

## CHARACTERIZATION OF AEROBIC AND ANAEROBIC MICROBIAL ACTIVITY IN HYDROCARBON-CONTAMINATED SOIL

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**ABSTRACT:** Microbial activity in hydrocarbon-contaminated soil was characterized and quantified to determine the potential for natural attenuation at a former oil field in California. Plate counts, direct microscopic counts, and carbon dioxide and methane production rates were used to quantify the populations and activity of soil microorganisms. Terminal restriction fragment (TRF) analysis provided preliminary identification of dominant microorganisms and community shifts as depth and contaminant concentrations changed. Plate counts under aerobic conditions resulted in 1.5 to  $22 \times 10^6$  colony-forming units (CFU) per gram of soil, and direct microscopic counts of total bacteria were 3 to  $33 \times 10^6$  cells per gram. Carbon dioxide production rates of 1.3 to 5.5  $\mu\text{L CO}_2/\text{g soil per day}$  were measured in the aerobic samples. Methane production rates in sealed anaerobic microcosms were higher in samples with more highly contaminated soil, and ranged from 0 to 20.9 ppmv  $\text{CH}_4/\text{day}$ . Terminal restriction fragment analysis revealed the presence of three distinct microbial communities at the site. The aerobic non-contaminated zone included *Actinomyces*, *Pseudomonas*, and other microorganisms. The transition zone included *Streptomyces*, and the zone with the highest TPH concentrations was characterized by microorganisms including *Mycobacteria* and *Actinobacteria*. Communities of both aerobic and anaerobic bacteria appear to be biodegrading hydrocarbons in situ.

### INTRODUCTION

Natural attenuation is often less expensive than other methods of remediation and can be implemented with minimal disturbance of the site. Extensive monitoring of parameters such as contaminant concentrations, electron acceptors, and appearance of breakdown products is needed to document the success of the method prior to full-scale implementation. Biodegradation of contaminants by in situ microorganisms must be demonstrated in the lab and in the field. This research investigates the potential application of natural attenuation to a field site in California with extensive petroleum hydrocarbon contamination.

Aerobic natural attenuation is expected to occur at the aerobic fringe of the plume under favorable environmental conditions for the microbes performing the degradation. Although aerobic biodegradation is the most energy-efficient respiration process, the subsurface environment at contaminated sites is often naturally anaerobic, especially within petroleum plumes where available oxygen has already been depleted. Anaerobic degradation of petroleum-related compounds such as benzene (Burland and Edwards, 1999) (Anderson et al., 1998), toluene (Ahad et al., 2000), polycyclic aromatic hydrocarbons, and alkanes (Coates et al., 1997) has been demonstrated.

This study involved aerobic and anaerobic quantification of bacteria and measurement of carbon dioxide and methane production in sealed microcosms of contaminated soil from the site. To evaluate the feasibility of natural attenuation at the field site,

microbial hydrocarbon-degrading activity was determined for soil samples under both aerobic and anaerobic (methanogenic) conditions for a range of TPH concentrations. Principal microbial populations were identified using terminal restriction fragment (TRF) analysis of DNA extracted from groundwater.

## **MATERIALS AND METHODS**

**Sample Handling.** Soil samples for aerobic experiments were collected as sleeves (6" long, 2" diameter) from two borings (Cores A and B). Soil from these samples was used for aerobic plate counts, direct counts, respirometry, and TRF analysis. For plate and direct counts, microbes were transferred from the soil particles into suspension by combining 10 g of the soil sample, 25 mL phosphate buffer solution (pH 7.2, Aldrich), 75 mL DI water, and 0.1 g sodium pyrophosphate in a 125 mL Erlenmeyer flask. The flask was covered and mixed for 15 minutes using a magnetic stir bar. Each milliliter of this suspension contains the bacteria from 0.1 g of soil.

Samples for anaerobic experiments were obtained from a boring and transported under a nitrogen blanket (Core C). Soil samples were collected from 20 depths at this location ranging from 11 to 42.5 feet below ground surface (bgs). In an anaerobic glove box, the Core C samples were distributed into 150-mL serum bottles sealed with Teflon<sup>®</sup>-lined septa to maintain an anaerobic environment within the microcosms. Anaerobic samples were used for TRF analysis and to determine methane production from the samples.

