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US EPA Demonstration of Steam Injection/Extraction Treatment of a DNAPL Source Zone at Launch Complex 34 in Cape Canaveral Air Force Station

Final Innovative Technology

Appendix E: Microbiological Assessment

E.1 Microbiological Evaluation Work Plan

Biological Sampling & Analysis Work Plan

The Effect of Source Remediation Methods on the Presence and Activity of Indigenous Subsurface Bacteria at Launch Complex 34, Cape Canaveral Air Station, Florida

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1.0 Purpose and Objectives

Overall purpose is to evaluate effects of three DNAPL source remediation treatments on the indigenous bacterial population. The three treatments in three different plots at LC34 are six-phase heating (SPH), chemical oxidation (OX), and steam injection (SI). The objectives of the biological sampling and analysis are:

1. To determine the immediate effect that each remediation technology has on the microbial community structure and specifically on TCE biodegraders.
2. To establish how quickly the microbial communities at the site recover and if any of the effects could be long-term.
3. To determine at what point that biodegradation could be used to complete remediation of the plume.
4. To establish if any of the technologies could cause and short-term effect on significant biogeochemical processes and the distribution and abundance of potential pathogens in the environment.

2.0 Background

Launch Pad 34 at Cape Canaveral Air Station has dense non-aqueous phase (DNAPL) concentrations of TCE over a wide aerial extent in relatively sandy soils with a shallow groundwater table (Resource Conservation and Recovery Act Facility Investigation Work Plan for Launch Complex 34, Cape Canaveral Air Station, Brevard County, Florida, 1996, Kennedy Space Center Report KSC-JJ-4277.). These conditions have made it an ideal site for side-by-side comparison of various DNAPL remediation technologies currently being conducted by the DNAPL Remediation Multi-agency Consortium. Initial sampling at the site revealed that there are also high concentrations of vinyl chloride and dichloroethylene indicating natural attenuation via biodegradation of the TCE plume has been occurring. Since these compounds are daughter products of the anaerobic reductive dechlorination of TCE by microbes (see discussion below) it is probable that these conditions could be greatly effected by the source remediation processes being tested. Since most of these processes will introduce air into the subsurface and are potentially toxic to many microbes they could have a variety of effects on the biological activity and biodegradation rates of contaminants in the source area and the surrounding plume. The effects could range from long-term disruption of the microbial community structure and biological activity at the site,

to a significant stimulation of biodegradation of TCE. Whatever the effect, it needs to be monitored carefully since the long-term remediation of this or any similar site will be significantly effected not only by the technologies ability to remove the DNAPL source but also by the rate of biodegradation both natural and stimulated that can occur in the aquifer after the source is removed. The rate and extent of biodegradation will effect how low the technology must lower the source concentration before natural or stimulated bioremediation can complete the remediation to the ppb levels normally used as cleanup goals. It could also have a major effect on the life-cycle costs of remediation of these sites.

Secondarily, unlikely as this is, it is also important to verify that these source remediation technologies do not cause any gross changes biogeochemistry, and distribution and abundance of potential pathogens. The pathogens are a possibility at this site since there was long-term sewage discharge at the edge of test plots. Studies at other sites have suggested that stimulation of pathogens especially by thermal increases could be a possibility and thus should be considered in the overall risk scenario for these remediation technologies.

Reductive Dechlorination of Chlorinated Solvents

Microbial degradation of chlorinated solvents has been shown to occur under both anaerobic and aerobic conditions. Highly chlorinated solvents are in a relatively oxidized state and are hence more readily degraded under anaerobic conditions than under aerobic conditions (Vogel et al., 1987). In subsurface environments where oxygen is not always available, reductive dechlorination is one of most important naturally occurring biotransformation reactions for chlorinated solvents. Microbial reductive dechlorination is a redox reaction that requires the presence of a suitable electron donor to provide electrons for dechlorination of chlorinated organic (Freedman and Gossett, 1989).

Highly chlorinated solvents, such as tetrachloroethylene (PCE) and trichloroethylene (TCE), are commonly detected in the subsurface. Under anaerobic conditions, PCE is reductively dechlorinated to TCE, which in turn may be dechlorinated to 1,2-dichloroethylene (cis-1,2-DCE, or trans-1,2-DCE), followed sequentially by vinyl chloride (VC) and finally ethylene (Freedman and Gossett, 1989) or ethane (Debruin et al. 1992). Further reductive dechlorination of DCE and VC to CO_2 and complete dechlorination of PCE to CO_2 are possible under anaerobic conditions (Bradley and Chapelle, 1996; Bradley and Chapelle, 1997; Bradley et al., 1998; Cabirol et al., 1998). However, complete dechlorination of PCE is often not achieved due to slow dechlorination process of its reduced intermediates, cis-1,2-DCE and VC, resulting the accumulation of these unfavorable intermediates in anaerobic environments. The accumulation of cis-1,2-DCE and VC is of great concern because they are known carcinogens. Such incomplete dechlorination is commonly observed in fields where reductive dechlorination of PCE and TCE is taking place (McCarty, 1996).

Reductive dechlorination reactions can be carried out by anaerobic microorganisms via either energy yielding or cometabolic processes. The energy-yielding process involves the use of chlorinated solvents as terminal electron acceptors (sometimes referred to as dehalorespiration). Anaerobic cultures that are capable of using PCE or TCE as terminal electron acceptors include the obligate anaerobes *Dehalospirillum multivorans* (Scholz-Muramatsu et al., 1995), *Dehalococcoides ethenogenes* (Maymo-Gattel et al., 1997), *Desulfitobacterium sp*. strain PCE1 (Gerritse et al ., 1996), *Desulfitobacterium sp*. strain PCE-S (Miller et al., 1997; Miller et al., 1998), *Desulfomonile tiedjei* (Fathepure et al., 1987; DeWeerd et al., 1990), *Dehalobacter restrictus* (Holliger and Schumacher, 1994; Holliger et al., 1998), strain TT4B (Krumholz et al., 1996), and the facultative organism strain MS-1 (Sharma and McCarty, 1996). With the exception of *Dehalococcoides ethenogenes* which dechlorinates PCE to ethene, and *Desulfitobacterium sp*. strain PCE1 which dechlorinates PCE to TCE, the end product of PCE dechlorination for all described pure cultures is cis-1,2, DCE. The end products of reductive dechlorination reactions vary depending on the physiological groups of bacteria involved. In acetogens, methanogens,

and some other anaerobic bacteria, reductive dechlorination is believed to be mediated by metallocoenzymes like the cobalt containing vitamin B12 and related corrinoids, and by the nickel containing cofactor F430. These metallocoenzymes are present as components of enzymes that catalyze normal physiological pathways in several anaerobic bacteria, and fortuitously are able to reductively dechlorinate several chlorinated compounds. Acetogenic and methanogenic bacteria contain high levels of these metallocoenzymes, the concentrations of which can be strongly dependent on growth substrates (Deikert et al., 1981; Krzycki and Zeikus, 1980).

