Microbial Responses to *In Situ* Chemical Oxidation, Six-Phase Heating, and Steam Injection Remediation Technologies in Groundwater

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The evaluation of microbial responses to three in situ *source removal remedial technologies—permanganate-based* in situ *chemical oxidation (ISCO), six-phase heating (SPH), and steam injection (SI)—was performed at Cape Canaveral Air Station in Florida. The investigation stemmed from concerns that treatment processes could have a variety of effects on the indigenous biological activity, including reduced biodegradation rates and a long-term disruption of community structure with respect to the stimulation of TCE (trichloroethylene) degraders. The investigation focused on the quantity of phospholipid fatty acids (PLFAs) and its distribution to determine the immediate effect of each remedial technology on microbial abundance and community structure, and to establish how rapidly the microbial communities recovered. Comprehensive spatial and temporal PLFA screening data suggested that the technology applications did not significantly alter the site's microbial community structure. The ISCO was the only technology found to stimulate microbial abundance; however, the biomass returned to predemonstration values shortly after treatment ended. In general, no significant change in the microbial community composition was observed in the SPH or SI treatment areas, and even small changes returned to near initial conditions after the demonstrations. © 2004 Wiley Periodicals, Inc.**

INTRODUCTION

Chlorinated aliphatics are among the most widespread dense nonaqueous phase liquid (DNAPL) contaminants in groundwater and soil due to their use for degreasing, dry cleaning, and as solvents (Azadpour-Keeley et al., 2001). As a result, a myriad of remediation technologies have been developed and applied at sites around the world for the restoration of these subsurface media. The U.S. Environmental Protection Agency (EPA), working with the Interagency DNAPL Consortium, completed an independent evaluation of microbial responses to remediation technology demonstrations at Launch Pad 34 at Cape Canaveral Air Station in Brevard County, Florida.

Launch Pad 34 is underlain by relatively sandy soils with a shallow water table. Because there is a relatively expansive area of the subsurface contaminated by DNAPL, the site was chosen for the comparison of three side-by-side DNAPL remediation technologies. The three selected technologies were permanganate-based *in situ* chemical oxidation (ISCO), six-phase heating (SPH), and steam injection (SI). The primary DNAPL contaminant at the site is trichloroethylene (TCE).

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Initial sampling indicated high concentrations of dichloroethylene and vinyl chloride, which are daughter products of the anaerobic reductive dechlorination of TCE. Aggressive remediation technologies typically remove a substantial portion of the DNAPL but often do not achieve regulatory clean-up levels. Since natural attenuation is often required to lower contaminant concentrations to ppb levels following enhanced treatment technologies, there were concerns that the treatment processes could have a variety of effects on the indigenous biological activity, including reduced biodegradation rates and a long-term disruption of community structure with respect to the stimulation of TCE degraders.

Fatty acids are known to differ in chemical composition depending upon microbial type and environmental conditions.

Microbiological evidence to support the natural attenuation of chlorinated solvents in the subsurface involves the characterization of microbial density, diversity, composition, and physiological and genetic/phylogenic traits. Analyses focused on the composition and diversity of bacterial community structures (Brigmon et al., 2002) cannot rely on traditional microbiological procedures alone. This is especially critical in subsurface ecological systems, because the vast majority of the microbial communities that reside in that environment cannot be cultivated using culture-dependent methods (Amann et al., 1995). Phospholipid fatty acid (PLFA) analysis is a popular biochemical assay founded on the identification and quantitation of individual fatty acids present in cell membranes (Ringelberg & White, 1996). Phospholipids are essential cellular components of the membranes of all cells and are composed of a hydrophilic glycerophosphate headgroup and two nonpolar fatty acid–derived tails (Petsch et al., 2003). Since intact phospholipids are hydrolyzed following cell death (Harvey et al., 1986), their presence is an indicator of living cells and their concentration is an expression of microbial biomass in soils and groundwater sediments (White et al., 1979).

Fatty acids are known to differ in chemical composition depending upon microbial type and environmental conditions. These differences allow a quantitative insight into three important attributes of microbial communities—viable biomass, community structure, and metabolic activity (Lehman et al., 1995). In practice, this chemotaxonomic tool is based on the extraction and separation of lipid classes followed by quantitative analysis using gas chromatography mass spectrometry (GC/MS; Tunlid et al., 1989; White & Ringelberg, 1997). The procedure is independent of the bias inherent in classical culturing techniques and provides a more accurate estimation of *in situ* microbial populations. PLFA analyses for the characterization of microbial ecologies have been reported for a variety of investigations, including the bioremediation of hazardous waste sites (Hansen et al., 2000; Pfiffner et al., 1997; Ringelberg et al., 1989), in deep strata (Lehman et al., 1995), and under natural conditions (Tunlid et al., 1989).

The objective of this investigation was to determine the effect of each DNAPL source remediation on microbial abundance and community structure (specifically TCE degraders). The total mass and fractional distribution of PFLA were used as an indicator of microbial changes.