**Plate Counts.** Each stock soil solution was serially diluted with filter-sterilized phosphate buffer. Then 100  $\mu$ L of each dilution was spread on plates with R2A growth agar using sterile technique. Three plates were prepared for each dilution. Controls were prepared by plating 100  $\mu$ L of filter-sterilized phosphate buffer. All plates were incubated aerobically at 20°C for four days.

**Direct Microscopic Counts.** To enumerate soil bacteria using direct counting methods, a method modified from Bhupathiraju et al. (1999) was used. 5-(4,6-dichlorotriazinyl) amino fluorescein (DTAF) was used to stain bacterial cell walls to obtain a total cell count. Separately, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was used to identify actively respiring cells. CTC is reduced by components of the electron transport system and/or dehydrogenases of bacteria, forming fluorescent formazan deposits within active cells.

A solution of DTAF dye was prepared using 10 mL DI water, 20 mg DTAF (Molecular Probes, Eugene, OR), and 47.3 mg Na<sub>2</sub>HPO<sub>4</sub>. In a 2.0 mL microcentrifuge tube, 1.0 mL of soil suspension was combined with 0.5 mL phosphate-buffered saline solution (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 138 mM NaCl, and 2.7 mM KCl) and 0.5 mL DTAF dye solution. The mixture was incubated aerobically for 20 minutes at 20°C while shaking at 200 rpm and protecting the samples from light. After incubation, the stained sample was passed through a 0.2  $\mu$ m black polycarbonate filter (25 mm diameter, 2.9 cm<sup>2</sup> effective filtration area, Millipore, Bedford, MA). The filter and support were sealed in a 50 mL autoclavable polysulfone filter funnel (Pall Corporation, East Hills, NY). The filtered and stained samples were rinsed with at least 200 mL of sterilized wash solution (50 mM

Na<sub>2</sub>HPO<sub>4</sub>, 145 mM NaCl). The filter was air-dried and mounted on a microscope slide using about 50 µL of Tris-buffered glycerol with 2 % 1,4-diazabicyclo [2,2,2] octane (0.2 g 1,4-diazabicyclo [2,2,2] octane, 5.0 mL glycerol, 5.0 mL 2 M Tris-buffer). The filter was covered with a cover slip and examined at 1000X. An Olympus BX50 Microscope and BX-FLA Reflected Light Fluorescence Attachment were used with Omega Optical filter XF115-2 in a darkened room. Stained cells within the eyepiece grid (0.01 mm<sup>2</sup>) on five randomly selected fields were counted, averaged, and used to calculate the total number of cells per gram of the original soil sample.

Separate serial dilutions of soil bacteria were stained with CTC (Polysciences, Inc., Warrington, PA). A phosphate-buffered saline solution was prepared with yeast extract as a bacterial substrate. In a 2.0 mL microcentrifuge tube, 1.0 mL of sample solution, 0.5 mL phosphate-buffered saline with yeast extract (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 138 mM NaCl, 2.7 mM KCl, and 0.5 mg/mL yeast extract) and 0.5 mL CTC dye solution (5.2 mg/mL CTC) were combined. The mixture was incubated at 28°C for 4 hours while shaking at 200 RPM and protecting the samples from light. After incubation, the stained samples were handled and viewed according to the same procedure as for the DTAF-stained samples, but were viewed with the Omega Optical XF43 optical filter.

**Aerobic Respirometry.** Carbon dioxide production in aerobic samples was measured using a Columbus Instruments (Columbus, OH) Micro-Oxymax respirometer with a CO<sub>2</sub>/CH<sub>4</sub> detector, sample pump, and expansion interface. Duplicate 50 g soil samples were placed in 250-mL chambers with gas feed lines through the lids. The respirometer drew and analyzed gas samples from each chamber every three hours during the 43-hour incubation. After every two samples, fresh, dry CO<sub>2</sub>-free air was provided to the sample chambers by passing ambient air through soda lime and Drierite<sup>®</sup> columns. The air from each of the chambers was analyzed with a calibrated CO<sub>2</sub> sensor.

