Derivative Classifier _________ WSRC-RD-91-23
D.B. Moore, Section Manager
Authorized Derivative Classifier

Test Plan for In Situ Bioremediation Demonstration of the Savannah River Integrated Demonstration Program DOE/OTD TTP No.: SR 0566-01 (U)

Terry C. Hazen

September 18, 1991

Authentication: _________

Approved by: _________
D.B. Moore-Sheadrow, Manager
Environmental Sciences Section
Savannah River Laboratory

WESTINGHOUSE SAVANNAH RIVER COMPANY
SAVANNAH RIVER SITE
Aiken, South Carolina 29808

Prepared for the U.S. Department of Energy under Contact No. DE-AC09-89R180035

Test Plan for In Situ Bioremediation Demonstration of the Savannah River Integrated Demonstration Program DOE/OTD TTP No.: SR 0566-01 (U)

Table of Contents

1.0 Test Plan Summary ... 1
2.0 Test Objectives ... 2
3.0 Background: Technology and Site. ... 3
 3.1 Technology Background. ... 3
 3.2 Technical Need. ... 4
 3.3 Alternatives. ... 5
 3.4 Benefits. ... 5
 3.5 Acceptability. ... 5
 3.6 Site Description. ... 6
 Figure 3.1. Integrated Demonstration Project Area Location Map. ... 8
4.0 Test Plan ... 9
 4.1 Criteria for Success. ... 9
 4.2 Pre-Test Characterization and Monitoring ... 9
 Figure 4.1. Map of Wells and Boreholes at Integrated Demonstration Project. ... 10
 4.3 Test Description. ... 12
  4.3.1 Injection Protocol. ... 12
  Figure 4.2. SRS Integrated Demonstration Project Site Plan. ... 13
  4.3.2 Tracer Studies. ... 14
  4.3.3 Groundwater Monitoring. ... 14
   4.3.3.1 Analysis of VOC. ... 14
   4.3.3.2 Acridine Orange Direct Counts (AODC). ... 15
   4.3.3.3 Aerobic Heterotrophic Plate Count. ... 15
   4.3.3.4 Methane Enrichment Plate Count. ... 15
   4.3.3.5 Anaerobic Plate Count. ... 15
   4.3.3.6 Community Diversity/Functionality ... 16
   4.3.3.7 Fluorescent Antibody Direct Counts. ... 16
   4.3.3.8 Signature Biomarkers ... 16
   4.3.3.9 Nucleic Acid Analysis. ... 17
   4.3.3.10 TCE/PCE Mineralization Analysis. ... 18
   4.3.3.11 Toxicity Analysis. ... 18
   4.3.3.12 Physical/Chemical Analysis. ... 19
   4.3.3.13 Fungal Analysis (Optional). ... 19
  4.3.4 Sediment Monitoring. ... 20
   4.3.4.1 Analysis of VOC. ... 20
   4.3.4.2 Acridine Orange Direct Counts (AODC). ... 20
   4.3.4.3 Aerobic Heterotrophic Plate Count. ... 21
   4.3.4.4 Methane Enrichment Plate Count. ... 21
   4.3.4.5 Anaerobic Plate Count. ... 21
   4.3.4.6 Community Diversity/Functionality ... 21
   4.3.4.7 Fluorescent Antibody Direct Counts. ... 22
   4.3.4.8 Phospholipid Fatty Acid Analysis and Other Physiological Measurements. ... 22
   4.3.4.9 Nucleic Acid Analysis. ... 23
   4.3.4.10 TCE/PCE Mineralization Analysis. ... 24
   4.3.4.11 Toxicity Analysis. ... 24
   4.3.4.12 Physical/Chemical Analysis. ... 24
   4.3.4.13 Protozoan Analysis. ... 25
   4.3.4.14 Fungal and Actinomycete Analysis (Optional). ... 25
  4.3.5 Offgas Monitoring. ... 26
  4.3.6 Laboratory Process Control Studies ... 26
   4.3.6.1 SRL. ... 27