METHODS AND MATERIALS

Microbiological Monitoring Points and Sampling Frequencies

As shown in Exhibit 1, five sampling locations were selected in each of three treatment plots (SPH, SI, and ISCO). In addition, five locations upgradient of the treatment

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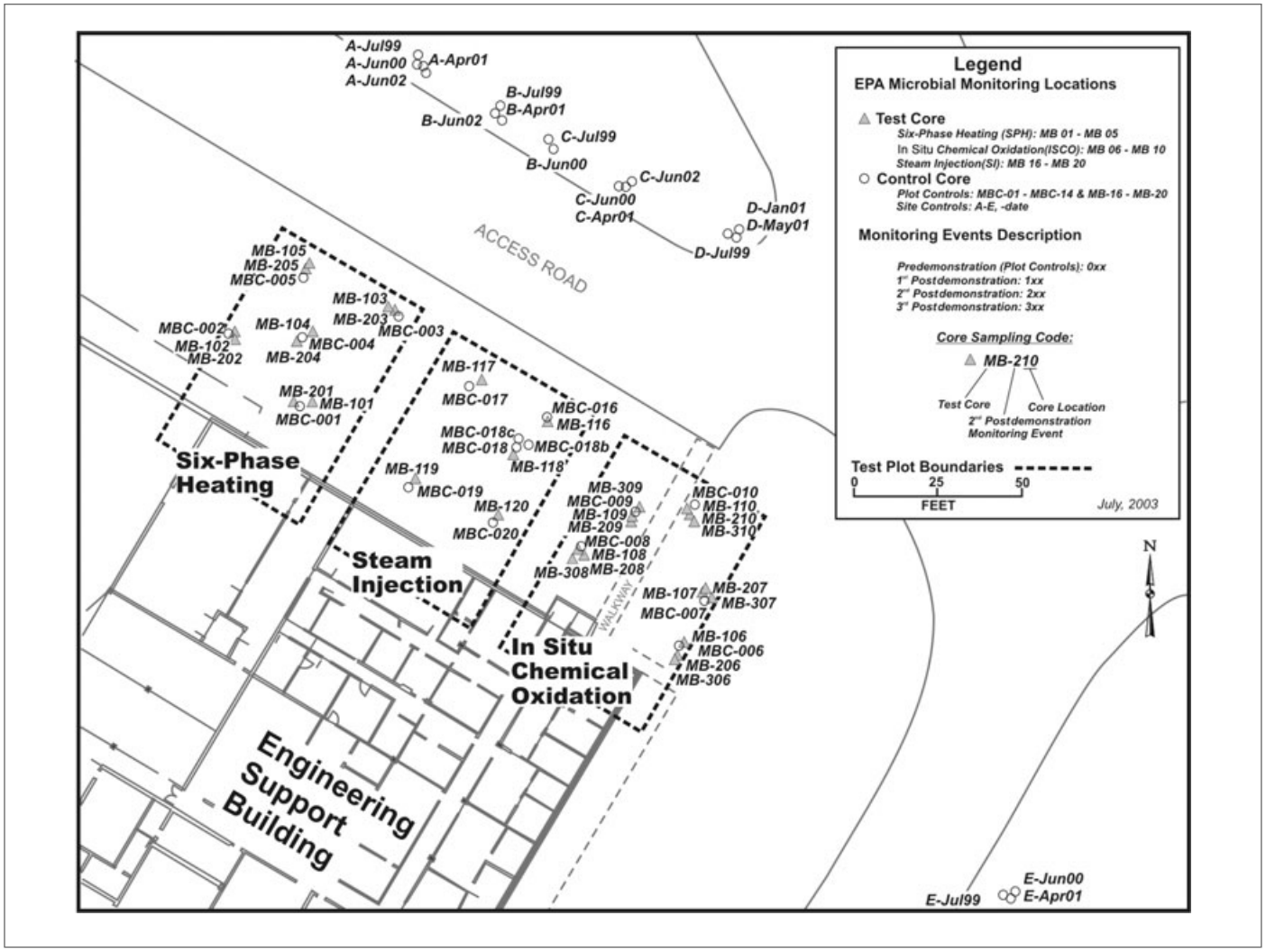

Exhibit 1. Map of microbiological sampling locations at LC34

plots, but still within the TCE-contaminated plume, were identified as controls. The geochemistry and TCE concentrations of the test plots and control sites were similar.

The three treatment plots were sampled during four events. Treatments occurred between the first and second sampling rounds in which two of the three remediation treatment technologies (ISCO and SPH) were demonstrated sequentially. The first sampling event occurred approximately one month prior to the treatments' initiation. The following three sampling events took place 1, 6, and 12 months after the treatments terminated. While the four rounds of sampling were completed for the ISCO, the fourth sampling round was not successfully accomplished for the SPH due to logistical constraints. The SI treatment plot data included two sampling rounds, one for the baseline predemonstration and the last occurring six months after the completion of the treatment technology.

The baseline predemonstration sampling events for the site controls were synchronized with sampling events for ISCO and SPH or SI. The experimental design for the remainder of the site controls' sampling was also synchronized with the treatment plots' sampling events.