**Anaerobic Microcosms.** Anaerobic soil samples from Core C were used to create anaerobic microcosms. Each microcosm contained 40 g of soil in a 150 mL serum bottle. Microcosms were incubated at 15 to 20°C and subsequently headspace gas was analyzed by Inland Empire Analytical (Norco, CA) for CH<sub>4</sub>, H<sub>2</sub>, O<sub>2</sub>, CO<sub>2</sub>, and N<sub>2</sub> using gas chromatography with a thermal conductivity detector. The headspace was sampled at time intervals of 42, 109, and 135 days.

**TRF Analysis.** TRF analysis is a polymerase chain reaction (PCR)-based method used to study microbial community structure and dynamics (Kitts, 2001). DNA is extracted from the soil and amplified using PCR. PCR and gel electrophoresis were performed on extracted DNA, using primers specific to archaeobacteria (group including methanogens) or eubacteria (group including hydrocarbon degraders). TRF patterns were generated for the samples to determine the relative abundance of archaeobacteria and eubacteria in each sample. The number of peaks in a TRF pattern indicates the level of microbial diversity in the sample (Kitts, 2001). The peak areas in the TRF patterns correlate with the relative abundance of individual species in the sample. Peaks can be matched with the identification of the original organism by comparing the TRF results with a clone library or database of sequences (Kitts, 2001).

## RESULTS AND DISCUSSION

**Aerobic Plate Counts.** Cell counts determined by plate counts increased with increasing depth, ranging from 7 to  $22 \times 10^6$  CFU/g soil in soil samples from Core A (Table 1). Samples from Core B had fewer CFU overall, ranging from about 2 to  $10 \times 10^6$  CFU/gram.

**TABLE 1. Aerobic plate and direct count results for Core A.**

| Soil Sample<br>Depth:<br>Core A | TPH     | Plate<br>Count<br>Results | Direct<br>Count<br>Results | Soil Sample<br>Depth:<br>Core B | TPH     | Plate<br>Count<br>Results | Direct<br>Count<br>Results |
|---------------------------------|---------|---------------------------|----------------------------|---------------------------------|---------|---------------------------|----------------------------|
| (feet)                          | (mg/kg) | $(10^6 \text{ CFU/g})$    |                            | (feet)                          | (mg/kg) | $(10^6 \text{ CFU/g})$    |                            |
| 54.0                            | 2900    | 7.43                      | 7.48                       | 57.0                            | 3200    | 1.6                       | 3.31                       |
| 54.5                            | 1200    | 9.17                      | 2.84                       | 57.5                            | 8900    | 6.3                       | 4.41                       |
| 55.5                            | 66000   | 22.5                      | 12.0                       | 58.5                            | 68000   | 4.4                       | 14.0                       |
| 56.0                            | 73000   | 10.4                      | 33.6                       | 59.0                            | 27000   | 10.5                      | 6.26                       |

**Direct Microscopic Counts.** Total cell counts ranged from 3 to  $33 \times 10^6$  cells/g for Core A and 3 to  $14 \times 10^6$  cells/g for Core B (Table 1). Higher cell counts correlated with higher depths and TPH concentrations (Table 1). The correlation between total cell counts, depth, and contaminant concentrations indicate degradation of TPH by microorganisms because higher substrate (TPH) levels can support higher microbial populations only if the microorganisms are able to use the hydrocarbon contaminants for growth processes.

Direct cell counts were higher than plate counts for some samples, but lower for others (Table 1). Total cell counts were expected to be higher since they include inactive and anaerobic bacteria, and count cells that cannot be cultured on plates in the lab. The results of these microbial assays were thus surprising because they indicate that few of the bacterial species present were not culturable.

Although staining with CTC was expected to allow identification of actively respiring cells, the method used did not work effectively for the soil from the field site. A killed (autoclaved 30 min per day, 3 days) control soil sample was subjected to the CTC staining method and displayed significant fluorescence. Abiotic processes within the soil sample apparently reduced CTC to extracellular precipitates indistinguishable from intracellular deposits. Results obtained using CTC were not used because of these false positive results.

**Aerobic Respiration Rates.** Carbon dioxide production rates for soil samples from Cores A and B varied from 7 to  $32 \mu\text{L/g/hr}$  (Figures 1 and 2). Aerobic respiration rates generally increased with increasing depth with the exception of the 57.0-foot sample from Core B (Figures 1 and 2). The  $\text{CO}_2$  production rate measured for the control chamber with no soil was extremely low ( $0.005 \mu\text{L/g/hr}$ ).