The presence of a suitable electron donor, such as hydrogen or reduced organic compounds including hydrocarbons, natural organic matter, glucose, sucrose, propionate, benzoate, lactate, butyrate, ethanol, methanol, and acetate have been reported serve as electron donors for reductive dechlorination (Bouwer and McCarty, 1983; Carr and Hughes, 1998; DiStefano et al., 1992; Fennell and Gossett, 1997; Freedman and Gossett, 1989; Gibson and Sewell, 1992; Holliger et al., 1993; Lee et al., 1997; Tandoi et al., 1994). However, since the microbial populations differ from site to site and their responses to substrates vary greatly, the addition of certain types of electron donors may or may not effectively enhance reductive dechlorination processes. Both laboratory studies and field observations suggest that the addition of electron donors for the enhancement of dechlorination can induce complex scenarios that are a function of the subsurface conditions (Carr and Hughes, 1998; Fennell and Gossett, 1997) and the indigenous microbial population (Gibson and Sewell, 1992). Although it is known that hydrogen serves as the specific electron donor for reductive dechlorination (Holliger et al., 1993; Holliger and Schumacher, 1994; Maymo-Gatell et al., 1995), different concentrations of hydrogen stimulate different groups of anaerobic microbial populations which may or may not be responsible for dechlorination, and may out compete the halorespirers, making the direct addition of hydrogen problematic. In fact, recent research has indicated that dechlorinating bacteria possess lower half-velocity coefficients for H_2 utilization than methanogens, suggesting that dechlorinating bacteria should out compete methanogens at low H_2 concentrations (Ballapragada et al., 1997; Smatlak et al., 1996). In short-term microcosm studies, the addition of slow-release H_2 donors butyrate and propionate was found to support complete dechlorination as well as to enrich PCE-degrading bacteria (Fennell and Gossett, 1997). In contrast, the addition of fast-release H_2 donors ethanol, lactate, and acetate did not result in complete dechlorination. However, both ethanol and lactate did support sustained dechlorination during long-term tests. In some cases, the addition of acetate and methanol to laboratory microcosms with PCE contaminated soil did not enhance dechlorination (Gibson and Sewell, 1992). Complex substrates such as molasses and yeast extract have been shown to result in higher dechlorination levels than simple substrates (Lee et al, 1997; Odem et al., 1995; Rasmussen et al., 1994). Apparently, the fate of amended electron donors and the dynamic changes of microbial populations responsible for reductive dechlorination within soils are still not well understood.

Aerobic Degradation of Chlorinated Solvents

Under aerobic conditions, microbial degradation of chlorinated solvents to non-toxic products can occur by metabolic or cometabolic transformation reactions. DCE and VC have both been shown to be aerobically degraded in energy-yielding reactions. Recently, several aerobic strains that are capable of using VC as primary carbon and energy source have been isolated. These aerobic microorganisms include Mycobacterium sp.(Hartmans and De Bont, 1992), *Rhodococcus sp*.(Malachowsky et al., 1994), *Actinomycetales sp*.(Phelps et al., 1991), and *Nitrosomonas sp*. (Vanelli et al., 1990). It is suggested that these VC-utilizers may not play significant roles in contaminated site remediation due to their long doubling time.

While there have been no reports of aerobic cultures that can oxidize TCE for growth, methanotrophs are one group of bacteria that can cometabolically oxidize chlorinated solvents such as TCE, DCE, and VC to carbon dioxide and chloride ions. These organisms utilize methane as their primary carbon and energy source and produce methane monooxygenase, a key enzyme that is involved in the oxidation of methane.

The same enzyme can also cometabolically oxidize chlorinated solvents. Typically, the chloroethenes are initially oxidized to chloroethene epoxides, which in turn decompose into various readily degradable chlorinated and non-chlorinated acids, alcohols or aldehydes, and carbon monoxide (Oldenhuis et al., 1989; Strandberg et al., 1989; Tsien et al., 1989; Little et al., 1988; Alvarez-Cohen and McCarty, 1991; Neuman and Wackett, 1991; Fox et al., 1990; Chang and Alvarez-Cohen, 1996). Anaerobic reductive dechlorination has also been shown to occur under bulk aerobic conditions dominated by aerobic co-metabolic biodegradation both in the field and in soil columns (Enzien et al., 1994)

3.0 Scope

Launch Complex 34 at Cape Canaveral Air Station in Florida is the test site for the remediation technology evaluation study. Separate testing plots will be established for each of the following three remediation technologies:

1. Six-Phase Heating™ (SPH)
2. In-Situ Oxidation (OX)
3. Steam Injection (SI)

Soil core samples and groundwater samples at different depths (subsurface layers) from each plot will be collected and analyzed by microbiology and molecular biology methods before and after remediation treatment in order to determine the effect of the treatments on the indigenous microbial population.

4.0 Analytical Approach and Justification

Several different microbiology and molecular analysis will be conducted to evaluate the effect of the remediation technologies used on the microbial community. The following analyses will be conducted:

- Total Heterotrophic Counts
- Viability Analysis
- Coliform and *Legionella* Analysis
- PLFA Analysis
- DNA Analysis

At this time, there are no fool-proof, broadly applicable methods for functionally characterizing microbial communities. The combination of assays we propose will provide a broadly based characterization of the microbial community by utilizing a crude phylogenetic characterization (PLFA), DNA-based characterization of community components, and microscopic counts of viable (aerobic and anaerobic) bacteria and total bacteria. We anticipate that this array of methods that we will help avoid some of the common pitfalls of environmental microbiology studies generally (Madsen, 1998).

Heterotrophic Counts Analysis. The concentration of culturable bacteria in a subset of samples collected from each plot at each event will be done using very low carbon availability media such as 0.1% PTYG or dilute soil-extract media amended with citrate and formate. This has been found to give the best overall recovery of subsurface bacteria (Balkwill, 1989). These viable counts can be done using either MPN or plating techniques for both soil and water. These analyses can be done both under aerobic or anaerobic conditions (Gas-Pak) to provide an estimate of changes in culturable bacteria. This analysis should be

used more as a check to verify changes in viable biomass changes, community shifts from anaerobic to aerobic, and direct effects that these remediation technologies may have on the culturability of indigenous bacteria. These data will help determine if these more conventional microbiological analyses can be used to monitor the effects of the remediation technologies in future applications.

Viability Analysis. In addition, the proportion of live and dead bacteria in these samples will be determined using a fluorescence-based assay (Molecular Probes, LIVE/DEAD® *Bac*Light™ Viability Kit). Since these technologies, especially the thermal ones, may kill bacteria it is important to determine the proportion of the total bacteria observed are dead and how this proportion is changed by the remediation technology being tested. Note: dead bacteria will still be visible by direct count, and thus you could have a total count of 10 billion cells/ml and yet no biological activity because they are all dead.

Coliform and Legionella Analysis. Water samples, collected near the sewage outfall and a few, will be analyzed for total coliforms. One-two liter samples will be collected specifically for this analysis. Samples will be shipped to BMI on ice for inventory and sample management. Coliforms are the primary indicator of human fecal contamination and thus the potential for presence of human pathogens. Since the site has a long-term sewage outfall at the edge of the test beds and since this environment is generally warm and contains high levels of nutrients it is possible that human pathogens may have survived and may be stimulated by the remediation technologies being tested. The coliform analyses of groundwater samples will verify it pathogens could be present. If initial screening indicates no coliforms than this sampling can be dropped; however, if coliforms are present it may be necessary to expand this analysis to determine the extent of their influence and the effect of that the remediation technology is having on them. *Legionella pneumophila* is a frank human pathogen that causes legionnaires disease (an often fatal pneumonia) that is found widely in the environment. It can become a problem in areas that are thermally altered, eg. nuclear reactor cooling reservoirs, pools, cooling towers, air conditioners, etc. A preliminary study done at SRS during a demonstration of radio frequency heating suggested that thermal alteration of the vadose zone could increase the density of legionella in the sediment. Since there is a sewage outfall nearby, since two of the remediation technologies are thermal, and since the remediation technologies are extracting VOC from the subsurface it would be prudent to test the subsurface for changes in *Legionella pneumophila.* This can be done by using commercially available DNA probes for *Legionella pneumophila* and testing both the soil and groundwater samples being analyzed for nucleic acid probes. This adds very little expense and can be done as part of that analyses, see below.