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Core Sample Collection, Preparation, and Analysis

Over 300 core samples were collected under the same aseptic sampling regime from July 1999 to June 2002. One duplicate sample was collected from one of the five boring locations in each plot and for every 20 site controls. From these, 266 samples were subjected to PLFA analyses. The collection of the core samples was accomplished using a Cone-Penetrometer (CPT) equipped with a Mostap™ sampler (20 inches long with a 1.5-inch diameter). The sampler contained three sterile sleeves (brass or stainless steel) and one spacer (Exhibit 2). Each sleeve was 6 inches long and held approximately 250 grams of soil. The sleeves were designated as A, B, and C for top, middle, and bottom positions, respectively.

The collection of the aquifer material for vertical profiling was accomplished by driving the sample barrel to four different depths. . .

The collection of the aquifer material for vertical profiling was accomplished by driving the sample barrel to four different depths: approximately 7 feet (Capilary fringe), 15 feet (upper sand unit below water table), 30 feet (middle fine grained unit), and 40 feet (lower sand unit) at each sampling location. Once the sample barrel was withdrawn, the three sleeves were extruded from the sample barrel. Care was taken not to disturb or contaminate the sample. The sleeves were tightly capped on both ends with plastic end caps and sealed as quickly as possible using sterile gloves. Each sleeve was labeled, with the percentage of recovery recorded and marked to designate the top and bottom sections. Two of the sleeves (B and C) were immediately frozen in liquid nitrogen (–150°C) and shipped overnight (–70°C) to the laboratory for DNA and PLFA analysis.

At the laboratory, the sleeves were cataloged and separated according to their designations (B or C). From each sampling location, the sleeve with the lower recorded percentage of recovery was transferred into a Whirl-Pak™ and stored (–70°C) for molecular analysis, while the other sleeve was sent frozen (–70°C) overnight to Microbial Insight, Inc. (Rockford, Tennessee) for PLFA analysis. Lipids were recovered using a modified Bligh and Dyer method (White et al., 1979). Extractions were performed using one-phase chloroform-methanol-buffer extractant. Lipids were recovered, dissolved in chloroform, and fractionated on disposable silicic acid columns into neutral-, glyco-, and polar-lipid fractions. The polar-lipid fraction was transesterified with mild alkali to recover PLFA as methyl esters in hexane. PLFAs were analyzed by gas chromatography with peak confirmation performed by electron impact mass spectrometry (GC/MS).

In order to minimize sample contamination, a strict aseptic sampling procedure adopted for the evaluation of microbiological samples included sterilization of brass or stainless sleeves with isopropanol (70 percent) bath dipping (15 minutes), air drying at ambient temperature (~ 1 hour), and aluminum foil wrapping. Each three individually foil-wrapped sleeves were placed in an autoclave bag. The bag was placed in a heat-resistant plastic container and autoclaved (121°C, 30 minutes). The container was tightly capped, packed, and shipped to the field. Polyethylene sleeve caps were not autoclaved but were surface-rinsed with isopropanol (70 percent) prior to use. Sterilization of drilling equipment involved steam cleaning between samples. After the samples were extruded, the sample barrel used to collect the soil sample was disassembled and decontaminated in Alconox® detergent–mixed water. The sample barrel was then rinsed with tap water, deionized water, and isopropanol (70 percent) prior to complete air drying (~ 1 hour) and before it was used again. Air exposure of the subsurface was minimized, since core sampling was continued immediately after each extraction using two sets of

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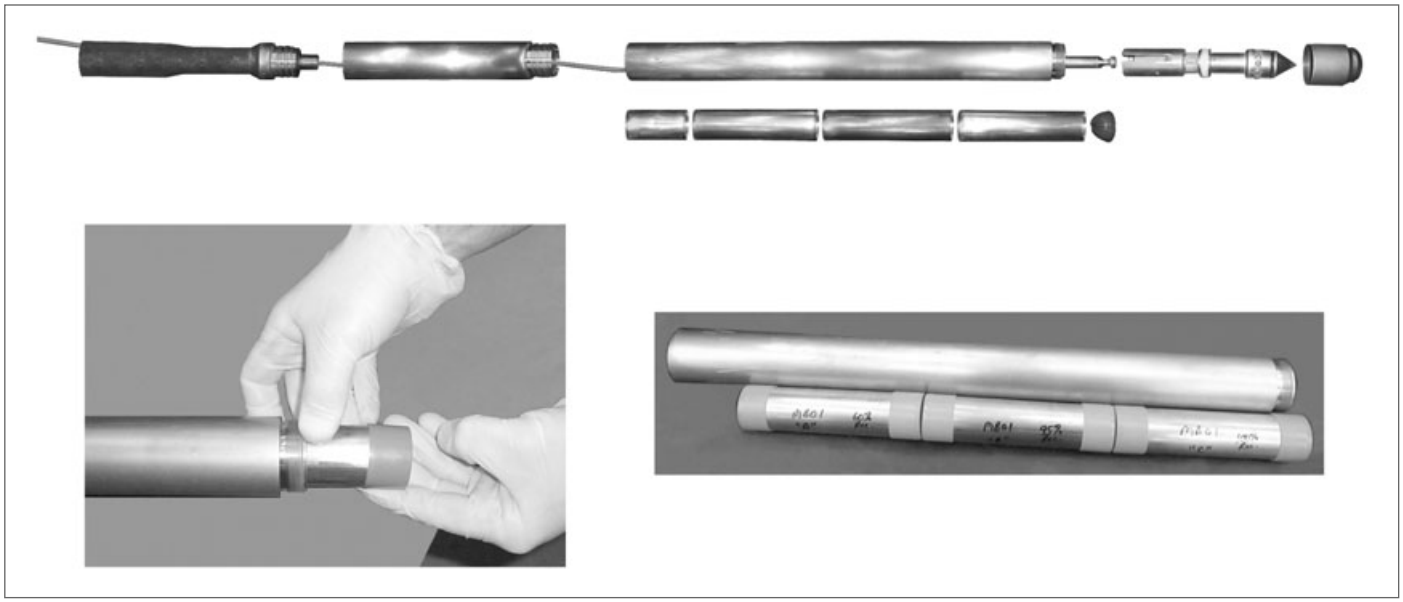