Carbon dioxide production was compared with direct counts and plate counts to determine the degree of correlation between cell counts and carbon mineralization, in addition to the agreement between the different methods used.

The methods used to quantify aerobic microbial populations and activity correlated well with each other (Figures 1 and 2). While the organic material being degraded may or may not be only the petroleum hydrocarbons, the increased levels of activity with increasing contamination levels suggest microbial degradation of TPH. The high aerobic respiration rates generated by the samples below the air/oil interface without a lag time indicate either the presence of a high in situ population of aerobic microorganisms or a rapid growth of these microorganisms in the lab when oxygen was provided.

**Methane Production from Anaerobic Microcosms.** Anaerobic microcosms were prepared from Core C. Significant quantities of methane were detected in microcosms with soil from below 38 feet bgs, ranging from 86 to 2700 ppmv. This indicates active fermentation in the microcosms with high TPH content. No significant amount of oxygen (>1.0%) was discovered in any microcosms, indicating little or no leakage. Methane concentrations were higher in samples with higher TPH (Table 2).

The strong correlation of headspace methane concentration with soil TPH concentration suggests the anaerobic bacteria are using TPH as a substrate. Increased levels of substrate allow more acetate to be produced and therefore support a higher population of methanogenic bacteria. Most samples had increasing methane concentrations with time over the sampling period (Table 2). Methane production rates (estimated using a linear regression) increased with increasing depth and correlated well with TPH concentration (Figure 3).

**Microbial Diversity (TRF Analysis).** TRF analyses were performed on Cores A and B to identify the presence of eubacteria and archaebacteria (methanogens) in the soil samples. Samples above the air/oil interface did not yield enough DNA to perform TRF analysis. Samples below the air/oil interface produced sufficient DNA to confirm the

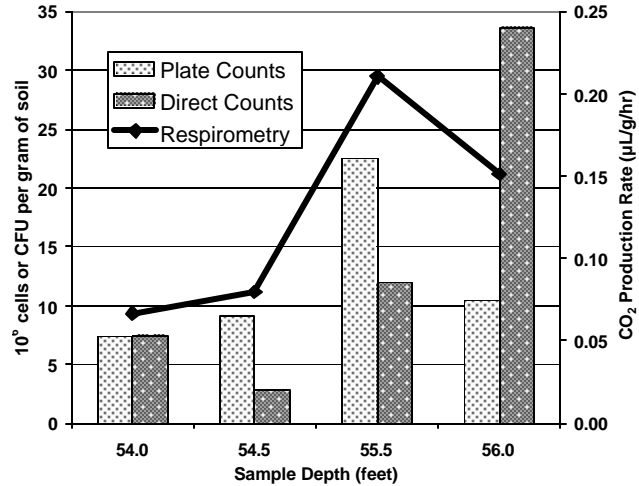


FIGURE 1. Comparison of Core A results.