PLFA/FAME Analysis. Phospholipid ester-linked fatty acids (PLFA) and Fatty Acid Methyl Ester (FAME) analysis can measure viable biomass, characterize the types of organisms, and determine the physiological status of the microbial community. Aliquots of each sample (100 g soil and 1-2 L water) will be shipped to frozen to EPA for analysis. The PLFA method is based on extraction and GC/MS analysis of "signature" lipid biomarkers from the cell membranes and walls of microorganisms. A profile of the fatty acids and other lipids is used to determine the characteristics of the microbial community. Water will be filtered with organic free filters in the field and shipped to EPA frozen. The filter can be used to extract both nucleic acids for probe analyses and lipids for PLFA/FAME analyses. Depending on the biomass in the water 1-10 liters will need to be filtered for each sample.

DNA Analysis. DNA probe analysis allow examination of sediment and water samples directly for community structure, and functional components by determining the frequency and abundance to certain enzyme systems critical to biogeochemistry and biodegradation potential of that environment. Sediment samples will be collected aseptically in sleeves and shipped frozen to EPA. These sediment samples will than be extracted and the DNA analyzed for presence of certain probes for specific genetically elements. Water samples will be filtered in the field to remove the microbiota and shipped frozen to EPA for subsequent extraction and probing. The Universal probe 1390 and Bacterial domain probe 338 will help quantify the DNA extracted from the samples. This information will be useful to determine the portion of

DNA that is of bacterial origin and the amount of DNA to be used in the analysis of specific bacterial groups. Transformation of chlorinated ethenes by aerobic methylotrophic bacteria that use the methane monooxygenase enzyme has been reported (Little et al., 1988). Methanotrophs can be separated into coherent phylogenetic clusters that share common physiological characteristics (Murrell, 1998) making the use of 16S rRNA probe technology useful for studying their ecology. Therefore, this study will use 16S rRNA-targeted probes, Ser-987 and RuMP-998, to detect Type II and Type I methanotrophs, respectively. Together, these probes will be used to monitor shifts in methanotroph population numbers that may result from the application of the oxidation technology. Reductive dechlorination of chlorinated ethenes has also been reported under anaerobic conditions. Therefore, we propose the use of archaea domain (Arch-915) and sulfate-reducing specific probes (Dsv-689) to assess microbial communities involved in reductive dechlorination. The characterization of enzymes capable of reductive dehalogenation such as the dehalogenase of *Dehalospirillum multivorans* (Neumann et al., 1995) or the PCE reductive-dehalogenase of *Dehalococcoides ethenogenes* (Maymo-Gatell et al., 1999) provides promise for future gene probe design. As these gene probes become available, they will be utilized for this study. The detection of *Legionella* has been improved using a combined approach of PCR primers and oligonucleotide probe that target the 16S rRNA gene has been reported (Miyamoto et al., 1997; Maiwald et al., 1998). These PCR primers and probes will be used in this study to assess the effects of steam injection on members of this species. The following table provides the list of 16S rRNA-targeted probes that we propose to use in this study.

Target	Probe/Primer Name	Target site[a]	Probe/Primer Sequence 5'--3'	Reference
Universal	S-*-Univ-1390-a-A-18	1407-1390	GACGGGCGGTGTGTACAA	Zheng et al., 1996
Bacteria domain	S-D-Bact-0338-a-A-18	338-355	GCTGCCTCCCGTAGGAGT	Amann et al., 1990a
Archeae domain	S-D-Arch-0915-a-A-20	915-934	GTGCTCCCCCGCCAATTCCT	Amann et al., 1990b
Desulfovibrio spp.	S-F-Dsv-0687-a-A-16	687-702	TACGGATTTCACTCCT	Devereux et al., 1992
Type II Methanotrophs	S-*-M.Ser-0987-a-A-22	987-1008	CCATACCGGACATGTCAAAAGC	Brusseau et al., 1994
Type I Methanotrophs	S-*-M.RuMP-0998-a-A-20	988-1007	GATTCTCTGGATGTCAAGGG	Brusseau et al., 1994
Legionella spp.	*Legionella* CP2 Probe	649-630	CAACCAGTATTATCTGACCG	Jonas et al., 1995
Legionella spp.	Primer LEG 225	225-244	AAGATTAGCCTGCGTCCGAT	Miyamoto et al., 1997
Legionella spp.	Primer LEG 858	880-859	GTCAACTTATCGCGTTTGCT	Miyamoto et al., 1997

[a] *Escherichia coli* numbering

In addition to hybridization of 16S rRNA gene probes hybridization to DNA extracted by a direct method, we will also utilize the denaturing gradient gel electrophoresis (DGGE) described in Muyzer et al., 1996. The DGGE method has been used to detect overall shifts in reductively dechlorinating microbial communities (Flynn et al., 2000). If significant shifts are observed, the DNA bands will be sequenced to analyzed the genetic diversity of the communities.

5.0 Sample Collection, Transport, and Storage

In each test plot, soil samples of approximately 500-g each (250 g frozen for DNA/PLFA analysis; 250 g ambient for microbial counts) will be collected using sterile brass core cylinders. Each clinder holds approximately 250 g of soil. Sterilization of soil sample containers will involve detergent wash, water wash, heating (100 C), and alcohol wash. Polyethylene caps will not be heated, just sterilized with alcohol. Sterilization of drilling equipment will involve steam cleaning between samples.

Five borings per test plot will be used to collect aquifer samples at four depths (capillary fringe, upper sand unit [USU], middle fine grained unit [MFGU], and lower sand unit [LSU]). In addition, groundwater samples will be collected from two well clusters at three depths per plot (USU, MFGU, and LSU). Control samples from an unaffected control area will be collected under the same sampling regime. Soil controls will be collected from five locations, four depths each for consistency with treatment plot samples. Similarly, groundwater controls will be collected from 2 well clusters, at 3 depths each, if available.

Samples will be collected at four events for each technology/plot within two phases:

Phase 1 (June '99 – Sep '00)
T<0 month (pretreatment for SPH and OX)
T= 0 months (post treatment; SPH and OX)
T<0 month (pretreatment; SI)

Phase 2 (Sep '00 – Sep '01)
T= 6 months (post-treatment; SPH, OX, and SI)
T= 12 months (post-treatment; SPH)

Tables 1 and 2 show the number of soil and groundwater samples involved. Table 3 shows the sampling requirements for this evaluation. Immediately after soil samples are retrieved from the borings, the collection cylinders will be tightly capped and sealed to minimize changes in environmental conditions, primarily oxygen content, of the samples. This will subsequently minimize adverse effects to the microbial population during sample transport. Samples for DNA/PLFA analysis will be frozen under nitrogen and shipped via express mail. Samples for microbial counts will be shipped at ambient temperature to an off-site lab designated by the IDC. Microbiology analysis will be conducted within 24 hours of sample collection. Approximately 5-10 g aliquots from each sample will be stored at $<-60^{o}C$ for molecular analysis. The study will be conducted over the course of 1.5 years in which two of the three remediation treatment methods will be demonstrated simultaneously.

Soil and groundwater sample from the region near the historical sewage outfall will be collected and analyzed as shown in Table 3.

As shown in Table 3, groundwater samples will include unfiltered groundwater (for microbial counts) and filters (for DNA/PLFA analysis) from filtration of 1 to 4 L of groundwater. Anodisc™ filters will be used and filtration apparatus will be autoclaved for 20 minutes between samples.