Exhibit 2. The aseptic extraction of the core samples using a Mostap™ sampler

the Mostap™ samplers. The sampler containing three cored sleeves was extracted and switched with another sterile one, and coring continued while the sampling crew conducted aseptic sample processing and decontamination procedures.

RESULTS AND DISCUSSIONS

During the three-year evaluation, 266 core samples were collected aseptically at four depths between 7 and 40 feet in the three treatment plots and site control locations. The study focused on the analysis of PLFA profiles, which provide an effective tool for monitoring microbial responses to their environment. Phospholipids are essential cellular components of the membrane of all cells and play a role as storage materials. The microbial membrane reflects the nature of both the intracellular components and extracellular environmental conditions. Therefore, the PLFA profiles simultaneously contain general information about the phylogenetic identity and physiological status of microbes, which are particularly crucial in an expression of microbial reaction to environmental stresses.

For purposes of this discussion, the interpretation of results is centered on data collected from the site control locations and the ISCO plot. The significance of the ISCO plot is due to the availability of a more comprehensive data set in terms of the number of sampling events and that they are fairly representative of the other treatment technologies, with the exception of an initial increase in microbial biomass during the ISCO demonstration. While lipid biomarker analyses are suitable for providing an overall picture of community structure (Pennanen et al., 1996; Schutter & Dick, 2000), they are incapable of identifying every microbial species in an environmental sample, since many species may contain overlapping PFLA (White & Ringelberg, 1990). Therefore, the results from Launch Pad 34 are discussed with an emphasis on changes within the community structure rather than species identification.

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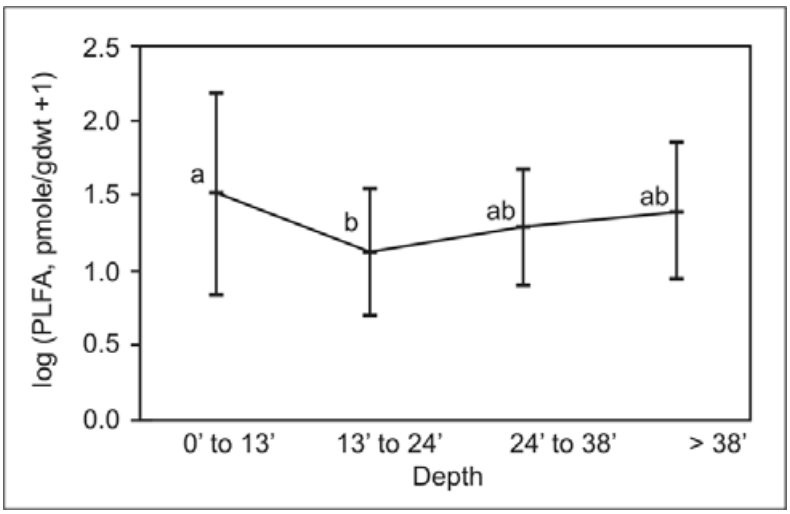

Note: Points with different letters are significantly different.

Exhibit 3. Variation in microbial biomass with depth in the control plot, expressed as log (PLFA pmole/gdwt) ± standard deviation

Seventy-eight core samples (A-E) were analyzed during the four sampling events from the site control locations. The microbial biomass, reported by treatment, date, and depth, is presented in Exhibit 3. The pattern typically observed in subsurface environments is for the biomass to decrease with depth, since, in most shallow sedimentary systems, oxygen and the major source of available carbon (photosynthate) are obtained from percolation from the surface. In this system, however, the minimum microbial biomass was at the second shallowest depth. The high variation of biomass at each depth is normal, but the lack of a biomass gradient is unusual when compared to the general pattern of microbial distribution in subsurface environments. One possibility for the presence of the minimum biomass at the second shallowest depth may be a result of reduced porosity. The lack of a biomass gradient was also reported by Reed et al. (2002) in characterizing the vertical profile of microbial communities in core samples collected from methane hydrate–bearing sediments at a considerably deeper strata (300 meters below the sea floor).