4.3.6.2 ORNL. 27
4.3.6.3 INEL. 27
4.3.7 Ancillary IDP Monitoring Activities. 27
4.3.8 Meteorological Data. 27
4.3.9 Ancillary Groundwater Data. 27
4.4 Post-Test Characterization and Monitoring. 28
4.4.1 Groundwater Monitoring. 28
4.4.2 Sediment Characterization. 28
4.5 Modelling 28
4.6 Schedule of Events and Reports. 28
4.6.1 Project Planning and Management. 28
4.6.2 Process Design and Modeling. 28
4.6.3 Operations Contract. 29
4.6.4 Permitting. 29
4.6.5 Analytical Facilities. 29
4.6.6 Pre-Test Characterization. 29
4.6.7 Finalization of Test Plan by BTSG. 29
4.6.8 Monitoring. 29
4.6.9 QA and Safety Report. 29
4.6.10 Mobilization. 29
4.6.11 Test Schedule. 29
4.6.12 Post-Test Monitoring. 30
4.6.13 Post-Test Characterization. 30
4.6.14 Demobilization. 30
4.6.15 Final Operations Report. 30
4.6.16 BTSG Review of Test and Final Test Report. 30
4.6.17 Post-Test Characterization Report. 30
4.6.18 BTSG Review and Recommendations. 30
4.6.19 Final Technology Report. 30
4.7 Communications and Technology Transfer. 30
4.7.1 WIN. 30
4.7.2 Reports and Sample Shipments. 30
4.7.3 Bioremediation Technical Support Group. 30
4.7.4 In Situ Bioremediation Demonstration Project Symposium. 30
4.7.5 Publications and Presentations. 30
5.0 Organizational Structure and Funding. 31
5.1 U.S. Department of Energy. 31
5.2 Westinghouse Savannah River Company. 31
5.3 Integrated Demonstration Project. 31
5.4 Bioremediation Technical Support Group. 31
5.5 Primary and Ancillary Technical Task Plans and Funding. 31
5.5.1 Savannah River Site 31
5.5.2 Oak Ridge National Laboratory 32
5.5.3 Idaho National Engineering Laboratory 32
5.5.4 Pacific Northwest Laboratory 32
5.6 Participants: Government, Industry, Academic 32
6.0 Permits, Patents, Licences and Contracts. 33
6.1 Permits 33
6.1.1 National Environmental Policy Act 33
6.1.2 Underground Injection Control Permit 33
6.1.3 Air Permit 33
6.1.4 US Department of Transportation Certification. 33
6.1.5 DOE SRS Site Use, Site Clearance, and Work Clearance Permits 33
6.2 Patents and Licences 33
6.2.1 Patent US 4660639 33
6.2.2 Patent US 4832122 33
6.2.3 Patent US 4713343 33
6.2.4 Patent US 5006250 33

6.2.5 Catalytic Destruction of Offgas Contaminants....34
6.3 Contracts and Agreements....34
6.3.1 Operations Contract....34
6.3.2 Methane Supply and Storage....34
6.3.3 Chemical Analysis of Water and Soil....34
6.3.4 Stanford University....34
6.3.5 ManTech Environmental Technology Inc. - USEPA....34
6.3.6 SCRUEF - University of South Carolina....35
6.3.6.1 Dr. James Yates....35
6.3.6.2 Dr. Charles Lovell....35
6.3.6.3 Dr. John Morse....35
6.3.7 Georgia State University (Optional)....35
7.0 Safety, Quality Assurance and Security....36
7.1 Safety....36
7.1.1 Savannah River Site....36
7.1.2 Savannah River Laboratory....36
7.1.3 Process Hazards Review....36
7.1.4 Other Safety Information....36
7.1.5 Subcontractor....36
7.2 Quality Assurance/Quality Control....36
7.2.1 Westinghouse Savannah River Company....36
7.2.2 Chemical Processes and Environmental Technology Department....36
7.2.3 Environmental Sciences Section....36
7.2.4 Subcontractor....36
7.3 Security....36
8.0 REFERENCES CITED....37
Appendix A. Lists of Analytical Parameters....43
Appendix B. Technical Task Plan....49
Appendix C. Scope of Work....71

Test Plan for In Situ Bioremediation Demonstration
of the
Savannah River Integrated Demonstration Program
DOE/OTD TTP No.: SR 0566-01

1.0 Test Plan Summary

This project is designed to demonstrate in situ bioremediation of groundwater and sediment contaminated with chlorinated solvents. Indigenous microorganisms will be stimulated to degrade trichloroethylene (TCE), tetrachloroethylene (PCE) and their daughter products in situ by addition of nutrients to the contaminated zone. In situ biodegradation is a highly attractive technology for remediation because contaminants are destroyed, not simply moved to another location or immobilized, thus decreasing costs, risks, and time, while increasing efficiency and public and regulatory acceptability. Bioremediation has been found to be among the least costly technologies in applications where it will work.