Table 1. Overall Soil Sample Collection Requirement

Plot (Remediation Treatment)	"Event" or Time Points (<0, 0, 6, 12 mo.)	Depths (5, 15, 30, 45 ft.)	Sampling Locations per Plot	Total # Soil Samples Collected Per Plot	Total # of Soil Samples Collected
SPH[a]	3	4	5	80	344
OX[b]	3	4	5	80	
SI	4	4	5	80	
Control	4	4	5	80	
Baseline (T<0 for SPH and OX)	1	4	3[c]	12	
Sewage Outfall	1	4	3	12	

a Fresh samples to be collected as baseline or T<0; shown in last row
b Fresh samples to be collected as baseline or T<0; shown in last row
c From undisturbed DNAPL area inside ESB

Table 2. Overall Groundwater Sample Collection Requirement

Plot (Remediation Treatment)	"Event" or Time Points (<0, 0, 6, 12 mo.	Depths (5, 30, 45 ft.)	Sampling Well Clusters per Plot	Total # of groundwater Samples Collected Per Plot	Total # of Groundwater Samples Collected
SPH[a]	3	3	2	18	87
OX[b]	3	3	2	18	
SI	4	3	2	24	
None (control)	3	3	2	18	
Sewage Outfall	3	3	1	9	

Table 3. Summary of Soil and Groundwater Sampling Requirements

		Native Microbes Analysis				Pathogens Analysis		
Medium	**Plot**	**PLFA/DNA[1]**	***Microbial[2]***	**Locations**	**Sample**	***Coliform/ Legionella***	***Locations***	**Sample**
Soil[3]	SPH	Freeze, store	***Ambient, 24 hrs***	5 cores per plot, 4 depths	2x250 g	*NA*		
	Oxidation	Freeze, store	***Ambient, 24 hrs***		2x250 g	*NA*		
	Steam Injection	Freeze, store	***Ambient, 24 hrs***		2x250 g	*NA*		
	Control	Freeze, store	***Ambient, 24 hrs***		2x250 g	*NA*		
	Baseline	Freeze, store	*Ambient, 24 hrs*	*Inside ESB; 3 cores 4 depths*	*2x250 g*	*NA*		
	Sewage Outfall	*NA*				*3 cores near sewage outfall at 4 depths each*		*2x250 g*
Ground -water[4]	*SPH*	*Filters from 1-4 L filtering, Freeze*	*500 mL unfiltered in Whirl-Pak, ambient*	*PA-13S/D and PA-14S/D*		*NA*		
	Oxidation	*Filters from 1-4 L filtering, Freeze*	*500 mL unfiltered in Whirl-Pak, ambient*	*BAT-2S/I/D and BAT-5S/I/D*		*NA*		
	Steam Injection	*Filters from 1-4 L filtering, Freeze*	*500 mL unfiltered in Whirl-Pak, ambient*	*PA-16S/I/D and PA-17S/I/D*		*NA*		
	Control	*Filters from 1-4 L filtering, Freeze*	*500 mL unfiltered in Whirl-Pak, ambient*	*IW-1I/D and PA-1S/I/D*		*NA*		
	Sewage Outfall	*NA*	*NA*	*NA*		*1 L unfiltered in Whirl-Pak*	*IW-17I/D and PA-15*	

Shaded and italicized text indicates new sampling and analysis scope that needs to be funded. Bold and italics indicates that the sampling is funded but the analysis is not funded.

NA: Not applicable

[1] DNA/PLFA: DNA/PLFA Analysis. Sleeves are frozen in Nitrogen before shipping.

[2] Microbial: Total Heterotrophic Counts/Viability Analysis. Sleeves are shipped at ambient temperature for analysis within 24 hrs.

[3] Soil samples will be collected in 6"-long 1.5"-dia brass sleeves, then capped. Brass sleeves need to be autoclaved and wiped with ethanol just before use. Caps need to be wiped with ethanol prior to use.

[4] 3 to 4 liters of groundwater will be filtered and filters will be shipped for analysis. Filters for DNA analysis will be frozen under N2 before shipping. Groundwater for microbial analysis will be shipped at ambient temperature for analysis within 24 hrs. Between samples, filtration apparatus needs to be autoclaved for 20 minutes.

References

Alvarez-Cohen, L., and P. L. McCarty. 1991. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. *Appl. Environ. Microbiol.* 57:228-235.

Amann, R.I., B.J. Binder, R.J. Olson, S.W. Chisholm, R. Devereux, and D.A. Stahl. 1990a. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56:1919-1925.

Amann, R.I., L. Krumholz, and D.A. Stahl. 1990b. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogentic, and environmental studies in microbiology. J. Bacteriol. 172:762-770.

Ballapragada, B. S., H. D. Stensel, J. A. Puhakka, and J. F. Ferguson. 1997. Effect of hydrogen on reductive dechlorination of chlorinated ethenes. *Environ. Sci. Tech.* 31:1728-1734.

Bouwer, E. J., and P. L. McCarty. 1983. Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. *Appl. Environ. Microbiol.* 45:1286-1294.

Bradley, P. M., and F. H. Chapelle. 1996. Anaerobic mineralization of vinyl chloride in Fe(III)-reducing, aquifer sediments. *Environ. Sci. Technol.* 30:2084-2086.

Bradley, P. M., and F. H. Chapelle. 1997. Kinetics of DCE and VC mineralization under methanogenic and Fe(III)-reducing conditions. *Environ. Sci. Technol.* 31:2692-2696.

Bradley, P. M., F. H. Chapelle, and D. R. Lovley. 1998. Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* 64:3102-3105.

Brusseau, G.A., E.S. Bulygina, and R.S. Hanson. 1994. Phylogenetic analysis of methylotrophic bacteria revealed distinct groups based upon metabolic pathways usage. Appl. Environ. Microbiol. 60:626-636.

Cabirol, N., F. Jacob, J. Perrier, B. Gouillet, and P. Chambon. 1998. Complete degradation of high concentrations of tetrachloroethylene by a methanogenic consortium in a fixed-bed reactor. *J. Biotech*. 62: 133-141.

Carr, C. S., and J. B. Hughes. 1998. Enrichment of high-rate PCE dechlorination and comparative study of lactate, methanol, and hydrogen as electron donors to sustain activity. *Environ. Sci. Technol.* 32: 1817-1824.

Chang, H-L., and L. Alvarez-Cohen. 1996. The Biodegradation of Individual and Multiple Chlorinated Aliphatics by Mixed and Pure Methane Oxidizing Cultures. *Appl. Environ. Microbiol.* 62:3371-3377.

Debruin, W. P., M. J. Kotterman, M. A. Posthumus, D. Schraa and A. J. Zehnder. 1992. Complete biological reductive transformation of tetrachloroethene to ethane. *Appl. Environ. Microbiol.* 58: 1996-2000.

Devereux, R., Kane, M. D., Winfrey, J., Stahl, D.A. 1992. Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. System. Appl. Microbiol. 15:601-609.

DeWeerd, K. A., L. Mandelco, S. Tanner, C. R. Woese, and J. M. Sulfita. 1990. *Desulfomonile tiedjei* gen. nov. and sp. nov., a novel anaerobic, dehalogenating, sulfate-reducing bacterium. *Arch. Microbiol.* 154:23-30.

Diekert, G., U. Konheiser, K. Piechulla, and R. K. Thauer. 1981. Nickel requirement and factor F_{430} content of methanogenic bacteria. *J. Bacteriol.* 148:459-464.

DiStefano, T. D., J. M. Gosset, and S. H. Zinder. 1991. Reductive dechlorination of high concentrations of tetrachloroethene by an anaerobic enrichment culture in the absence of methanogenesis. *Appl. Environ. Microbiol.* 57:2287-2292.

Enzien, M. V., F. Picardal, T. C. Hazen, R. G. Arnold, and C. B. Fliermans. 1994. Reductive Dechlorination of trichloroethylene and tetrachloroethylene under aerobic conditions in a sediment c olumn. Appl. Environ. Microbiol. 60:2200-2205.

Fathepure, B. Z., J. P. Nengu, and S. A. Boyd. 1987. Anaerobic bacteria that degrade perchloroethene. *Appl. Environ. Microbiol.* 53:2671-2674.