Although the site control locations were generally distributed along a transect upgradient of the technology demonstrations to provide a description of the background population, the locations were within the TCE-contaminated plume and therefore impacted by the presence of chlorinated solvents. In this respect, the microbial community structures found in samples from the site controls were also unusual (Exhibit 4). PLFAs were broken down into six groups on the basis of their chemical structure, which followed phylogenic relatedness. Usually, monounsaturated PLFAs are the most abundant fatty acids and long saturates (defined in the legend for Exhibit 4) are less than a few percent of the total. However, at this site, the short saturates were generally the largest group, and the long saturates were much higher than usual.

Among the ISCO cores, samples from the 15-foot depth at location 8 (MBC-008, MB-108, MB-208), and the 40-foot depth at location 10 (MBC-010, MB-110, MB210)

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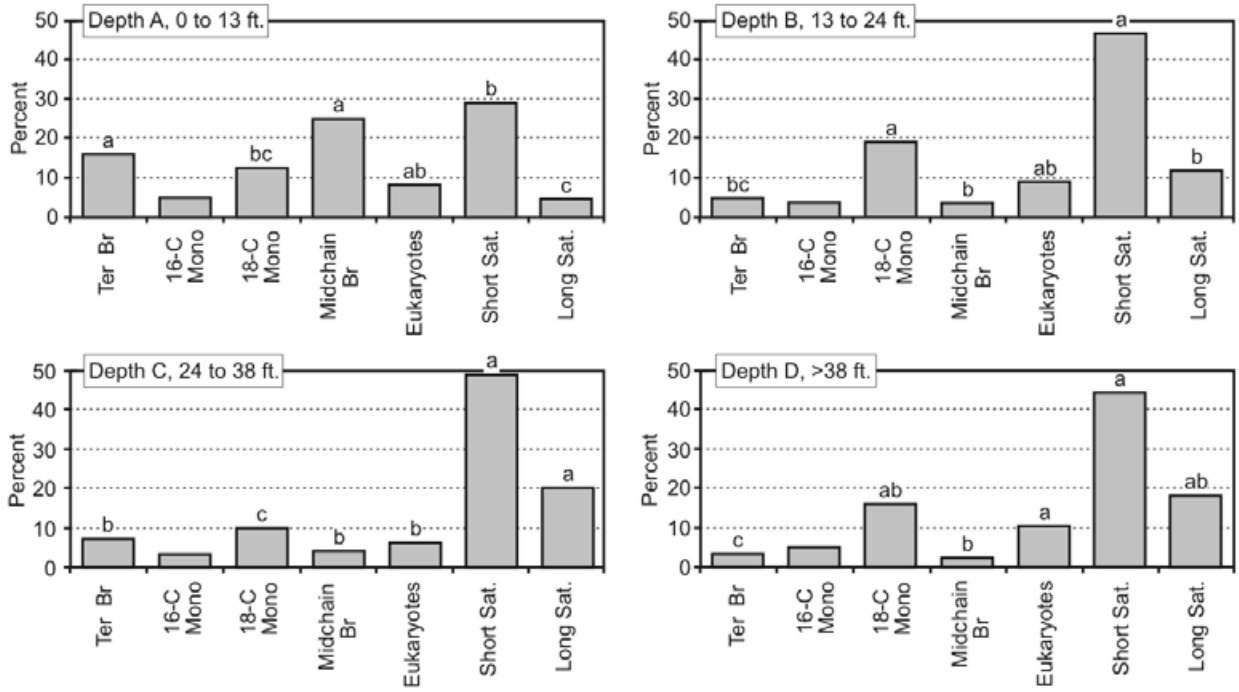

Legend: Ter Br = terminally branched, 16-C Mono = 16-carbon monounsaturated, 18-C Mono = 18-carbon monounsaturated, Midchain Br = midchain branched, Short Sat. = short (13- to 18-carbon) saturated, and Long Sat. = long (20- to 24-carbon) saturated fatty acids.

Note: The Eukaryote biomarkers were 18:ω9c and polyunsaturates. Bars of a variable marked with different letters are statistically different.

Exhibit 4. Microbial community structure at four depths of the control plot

were collected in duplicate for all sampling events, except the final post demonstration event. Exhibit 5 shows the variation of viable microbial biomass of PLFA with sample location, depth, date, and percent moisture. The values are expressed as the natural logarithms of picomols of PLFA per dry weight grams (i.e., Lnpmols PLFA/g). The data indicate that the sample location or percent moisture had no detectible effect on the microbial biomass. Biomass was minimum at the 15-foot depth, maximum at the shallowest depth, while at the 30-foot and 40-foot depth, the samples were intermediate and indistinguishable. Microbial biomass clearly increased from the baseline control samples (MBC-06 to MBC-10) to the oxidation samples (first postdemonstration; MB-106 to MB-110) ranging from 2.92×10^4 to 1.85×10^8 cells per gram dry weight. The sharpest increase in the biomass occurred in the shallowest core samples. The metabolic status of the populations suggested that they were in the stationary phase. This increase in the biomass, however, was followed by decreases in subsequent sampling events during recovery. The action of permanganate on biologically unavailable organic carbon, such as lignin and humics, is anticipated to release small soluble organic molecules that can serve as a microbial carbon source. Thus, an increase in microbial biomass and in the proportion of monounsaturates is expected to occur subsequent to chemical oxidation