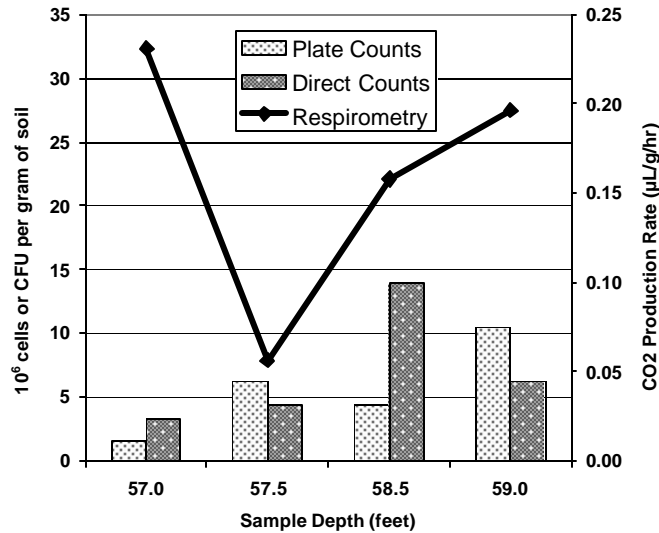
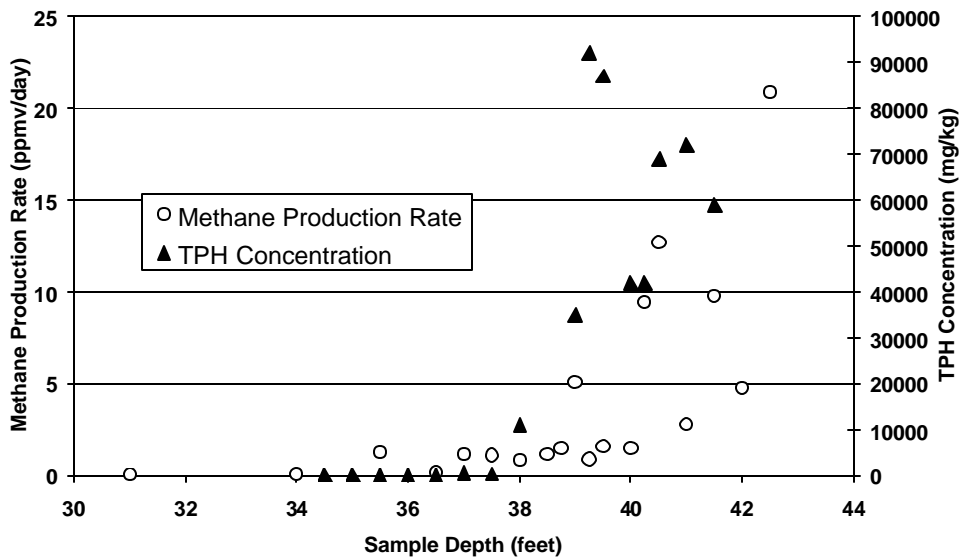


FIGURE 2. Comparison of Core B results.

**TABLE 2. Gas concentrations over time in anaerobic microcosms.**

| Day    | 42    | 109              | 135             | 42              | 109             | 135             | 42              | 109            | 135            |                |
|--------|-------|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|----------------|----------------|
|        | TPH   | CH <sub>4</sub>  | CH <sub>4</sub> | CH <sub>4</sub> | CO <sub>2</sub> | CO <sub>2</sub> | CO <sub>2</sub> | H <sub>2</sub> | H <sub>2</sub> | H <sub>2</sub> |
| Sample | ppm   | ppmv             | ppmv            | ppmv            | ppmv            | ppmv            | ppmv            | ppmv           | ppmv           | ppmv           |
| 11     |       | 102 <sup>a</sup> | 69              | 12              |                 | 13010           | 15200           |                | 5538           | <0.5           |
| 31     |       | <15 <sup>a</sup> | <5              | <5              |                 | 13770           | 14270           |                | 1              | <0.5           |
| 34     |       | <15 <sup>a</sup> | <5              | 10              |                 | 16490           | 14920           |                | 9289           | 10315          |
| 34.5   | ND    | <15 <sup>a</sup> | <5              | <5              |                 | 14530           | 14050           |                | 2239           | 553            |
| 35     | ND    | <15 <sup>a</sup> | <5              | <5              |                 | 14100           | 13880           |                | 3496           | 2726           |
| 35.5   | 48    | 228 <sup>a</sup> | 116             | 130             |                 | 7000            | 6860            |                | 95             | 14             |
| 36     | ND    | <15 <sup>a</sup> | <5              | <5              |                 | 11840           | 11640           |                | 1              | <0.5           |
| 36.5   | 73    | <15 <sup>a</sup> | <5              | 26              |                 | 21680           | 19150           |                | 128            | 26             |
| 37     | 600   | 170 <sup>a</sup> | 121             | 115             |                 | 10680           | 7710            |                | 1363           | 25             |
| 37.5   | 180   | 126              | 114             | 125             | 11530           | 10590           | 11220           | 7152           | 1989           | 5125           |
| 38     | 11000 | 109              | 86              | 93              | 14250           | 20640           | 19510           | 8              | 5              | 1              |
| 38.5 T |       | 120              | 123             | 131             | 6390            | 6770            | 7300            | 1613           | <0.5           | <0.5           |
| 38.5 B |       | 139              | 156             | 178             | 4640            | 3770            | 2980            | 23915          | 20299          | 13791          |
| 39 T   | 35000 | 148              | 593             | 667             | 10570           | 12280           | 12830           | 1              | 6              | 1              |
| 39 B   | 92000 | 92               | 96              | 101             | 8400            | 10370           | 11010           | 1759           | 1              | <0.5           |
| 39.5   | 87000 | 151              | 158             | 191             | 9550            | 12490           | 12350           | 5              | <0.5           | <0.5           |
| 40 T   | 42000 | 105              | 167             | 175             | 11050           | 11590           | 13360           | <0.5           | 1              | 1              |
| 40 B   | 42000 | 266              | 964             | 1369            | 9120            | 9110            | 8970            | 48             | 5821           | 4694           |
| 40.5   | 69000 | 719              | 1175            | 1817            | 10500           | 11600           | 11480           | 2              | 23             | 40             |
| 41     | 72000 | 265              | 304             | 319             | 8350            | 9980            | 11740           | <0.5           | 1              | 1              |
| 41.5   | 59000 | 497              | 738             | 1561            | 12050           | 10430           | 10520           | 17             | 53             | 15             |
| 42     |       | 594              | 155             | 814             | 9090            | 13910           | 11470           | 1              | 1              | 1              |
| 42.5   |       | 576              | 2699            | 2568            | 21110           | 19760           | 22100           | 11804          | <0.5           | <0.5           |