Fennell, D. E., J. M. Gossett, and S. H. Zinder. 1997. Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of tetrachloroethene. *Environ. Sci. Tech.*. 31:918-926.

Flynn, S.J., F.E. Loffler, and J.M. Tiedje. 2000. Microbial community changes associated with a shift from reductive dechlorination of PCE to reductive dechlorination of cis-DCE and VC Trichloroethylene biodegradation by a methane-oxidizing bacterium. *Appl. Environ. Microbiol.* 54:951-956.

Fox, B. G., J. G. Borneman, L. P. Wackett, and J. D. Lipscomb. 1990. Haloalkane oxidation by soluble methane monooxygenase from Methylosinus trichosporium OB3b-mechanistic and environmental implications. *Biochem.* 29:6419-6427.

Freedman, D. L., and J. M. Gosset. 1989. Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. *Appl. Environ. Microbiol.* 55:2144-2151.

Gerritse, J., V. Renard, T. M. Pedro Gomes, P. A. Lawson, M. D. Collins, and J. C. Gottschal. 1996. *Desulfitobacterium* sp. strain PCE1, an anaerobic bacterium that that can grow by reductive dechlorination of tetrachloroethane or *ortho*-chlorinated phenols. *Arch. Microbiol.* 165:132-140.

Gibson, S. A., and G. W. Sewell. 1992. Stimulation of reductive dechlorination of tetrachloroethene in anaerobic aquifer microcosms by addition of short-chain organic acids or alcohols. *Appl. Environ. Microbiol*. 58(4):1392-1393.

Hartmans, S. and J. A. M. De Bont. 1992. Aerobic vinyl chloride metabolism in *Mycobacterium*-aurum L1. Appl. Environ. Microbiol. 58: 1220-1226.

Holliger, C., and W. Schumacher. 1994. Reductive dehalogenation as a respiratory process. *Antonie van Leeuwenhoek* 66:239-246.

Holliger, C., D. Hahn, H. Harmsen, W. Ludwig, W. Schumacher, B. Tindal, F. Vasquez, N. Weiss, and A. J. B. Zehnder. 1998. *Dehalobacter restrictus* gen. nov. and sp. nov., a strictly anaerobic bacterium that reductively dechlorinates tetra- and trichloroethene in an anaerobic respiration. *Arch. Microbiol.* 169:313-321.

Holliger, C., G. Schraa, A. J. M. Stams, and A. J. B. Zehnder. 1993. A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethane to growth. *Appl. Environ. Microbiol.* 59:2991-2997.

Krumholz, L. R., R. Sharp, and S. S. Fishbain. 1996. A freshwater anaerobe coupling acetate oxidation to tetrachloroethylene dehalogenation. *Appl. Environ. Microbiol.* 62:4108-4113.

Krzycki, J., and J. G. Zeikus. 1980. Quantification of corrinoids in methanogenic bacteria. *Curr. Microbiol.* 3:243-245.

Lee, M. D., G. E. Quinton, R. E. Beeman, A. A. Biehle, R. L. Liddle, et al. 1997. Scale-up issues for in situ anaerobic tetrachloroethene bioremediation. *J. Ind. Microbiol. Biotechnol.* 18:106-115.

Little, C. D., A. V. Palumbo, S. E. Herbes, M. E. Lidstrom, R. L. Tyndall, and P. J. Gilmer. 1988. Maiwald, M., J.H. Helbig, P.C. Luck. 1998. Laboratory methods for the diagnosis of Legionella infections. J. Microbiol. Meth. 33:59-79.

Malachowsky, K. J., T. J. Phelps, A.B.Tebolic, D. E. Minnikin, and D.C. White. 1994. Aerobic mineralization of trichloroethylene, vinyl chloride, and aromatic compounds by *Rhodococcus* species. *Appl. Environ. Microbiol.* 60: 542-548.

Maymo-Gatell, X., Y. Chien, J. M. Gossett, and S. H. Zinder. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* 276:1568-1571.

McCarty, P.L. 1996. Biotic and abiotic transformation of chlorinated solvents in ground water. *EPA/540/R-96/509.* p5-9.

Miller, E., G. Wohlfarth, and G. Diekert. 1997. Comparative studies on tetrachloroethene reductive dechlorination mediated by *Desulfitobacterium* sp. strain PCE-S. *Arch. Microbiol.* 168:513-519.

Miller, E., G. Wohlfarth, and G. Diekert. 1998. Purification and characterization of the tetrachloroethene reductive dehalogenase of strain PCE-S. *Arch. Microbiol.* 169:497-502.

Miyamoto, H., H. Yamamoto, K. Arima, J. Fujii, K. Maruta, K. Izu, T. Shiomori, and S. Yoshida. 1997. Development of a new seminested PCR method for detection of Legionella species and its application to surveillance of Legionellae in hospital cooling tower water. Appl. Environ. Microbiol. 63:2489-2494.

Murrell, J.C., I.R. McDonald, D.G. Bourne. 1998. Molecular methods for the study of methanotroph ecology. FEMS Microbiol. Ecol. 27:103-114.

Muyzer, G., S. Hottentrager, A. Teske and C. Wawer. 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA- A new molecular approach to analyse the genetic diversity of mixed microbial communities. Molecular Microbial Ecology Manual. 3.4.4:1-23.

Neuman, L. M., and L. P. Wackett. 1991. Fate of 2,2,2-trichloroacetaldehyde (chlora hydrate) produced during trichloroethylene oxidation by methanotrophs. *Appl. Environ. Microbiol.* 57:2399-2402.

Neumann, A., G. Wohlfarth and G. Diekert, 1995. Properties of tetrachlorethene and trichloroethene dehalgenase of Dehalospirillum multivorans. Arch. Microbiol. 163:276-281.

Odem, J. M., J. Tabinowaski, M. D. Lee, and B. Z. Fathepure. 1995. Anaerobic biodegradation of chlorinated solvents: comparative laboratory study of aquifer microcosms. In eds., Hinchee, R. E., A. Leeson, and L. Semprini, *Bioremediation of chlorinated solvents, Third International In Situ and On-Site Bioreclamation Symp.*, Batelle Press, Columbus, OH, pp.17-24.

Oldenhuis, R., J. Y. Oedzes, J. J. van der Waarde, and D. B. Janssen. 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Appl. Environ. Microbiol.* 57: 7-14.

Phelps, T. J., K. Malachowsky, R. M. Schram, and D. C. White. 1991. Aerobic mineralization of vinyl chloride by a bacterium of the order *Actinomycetales*. *Appl. Environ. Microbiol.* 57: 1252-1254.

Rasmussen, G., S. J. Komisar, J. F. Ferguson. 1994. Transfomation of tetrachloroethene to ethene in mixed methanogenic cultures: effect of electron donor, biomass levels, and inhibitors. In eds., Hinchee, R. E., A. Leeson, and L. Semprini, *Bioremediation of chlorinated solvents, Third International In Situ and On-Site Bioreclamation Symp.*, Batelle Press, Columbus, OH, pp309-313.

Scholtz-Muramatsu, H., A. Neumann, M. Meßmer, E. Moore, and G. Diekert. 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov. sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. *Arch. Microbiol.* 163:48-56.

Sharma, P. K. and P. L. McCarty. 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to cis-1,2-dichloroethene. *Appl. Environ. Microbiol*. 62: 761-765.

Smatlak, C. R., J. M. Gossett, and S. H. Zinder. 1996. Comparative kinetics of hydrogen utilization for reductive dechlorination of tetrachloroethene and methanogenesis in an anaerobic enrichment culture. *Environ. Sci. Tech.*. 30:2850-2858.

Strandberg, G. W., T. L. Donaldson, and L. L. Farr. 1989. Degradation of trichloroethylene and *trans*-1,2-dichloroethylene by a methanotrophic consortium in a fixed-film, packed-bed bioreactor. *Environ. Sci. Technol.* 28:973-979.