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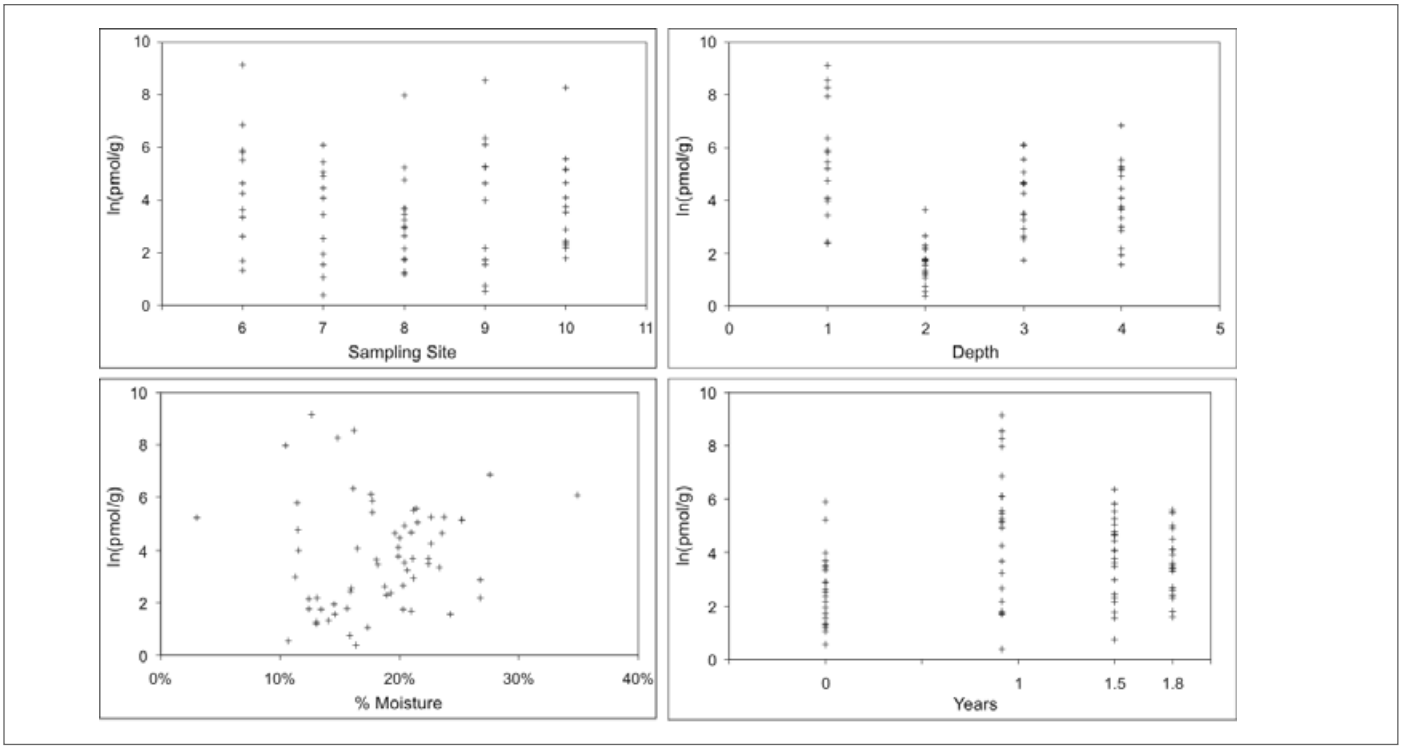

Exhibit 5. Sampling site, sample depth, percent moisture, and sampling data versus the natural logarithm of pmol PLFA/gram dry weight

treatment with permanganate. If this were the sole reason for the increase in biomass, the system would be expected to quickly become anaerobic due to the demand for slightly soluble oxygen, which did not appear to happen. This suggests that oxygen is supplied by the breakdown of permanganate; therefore, the ISCO supplied both oxygen and available carbon to support microbial growth.

As shown in Exhibit 6, there was a significant spatial and temporal variation in the microbial biomass from 1.5 picomol/g to 9.2 picomol/g. It is noted that the fatty acid content of one sample was below the detection limit. The increase in microbial biomass from baseline control locations to postoxidation sampling events is shown to illustrate the pattern of biomass distribution down each well as it is replicated across sampling times. For example, the biomass at sample location 6 in the baseline control (MBC-006) decreases in the order 7, 40, 30, and 15 feet. This same pattern is replicated in the first and second postoxidation sampling events (MB-106 and MB-206, respectively). This consistency of the sampling locations allowed pooling for statistical analysis.