The horizontal wells that form the basis for the SRS Integrated Demonstration are expected to provide significant advantages over conventional bioremediation nutrient delivery techniques. The increased surface area will allow better delivery of nutrients and easier recovery of gas and water, as well as minimizing formation clogging and plugging phenomena. The principal nutrient to be supplied via the horizontal wells in this test is methane, at a low concentration in air (4%). The lower horizontal well will provide a very efficient delivery of gas throughout the contaminated region. A vacuum will be applied to the upper well (vadose zone) to encourage air/methane movement through the upper saturated zone and lower vadose zone and inhibit spreading of the plume. Air/methane mixtures have been demonstrated to stimulate selected members of the indigenous microbial community that have the capability to degrade TCE. An extensive characterization and monitoring program using existing monitoring wells and periodic borings for sediment will be used to measure the response of the soil and water following injection of air/methane. In addition, off-gas from the upper horizontal well will be assayed for methane, total VOC, TCE, PCE, potential break down products of TCE/PCE (eg. DCE, VC, and carbon dioxide). Data from the previous demonstration of in situ air stripping, where air alone was injected at different rates for 19 weeks will be used to provide base line geological, hydrological, chemical, and biological characteristics. An extensive pre-test and post-test characterization of the site via sediment borings was done for the in situ air stripping test. The post-test characterization study for the in situ air stripping test will also serve at the pre-test characterization for the in situ bioremediation test. The previous characterization and monitoring data will also establish the effect of air injection without nutrients on the hydrological, chemical, and biological characteristics of the site, in effect providing a unique and dramatic control experiment for the first bioremediation demonstration. After the test is complete another post-test characterization will be done at the site.

Initially 4% air/methane will be injected continuously; however, in order to insure process optimization, ie. to further stimulate the indigenous microorganisms to peak biodegradation rates and efficiencies, the injection protocol may be altered. At three month intervals during the twelve month demonstration the data from the test and process support activities will be examined by the technical support group and a decision made as to whether the injection protocol should be altered. These alterations could include changes in injection rates, extraction rates, concentrations of methane, pulsing of air/methane to stimulate stress biodegradation, vadose zone wetting to inhibit the potential drying effect of vacuum extraction from the vadose zone, and periodic addition of other nutrients, such as phosphate, that may prove to be limiting factors. It is anticipated that more than one of these alternatives will be tried during the twelve month demonstration.

2.0 Test Objectives: The principal objective is to demonstrate the utility of in situ methanotrophic bioremediation for cleanup of non-arid waste sites contaminated with chlorinated solvents. The ancillary objectives are, 1. to establish the optimal conditions for complete biodegradation of chlorinated solvents by in situ nutrient stimulation of microbes, 2. to demonstrate the utility of horizontal wells as a nutrient delivery technique for in situ bioremediation, 3. to demonstrate the utility of biomolecular probes (nucleic acids, antibodies and enzymes) and other direct analysis assays for

characterization, monitoring and controlling the biological aspects of an in situ bioremediation, and 3. to establish, via process optimization studies (bioreactors) compared with in situ data, an explanatory and deterministic environmental model of the in situ methanotrophic bioremediation process.

3.0 Background: Technology and Site.

3.1 Technology Background. Biodegradation of TCE by methanotrophs (methane-oxidizing bacteria) has been demonstrated in microbiological studies and in methanotrophic laboratory-scale bioreactors. J. T. Wilson at the U.S. Environmental Protection Agency laboratory in Ada, Oklahoma was among the first to observe TCE degradation in laboratory soil columns in the presence of methane (Wilson and Wilson, 1985; Wilson et al., 1986). Investigators at Stanford University were demonstrated TCE degradation by methanotrophs in laboratory columns of saturated aquifer material (Mayer et al., 1988). Little et al. (1988) at ORNL isolated a mixed methanotrophic culture from a TCE-contaminated well on the Oak Ridge Reservation. This culture was subsequently used in a prototype lab-scale continuous flow bioreactor at ORNL (Donaldson et al., 1988). Fliermans et al. (1988) at SRL isolated consortia and species capable of aerobic degradation of TCE with methane as the primary nutrient from TCE contaminated soil and groundwater from the Savannah River Site. These organisms have also been successfully used in laboratory scale fluidized bed bioreactors to treat TCE/PCE contaminated groundwater (Phelps et al., 1990).

Methanotrophs, methane-oxidizing bacteria, oxidize methane via a series of enzymes that are unique to this group. The primary enzyme in this oxidation chain is methane monooxygenase. Methane monooxygenase is an extremely powerful oxidizer, thus giving it the capability of oxidizing a wide variety of normally recalcitrant compounds including TCE. Wackett (Newman and Wackett, 1991; Tsien et al., 1989) and others (Chaudhry and Chapalamadugu, 1991; Wilson and Wilson, 1985; Fogel et al., 1986; Little et al., 1988) have shown that the soluble methane monoxygenase type 1 induces formation of TCE-epoxide from TCE. TCE-epoxide is extremely unstable and therefore spontaneously breaks down to simpler compounds like formate, etc. All of the daughter compounds are either unstable or small and easily metabolizable compounds, thus making the final and almost immediate end products of TCE-epoxide formation, carbon dioxide and chloride. Several investigators have also shown that even though TCE is degraded by methanotrophs they achieve no measurable benefit from the reaction making it a fortuitous metabolism or as some investigators prefer, co-metabolism/co-oxidation.