Tandoi, V., T. D. DiStefano, P. A. Bower, J. M. Gossett. and S. H. Zinder. 1994. Reductive dechlorination of chlorianted ethenes and halogenated ethanes by a high-rate anaerobic enrichment culture. *Environ. Sci. Tech.*. 28:973-979.

Tsien, H-,C., G. A. Brusseau, R. S. Hanson, and L. P. Wackett. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. *Appl. Environ. Microbiol.* 55:3155-3161.

Vanelli, T., M. Logan, D. M. Arciero, and A. B. Hooper. 1990. Degradation of halogenated alophatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas euriopaea*. *Appl. Environ. Microbiol.* 56: 1169-1171.

Vogel, T. M., C. S. Criddle, and P. L. McCarty. 1987. Transformation of halogenated compounds. *Environ. Sci. Technol.* 21:722-736.

Zheng, D., L. Raskin, E.W. Alm, and D.A. Stahl. 1996. Characterization of universal small-subunit rRNA-targeted oligonucleotide hybridization probes. Appl. Environ. Microbiol. 62:4504-4513.

E.2 Microbiological Evaluation Sampling Procedures

Work Plan for Biological Soil and Groundwater Sampling and Procedure

Battelle
January 4, 2001

Soil Sampling

Soil samples are collected at four discrete depths in the subsurface with a 2-inch diameter sample barrel containing sample sleeves. Once the sample is retrieved, the sleeves are removed from the sample barrel, capped at both ends, and preserved accordingly. The sleeves are then transported to off-site analytical laboratories for analyses. Field personnel should change their gloves after each sample to prevent cross-contamination. The details of the sampling are provided below:

Samplers: The Mostap™ is 20-inch long with a 1.5-inch diameter and the Macro-core™ sampler is about 33-inch long with a 2-inch diameter. Sleeves (brass or stainless steel) are placed in a sample sampler (Macro-core™ or Mostap™). Brass sleeves with 1.5-inch diameter and 6-inch long are used for a Cone-Penetrometer (CPT) rig from U.S. EPA. Stainless steel sleeves with 2-inch diameter and 6-inch long are used with a rig from a contracted drilling company rig.

For Mostap™, three of these brass sleeves and one spacer will be placed in the sampler. For the Macro-Core™ sampler, five 6-inch long stainless sleeves and one spacer are required. All sleeves and spacers need to be sterilized and the procedure is as follows.

Procedures: sampling preparation procedures are as follows:

1. Preparation for sterilization:

    - Dip sleeves in an isopropyl alcohol bath to clean surface inside and outside
    - Air-dry the sleeves at ambient temperature until they are dried
    - Wrap up the sleeves with aluminum foil
    - Place the aluminum foil-wrapped sleeves in an autoclavable bag and keep the bag in a heat-resistant plastic container
    - Place the container in an autoclave for 30 minutes at about 140 °C
    - Once the autoclaving is completed, let the sleeves sit until the materials are cool, and then pack and ship to the field site.

2. In the field, drive the sample barrel down to four different depths: approximately 8 (capillary fringe), 15 (USU below water table), 23 (MFGU), and 45 (LSU) ft below ground surface (bgs). Once the sample barrel is withdrawn, the sleeves are extruded from the sample barrel. Each sleeve immediately capped with plastic end caps that have been previously wiped with isopropyl alcohol. After capping, clear labeling of the sleeve is required including sample site, sample ID, actual depth of the sample, collection date and time, percentage of recovery in each sleeve, and markings for top and bottom of the sample sleeves.

    Sample Preservation: one of the sleeves is kept at ambient temperature. At least, two of the sleeves need to be frozen in liquid nitrogen immediately then stored in a freezer at temperature below freezing point.

Off-site Laboratories: The sample sleeve at ambient temperature is to be shipped off to Florida State University for analyses of ***live/dead stain test*** and ***aerobic and anaerobic heterotrophic counting***. The frozen samples are shipped off to EPA Ada Laboratory, an off-site laboratory for ***DNA*** and ***Phospholipids Fatty Acid Analyses (PLFA)***.

3. Decontamination Procedure: after the samples are extruded, the sample barrel used to collect the soil samples needs to be disassembled and cleaned in Alconox® detergent mixed water. The sample barrel is then rinsed with tap water, followed by de-ionized (DI) water. The sample barrel is air-dried and rinsed with isopropyl alcohol before the next sampling.

Groundwater Sampling

Groundwater sampling involves collection of groundwater from performance monitoring wells using a peristaltic pump and Teflon® tubing. During the groundwater sampling, unfiltered water samples will be collected. Large volume of groundwater will be filtered through in-line filtration unit and the filter will be retrieved and this filter will be preserved necessarily.

1. Preparation for Sterlization
   - Dip in-line filter holders in an alcohol bath and air-dry
   - Wrap each filter unit up in aluminum foil
   - Place them in an autoclavable bag and keep the bag in a heat resistant container
   - Autoclave the container with filters for 30 minutes at 140°C
   - Once the autoclaving is completed, let the sleeves sit until the materials are cool, and then pack and ship to the field site.

2. Materials and Equipments: Non-carbon Anodisc® 0.2 µm pore size supported filters, filtration equipment, a low-flow pump, Teflon tubing and Viton® tubing and a vacuum (or pressure) pump.

The dimensions of the Anodisc® filters are 0.2 micron pore size and 47-mm diameter. The filters are pre-sterilized by the manufacturer. Each filter is carefully placed inside a filter holder case. A forcep is used to place a filter in either an in-line polycarbon filter holder or in an off-line filter holder. The filter is very brittle and should be handled delicately.

3. Filter samples by using an in-line filter holder: An Anodisc® filter is wetted with D.I. water and placed on the influent end of the filter holder. A rubber o-ring is gently placed on the filter holder. The filter holder is connected to the effluent end of the peristaltic pump with Teflon® tubing and approximately one liter of groundwater is filtered through it. The filter is retrieved from the filter holder carefully with forceps and placed in a Whirl-Pak®. The filter, along with the bag, is deep frozen under liquid nitrogen and stored in a freezer until shipping.

4. Filter Samples by using an filtration unit: To use this filtration device, a vacuum or pressure pump is required to pull or push the water through. Influent water from a low-flow peristaltic pump goes into a funnel-shaped water container. The filter will be retrieved after water filtration and the filtrated water can be disposed. The filter is frozen immediately in liquid nitrogen and stored then kept in a freezer.

5. Unfiltered Groundwater Samples: unfiltered groundwater samples are collected into each 500-mLWhirl-Pak® bag. This water sample is kept at ambient temperature.

6. Labeling includes sample ID, same date and time, and site ID on the Whirl-Pak® after the sample is placed with a permanent marker.

7. Sterilization of the filter holders may be done as follows:

   - Clean forceps and filter holder in warm detergent mixed water, then rinse with isopropyl alcohol and air-dry at room temperature.
   - The cleaned forceps and filter holders are wrapped in aluminum foil and taped with a piece of autoclave tape that indicates when the autoclaving is completed.
   - These items are then placed in an autoclavable bag and the bag is placed in an autoclave for about 30 minutes at 140 °C. After taking them out of the autoclave, the items sit until cool.

8. Off-site laboratories: The unfiltered water samples are shipped off to Florida State University for ***aerobic and anaerobic heterotrophic count tests and viability analysis*** at ambient temperature within 24 hours**.** The filter samples are shipped off in dry-ice condition to EPA Ada Lab for ***DNA, PLFA, and Legionella analyses***.

Sample Locations

Soil Sampling

Five biological sampling locations will be located in each of three plots in January 2001. One duplicate samples will be collected from one of the five boring locations in each plot (Figure 1). At each location, soil samples will be collected at four depths (Capillary fringe, USU, MFGU and LSU). Soil sampling procedures are described in previous sections. Summary of the biological soil sampling is shown in Table 1.