Analyses of the data from the three treatment technologies as well as the site control locations were compared by constructing graphs demonstrating changes in Ln(pmols PLFA/g) versus time at each sampling depth. The graph lines also included $\pm$ 1 standard deviation confidence intervals. The variation of community structure with treatment and time was also demonstrated in terms of the percentage of terminally branched fatty acids, 16-carbon monounsaturates, 18-carbon monounsaturates, midchain branched fatty acids, branched unsaturates, eukaryote biomarkers, short saturates, and long saturates. Results suggested that terminally branched fatty acids did not show clear differences with respect to treatment or time, except a decrease with depth within the

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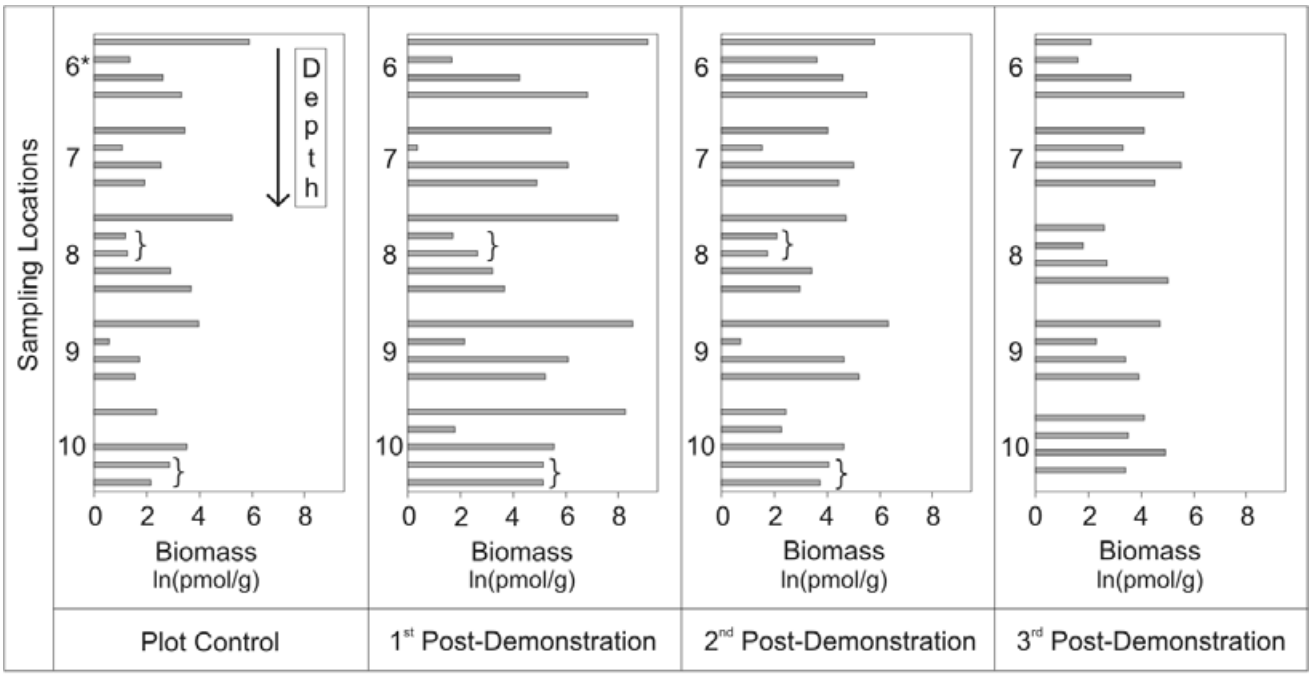

* 6, 7, 8, 9, and 10 represent MBC-006 to MBC-010 for plot control, MB-106 to MB-110 for 1st post-demonstration, MB-206 to MB-210 for 2nd post-demonstration, and MB-306 to MB-310 for 3rd post-demonstration.

Note: Duplicate samples are enclosed in a curly bracket.

Exhibit 6. ISCO microbial biomass by location, depth, and date

site control populations. Often, a difference from the controls was associated with an increase in variation and was also seen in the 16- and 18-carbon monounsaturates and the branched unsaturates.

The 16-carbon monounsaturates demonstrated a sharp increase in the chemical oxidation treatment at the second time point for the two deepest horizons, and less for the shallowest depth. The 18-carbon monounsaturates showed increases over site controls for the two shallowest depths. Most of these same points (treatment × time × depth) had an increase in biomass, which is associated with an increase in the proportions of monounsaturates—mostly 16-carbon monounsaturates at the two deepest horizons and mostly 18-carbon monounsaturates in the two shallowest. Monounsaturates are largely due to *Proteobacteria*, abundant in soils and often found to rapidly increase when carbon and oxygen are available. While proportions of 16- and 18-carbon monounsaturates often differ with treatment, it is not known at this time which *Proteobacteria* are responsible for producing the difference. It is expected that the ongoing molecular microbial characterization of the Launch Pad 34 samples using 16S rDNA sequences will determine the phylogenic relationships of the predominant species at this site.