<sup>a</sup> Measured using HP 6890 gas chromatograph and flame ionization detector. All other concentrations were measured by Inland Empire Analytical.



**FIGURE 3. Methane production rates and TPH concentrations as a function of depth below ground surface in Core C.**

presence of both eubacteria and archaeobacteria, especially in the samples with higher TPH concentrations. Microbial diversity, indicated by larger numbers of peaks, generally increased with depth in both Core A and Core B samples. Higher diversity and higher populations of archaeobacteria were observed below the air/oil interface than above it. Archaeobacteria are expected in the more contaminated soil because they become more competitive when alternate electron acceptors are not available.

For the anaerobic Core C, TRF analyses indicated that archaeobacteria were present in all samples from at or below the air/oil interface. Dominant eubacterial organisms were tentatively identified and ranked according to their relative abundance in the soil for three soil zones: top (34' – 37.5' bgs), middle (38' – 40' bgs), and bottom (40' – 42.5' bgs). Each contained very different types of dominant species. The top (most aerobic) zone had low TPH contamination (from less than 10 ppm to a maximum of 180 ppm at 37.5' bgs). The microbial community populating this zone included *Actinomyces* and *Pseudomonas*. The middle zone was the transition or “smear” zone with TPH ranging from 5500 to 87,000 ppm and generally increasing with depth. The transition zone microbial community included *Streptomyces*. The bottom zone was characterized by high TPH contamination (42,000 to 92,000 ppm) and anaerobic conditions. This zone was dominated by microorganisms including *Mycobacteria* and *Actinobacteria*. Both species are known to degrade large organic compounds (such as petroleum hydrocarbons). The presence of these organisms and the increased methane production in highly contaminated soil samples indicate the utilization of TPH as a substrate for microbial activity.

## CONCLUSIONS AND RECOMMENDATIONS

The several methods used to quantify microbial populations and activity have provided evidence of natural attenuation and a meaningful characterization of the microbial communities associated with TPH contamination at the field site. The identification of dominant organisms by TRF analysis provided valuable information about the microbial community. While several limitations may reduce the applicability of each method, combining several analytical techniques overcomes the limitations of each individual method and resulted in a more reliable characterization of the microbial population.

Microbial activity (carbon dioxide and methane production), populations (direct counts and plate counts), and TPH concentrations increased with sample depth at all three sample locations. These results indicate that both aerobic and anaerobic TPH degradation are operative at the site.

Three distinct microbial communities with different dominant organisms are present at the Core C location: the aerobic area with little contamination, the transition zone near the air/oil interface, and the anaerobic zone with high TPH concentrations. Microorganisms known to be associated with petroleum degradation and degradation of large molecules are dominant in the contaminated soil from Core C.

Recommendations include: (1) Determine the relationships between TPH degradation and carbon dioxide and methane production so that respiration rates can be interpreted in terms of TPH degradation rates; (2) Evaluate and quantify natural attenuation more extensively at the site to determine whether it is feasible as a full-scale remediation strategy; (3) Check anaerobic samples for alternate electron acceptors in non-methane producing samples; and (4) Quantify the disappearance of electron acceptors and TPH at the site.

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