Table 1. Biological Soil Sampling in January-February 2001

Plot	Event	Number of Coring	Total Number of Samples
Steam Injection	Pre-Demo (T<0)	5	20 + 1 (Dup)
Oxidation	6 Months After (T=6)	5	20 + 1 (Dup)
Control	-	5	20
SPH*	Post-Demo (T=0)	5	20 + 1 (Dup)

* In February along with chemical coring in Oxidation plot.

Groundwater Sampling

Biological groundwater samples will be collected from wells within the Steam Injection plot, the Oxidation plot, and the Six-Phase Heating plot in January 2001 in conjunction with the biological soil sampling. Groundwater sampling will be completed as described previously. One QA groundwater sample will be completed at a random well location. Table 2 summarizes the performance monitoring wells (Figure 1) to be sampled.

Table 2. Biological Groundwater Sampling in January-February 2001

Plot	Event	Well ID	Total Number of Samples
Steam Injection	Pre-Demo (T<0)	PA-16S/I/D PA-17S/I/D	6
Oxidation	6 Months After (T=6)	BAT-2S/I/D BAT-5S/I/D	6
SPH	Post-Demo (T=0)	PA-13S/D PA-14S/D	4
Control	-	PA-18S/I/D	3
QA	-	random	1

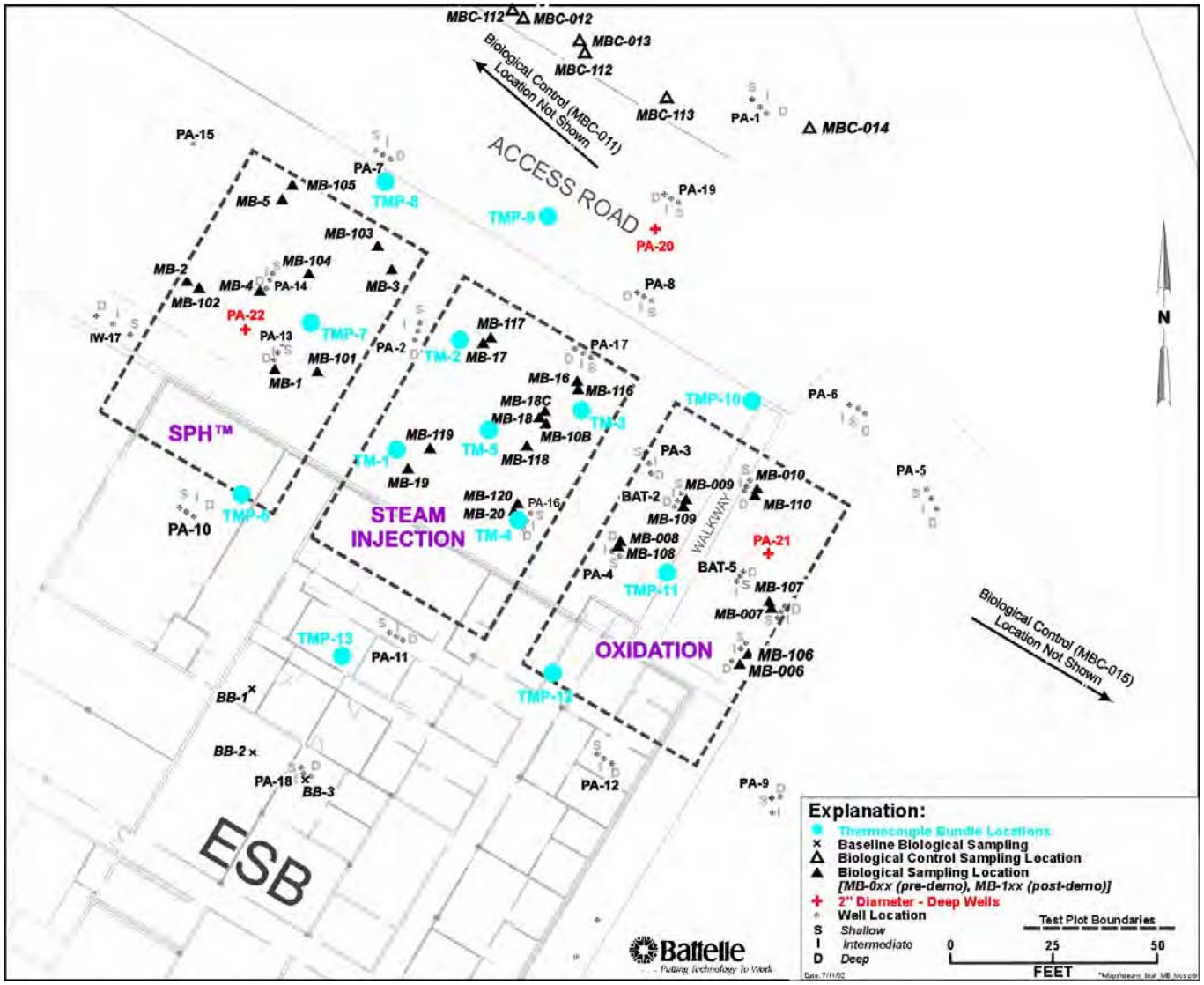

Figure 2. Map of Biological Sampling Location at LC34

Table E-1. Results of Microbial Counts of Soil Samples

Sample ID	Top Depth ft bgs	Bottom Depth ft bgs	Aerobic Heterotrophic Counts CFU/g* or MPN/g	Anaerobic Heterotrophic Counts Cells/g or MPN/g	BacLight Counts/ Live dead stain %live/%dead
Soil Core Samples					
Baseline Samples (August 2000)					
BB1-A	7	9	15,849	7,943	59/41
BB1-A	15.5	17	<316.23	158	25/75
BB2-A	7	9	19,953	31,623	70/30
BB3-A	9	11	12,589	3,162	39/61
BB3-A	15	17	<316.23	<1.78	28/72
BB-1-7.0	6.5	7.0	79,432.8	1,584,893.2	40/60
BB-1-14.0	13.5	14.0	<316.2	631.0	32/68
BB-1-24.0	23.5	24.0	199.5	1,584.9	28/72
BB-1-44.0	43.5	44.0	<316.2	316.2	82/18
BB-2'-7.0	6.5	7.0	19,952.6	19,952.6	43/57
BB-2-7.0	6.5	7.0	31,622.8	10,000.0	27/73
BB-2-16.5	16.0	16.5	2,511.9	3,162.3	15/85
BB-2-23.0	22.5	23.0	1,584,893.2	1,258,925.4	24/76
BB-2-24.0	23.5	24.0	<316.2	No Growth	10/90
BB-2-44.0	43.5	44.0	<316.2	251.2	92/08
BB-3-7.0	6.5	7.0	199,526.2	158,489.3	99/01
BB-3-14.0	13.5	14.0	6,309.6	50,118.7	84/16
BB-3-24.0	23.5	24.0	631.0	501.2	100/0
BB-3-44.0	43.5	44.0	25,118.9	63,095.7	56/44
Control Samples, Untreated (June 2000 except MBC014 in January 2001)					
MBC011-A-1	6	7.5	1,584,893	1,584,893	77/23
MBC011-A-2	15	16.5	501,187	794,328	79/26
MBC011-A-3	30	31.5	15,849	7,943	75/25
MBC011-A-4	40	41.5	316,228	63,096	26/74
MBC012-A-1	6	7.5	25,119	50,119	43/57
MBC012-A-3	30	31.5	125,893	6,310	48/52
MBC012-A-4	40	41.5	1,585	794	59/41
MBC013-A-1	6	7.5	125,893	19,953	50/50
MBC013-A-2	15	16.5	1,259	2,512	61/39
MBC013-A-3	30	31.5	501	794	44/56
MBC013-A-4	40	41.5	7,943	5,012	18/82
MBC014	7	7.5	63,095.73	79,432.82	47/53
MBC014	16	16.5	100,000.00	316,227.77	43/57
MBC014	31	31.5	39,810.72	79,432.82	55/45
MBC014	41	41.5	7,943.28	25,118.86	50/50
MBC015-A-1	6	7.5	3,981	5,012	53/47
MBC015-A-3	35	36.5	316	251	41/59
Control Samples, Untreated (April 2001)					
MBC-011	7	7.5	15,848,932	7,943,282	94/06
MBC-011	20	20.5	25,119	10,000	86/14
MBC-011	24.5	25	3,981	2,512	88/12