In soils and sediments, the midchain branched PLFA are mostly due to *Actinobacteria*, the parent group of *Actinomycetes*, and/or metal-reducing bacteria. The data suggest that this group decreased with depth and showed a modest response to treatment. The branched unsaturates are a minor group of PLFA also associated with metal-reducing bac-

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teria. There was a large difference at the shallowest depth between the chemical oxidation plot and site controls. However, since this difference was detected both before and after the oxidation treatment, the difference is apparently between the plots, and not due to the treatments.

Eukaryote biomarkers in soils are largely due to fungi. Therefore, they tend to decrease with depth away from the oxic atmosphere and photosynthesis of the organic carbon they are dependent upon. At this site, there was no apparent relationship between the proportion of eukaryotic microorganisms and depth among the control populations (plot or site). There appears to be some loss of eukaryote biomarkers after chemical oxidation and steam treatments; however, the variation is high relative to these differences.

Overall, the total PLFA abundance from the three treatments is indicative of the presence of a fairly substantial indigenous microbial population.

The saturated PLFAs are usually analyzed as one group. The unusually high amounts of 20- to 24-carbon saturates in this sample set suggested a benefit from analyzing them separately. The short and long saturates had different patterns. Although the long saturates increased with depth, they showed no significant response to treatment activities. Short saturates at the second depth were different from the site controls for chemical oxidation and six-phase resistive heating treatments, but only before treatment.

In order to characterize the microbial community composition in SPH and SI and to assess their treatment effect, terminally branched, 16-carbon monounsaturates, 18-carbon monounsaturates, midchain branched, branched unsaturated, eukaryote biomarkers 18:ω9c and polyunsaturated, short saturates, and long saturates fatty acids were also analyzed. These PLFAs are routinely used to detect treatment differences. In general, the results showed no significant variation in microbial community composition for the resistive heating or steam-extraction treatments. This finding could be due to any combination of three possible scenarios: (1) there might not be any detectible difference between the microbial communities as a result of resistive or steam injection and that the communities may have nearly recovered to initial conditions by the time of the second sampling event (first posttreatment); (2) the fatty acid structural groups used above might have missed the differences; and (3) samples with very low biomass, and therefore unreliable patterns of PLFAs, may have interfered with the analysis.

The data for SPH and SI treatment plots suggested a significant increase in terminally branched saturated PLFA, indicating either gram-positive or obligate anaerobic gram-negative bacteria. Terminally branched saturates in the *iso* form have shown to be greater in gram-negative obligate anaerobes such as sulfate-reducing bacteria (Dowling et al., 1986; Rutters et al., 2002). In contrast, *anteiso* forms have been seen at higher concentrations in gram-positive bacterial species (i.e., *Arthrobacter*). A number of thermophilic bacterial species have also been shown to contain *iso* and *anteiso* PLFA, with the iso configuration having the more substantial branching (Lehman et al., 1995). Among the SPH and SI samples, the *iso* terminally branched saturates increased after the treatments. The increase was generally observed throughout the four depths, suggesting that the treatments increased the populations of thermophilic bacteria.

Overall, the total PLFA abundance from the three treatments is indicative of the presence of a fairly substantial indigenous microbial population. The types of PLFAs contained in the samples indicated a mixed community of anaerobic and aerobic bacteria that is consistent with other reports (Colwell et al., 1997; Rajendran et al., 1992; Ringelberg et al., 1997). The numbers of viable microbes can, if stimulated, influence the biogeochemistry of their environment.

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CONCLUSIONS

Comprehensive spatial and temporal screening data suggested that the remedial technology applications did not significantly alter the site's microbial community structure. The PLFA distribution prior to the treatment demonstration (site controls) suggested that the microbial community structures were atypical. In particular, the biomass gradient did not decrease with increasing depth. The high level of biomass variation identified at each depth, however, was consistent with the extensive heterogeneity of biota expected in subsurface environments. Analysis of fatty acid structure groups indicated that short saturates constituted the largest group and that the number of long saturates was significantly higher than usual. These findings contrasted with other studies indicating that monosaturated PLFAs usually are the most abundant, while long saturates constitute a smaller percentage of the total fatty acids.

PLFA distribution for each of the three demonstration areas also indicated a high variation in biomass at each sampling event and depth. As mentioned above, ISCO was the only technology found to stimulate microbial abundance: a significant initial increase in biomass was observed following the ISCO demonstration. This behavior is consistent with findings from other permanganate-based chemical oxidation applications. Biomass and the proportion of monounsaturates returned to normal levels shortly after chemical injections ceased. In general, no significant change in the microbial community composition was observed in the SPH or SI treatment areas, and even small changes returned to near initial conditions after the demonstrations.

Limiting factors for this evaluation may include the selection of fatty acid structural groups that may have been insufficiently sensitive to subtle differences in microbial populations, or the use of samples with very low biomass and corresponding patterns of PLFA. Although the PLFA tests suggest that the indigenous microbial populations recovered after treatments, further investigations are required to demonstrate conclusively that the population can be used as a finishing remediation step.

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