterial organisms were isolated. A number of bacteria grouped within the Proteobacteria and were tentatively identified as *Ralstonia*, *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* spp. The most commonly encountered bacterial species had high nucleotide sequence similarity to *Ralstonia* sp. KN1. Several bacteria grouped within the Actinobacteria and Firmicutes. Some bacterial strains expressed dihydroxylating dioxygenase activity and were able to grow on catechol as a sole carbon source. A bacterial isolate with sequence similarity to *Ralstonia* KN1 was able to grow on naphthalene and degrade phenanthrene. Based on these results twelve strains were selected for bioaugmentation in the bioreactor. These strains were identified as *Achromobacter*, *Pseudomonas* and *Burkholderia* spp. and *Sphingomonas* spp. Three strains also characterized as biosurfactant producers were found to be *Ralstonia* spp.

Total petroleum contaminated hydrocarbon (TPH) degradation was being monitored from carbon dioxide measurements on the influent and effluent gasses (Figure 1). The carbon dioxide production rates showed first order degradation of TPH using this approach. Degradation rates were verified from periodic soil samples. The soil samples were also used to monitor nutrient levels and moisture levels in the system. Nutrient levels were not amended during the test. Moisture levels were amended periodically during the investigation. Degradation was quicker with higher air flow and soil temperatures. A relationship between the soil temperature, air flow rate, and degradation rate was observed. Based on the increase in carbon dioxide production it is conservatively estimated that over 12,000 mg/kg of petroleum were degraded in the first 110 days. This was verified with soil samples. The system was also modified to operate using solar powered pumps and the lid was modified so it could be removed using a forklift. The efficient, safe, system operation is ongoing and other similar waste streams are being evaluated for similar treatment.

**Figure 1. Bioreactor TPH degradation vs Time****References**

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