Table E-1. Results of Microbial Counts of Soil Samples (continued)

Sample ID	Top Depth ft bgs	Bottom Depth ft bgs	Aerobic Heterotrophic Counts CFU/g* or MPN/g	Anaerobic Heterotrophic Counts Cells/g or MPN/g	BacLight Counts/ Live dead stain %live/%dead
MBC-011	41.5	41.75	25,119	79,433	89/11
MBC-011	41.75	42	25,119	10,000	80/20
MBC-012	20.5	21	1,995	794	95/05
MBC-012	24.5	25	19,953	31,623	91/09
MBC-012	41	41.5	126	158	98/02
MBC-013	6.5	7	1,000,000	316,228	47/53
MBC-013	10	10.5	15,849	25,119	80/20
MBC-013	20.5	21	6,310	1,585	100/0
MBC-013	24	24.5	631	1,259	76/24
MBC-013	41.5	42	2,512	2,512	73/27
MBC-214	32	32.5	501,187	316,228	90/10
MBC-214	40	40.5	79,433	10,000	96/04
MBC-015	6.5	7	316,228	1,584,893	100/0
MBC-015	20.5	21	39,811	5,012	82/18
MBC-015	24	24.5	794	1,585	85/15
MBC-015	41.5	42	6,310	12,589	94/06
Control Samples, Untreated (June 2002) (MPN/g)					
MBC111	6	6.5	85,000	19,000	40/60
MBC111	15.5	16	5	42,000	48/52
MBC111	30	30.5	150	5,700	18/82
MBC111	40	40.5	5	1,500	50/50
MBC112	6	6.5	48,000	30,000	28/72
MBC112	15	15.5	8,500	550	37/63
MBC112	30	30.5	29	4,800,000	30/70
MBC112	40	40.5	19	220,000	37/63
MBC-113	6	6.5	480,000	85,000	58/42
MBC-113	15	15.5	48	190	65/35
MBC-113	30	30.5	85,000	480,000	50/50
MBC-113	40	40.5	8,500	4,800,000	49/51
Steam Plot, Untreated T<0 (January 2001)					
MB16-A	15	15.5	15,848.93	100,000.00	17/83
MB16-B	31	31.5	794.33	251.19	55/45
MB16-C	32	33.5	<316.23	100.00	30/70
MB16-D	41	41.5	1,258.93	501.19	34/66
MB17-B	16	16.5	2,511.89	2,511.89	95/05
MB17-C	31	31.5	<316.23	316.23	33/67
MB17-D	33	33.5	158.49	501.19	28/72
MB18A	7	7.5	19,952.62	501,187.23	42/58
MB18B	21.5	22	<316.23	251.19	48/52
MB-18	31	31.5	10,000.00	1,995.26	60/40
MB-18	41	41.5	158,489.32	501,187.23	73/27
MB-19A	7	7.5	398,107.17	100,000.00	53/47
MB-19B	16	16.5	<316.23	39.81	36/64

Table E-1. Results of Microbial Counts of Soil Samples (continued)

Sample ID	Top Depth ft bgs	Bottom Depth ft bgs	Aerobic Heterotrophic Counts CFU/g* or MPN/g	Anaerobic Heterotrophic Counts Cells/g or MPN/g	BacLight Counts/ Live dead stain %live/%dead
MB-19C	31	31.5	<316.23	<1.78	76/24
MB-19D	41	41.5	<316.23	251.19	71/29
MB-20A	7	7.5	50,118.72	158,489.32	82/18
MB-20B	21.5	22	<316.23	158.49	47/53
MB-20C	31	31.5	12,589.25	31,622.78	80/20
MB-20D	41	41.5	158,489.32	158,489.32	22/78
Steam Plot, Treated T=0 (June 2002) (MPN/g)					
MB16-A	7	7.5	85,000.0	1,500.0	69/31
MB16-B	15	15.5	1.8	<0.6	23/77
MB16-C	30	30.5	85.0	4.6	46/54
MB16-D	40	40.5	150.0	48.0	66/34
MB17-A	6	6.5	41,000.0	850.0	66/34
MB17-B	15	15.5	57.0	8.5	81/19
MB17-C	31.5	32	85.0	4.6	44/56
MB17-D	40	40.5	8.5	8.5	44/56
MB-20A	6	6.5	220,000.0	410,000.0	45/56
MB-20B	15	15.5	300,000.0	550,000.0	60/40
MB-20C	30	30.5	4.6	4,800,000.0	33/67
MB-20D	40	40.5	85.0	410,000.0	17/83

bgs: Below ground surface.

*CFU: Colony-forming units (roughly, number of culturable cells).

Table E-2. Results of Microbial Counts Groundwater Samples

Sample ID	Top Depth ft bgs	Bottom Depth ft bgs	Aerobic Heterotrophic Counts CFU/mL or MPN/mL	Anaerobic Heterotrophic Counts Cells/mL or MPN/mL
Groundwater Samples				
Control Samples, Untreated, Distant Wells				
IW-1I	NA	NA	79,433	>1,584,893.19
IW-1D	NA	NA	5,012	15,849
PA-1S	NA	NA	15,849	158,489
PA-1I	NA	NA	501,187	>1,584,893.19
PA-1D	NA	NA	39,811	1,584,893
Control Samples, Untreated, Distant Wells, T=0 (June 2002) (in MPN/L)				
PA-1S	NA	NA	2,200	4
PA-1S-DUP	NA	NA	2,200	300
PA-1I	NA	NA	48,000	48
PA-1D	NA	NA	92,000	67
Steam Injection Plot Wells, Untreated, T<0 (February 2001)				
PA-16S	NA	NA	158,489	602
PA-16I	NA	NA	1,000	12
PA-16D	NA	NA	16	7
PA-17S	NA	NA	<31.7	6.31
PA-17I	NA	NA	501	5,011.87
PA-17I -Dup	NA	NA	398	10,000.00
PA-17D	NA	NA	159	1,000.00
Steam Injection Plot Wells, Treated, T=0 (June 2002) (in MPN/L)				
PA-16S	NA	NA	92,000	920
PA-16I	NA	NA	48,000	4,800
PA-16D	NA	NA	92,000	48
PA-17S	NA	NA	48,000	9
PA-17I	NA	NA	920,000	48
PA-17D	NA	NA	48,000	430
Steam Injection Perimeter Wells, Untreated, T<0 (February 2001)				
PA-18S	NA	NA	<31.7	2
PA-18I	NA	NA	<31.7	<1.3
PA-18D	NA	NA	13	5
Steam Injection Perimeter Wells, Untreated, T=0 (June 2002) (in MPN/L)				
PA-13S	NA	NA	220,000	8.5
PA-13I	NA	NA	48,000	92
PA-13D	NA	NA	3,000	0.61
PA-14S	NA	NA	48,000	4.6
PA-14I	NA	NA	48,000	2.9
PA-14D	NA	NA	48	48

bgs: Below ground surface.

*CFU for Colony-forming units (roughly, number of culturable cells).