Other leading investigators in the development of TCE bioremediation technology include W. Jewell at Cornell University, P. McCarty at Stanford University, D. White and T. Phelps at The University of Tennessee (UT), S. Fogel at CAA, Inc., and a group at Battelle Columbus. These investigators comprise a consortium for development of this methanotrophic treatment technology under the auspices of the Gas Research Institute and the Savannah River Laboratory (SRL). The investigators meet regularly to exchange technical information, and Radian Corporation is serving as a data repository and process evaluation function under contract to the Gas Research Institute. The combined expertise and knowledge base of this consortium will be essentially an ad hoc resource to this present DOE in situ remediation project since Oak Ridge National Laboratory (ORNL), UT, and SRL are charter members of the consortium.

In addition to the laboratory bioreactor studies at ORNL, UT, and elsewhere, one pilot-scale bioreactor system has been operated by Battelle Columbus at Tinker Air Force Base, Oklahoma (Wickramanayake et al., 1990). This project was funded by the Air Force Engineering and Services Center, Tyndall Air Force Base, Florida. This pilot-scale study demonstrated that actual TCE-contaminated groundwater can be treated in a trickle-bed bioreactor. The culture used in this test was provided by ORNL. Tyndall AFB is continuing to support development of TCE bioreactor technology at ORNL and UT and Savannah River Site (SRS). The bioreactors used at Tinker Air Force Base are being provided by the Air Force for further field tests at Oak Ridge and Savannah River.

Although development of methanotrophic bioreactors for TCE bioremediation is progressing well, in situ biodegradation of TCE is an emerging technology that has not yet been

demonstrated at a practical scale. Tests on a small area of a shallow aquifer at the Moffett Naval Air Station in California (Semprini et al., 1988) have shown that indigenous microorganisms can be stimulated with methane and oxygen to degrade TCE. These results are very encouraging. Their experiences in these studies are a large part of the basis for the process design for this in situ demonstration at the SRS.

Methane itself is generally recognized as a natural compound found universally in subsurface environments. Years of experience by the Oil and Gas industries has shown that subsurface environments and groundwater can be exposed to high concentrations of methane for many years with no adverse effects. In addition, the U.S. Geological Survey has used methane as a conservative tracer in groundwater at Cape Cod for several years at their Groundwater Flow Study Facility with no adverse effects (Harvey and George, 1987; Garabedian, 1990). Thus we are confident that methane can be injected safely with extremely low probability of any adverse environmental effects of any kind.

3.2 Technical Need. Organic xenobiotic chemical contamination of groundwater has become the most important pollution problem of industrialized nations of the world. More than 15% of community drinking water supplies in the United States are contaminated with carcinogenic, chlorinated hydrocarbons (Craun, 1986; Patrick et al., 1983). Identification of previously unknown waste disposal sites that are impacting groundwater occurs almost daily, thus the extent of the problem is undoubtedly greater than any of the current data suggest. Indeed, our reliance on groundwater in the United States has steadily increased over the past 30 years, not only for drinking water but also for industrial processes, agricultural irrigation, etc. (Craun, 1986; Patrick et al., 1983). As sources of clean surface water steadily decline, our reliance on groundwater will undoubtedly continue to increase far into the next century. Thus, with increasing urgency ways have been sought to clean-up, i.e. remediate, contaminated groundwater. The major organic contaminate of waste sites at DOE facilities is also chlorinated solvents.

Subsurface soils and water adjacent to an abandoned process sewer line at the SRS have been found to have elevated levels of TCE/PCE. This area of subsurface and groundwater contamination is the focus of a current integrated demonstration of in situ air stripping technology utilizing horizontal wells. Bioremediation has the potential to enhance the performance of in situ air stripping as well as offering stand-alone remediation of this and other contaminated sites. Horizontal wells could also be used to enhance the recovery of groundwater contaminants for bioreactor conversions from deep or unaccessible areas (e.g., under buildings) and to enhance the distribution of nutrient or microbe additions in an in situ bioremediation.

The basic concepts of this technology are expected to be applicable to other sites having TCE-contaminated soils and water. However, the particular process designs will be site specific. The experience gained at the SRS Integrated Demonstration will provide the basis for designs for other sites. The generic needs for this technology are described in Sections 3.1.3 and 3.1.4 of the USDOE Environmental Restoration and Waste Management Five-Year Plan (1989). Regulatory drivers for this activity are RCRA (40 CFR 264 and 265 Subparts F and G), CERCLA (40 CFR 300 1986 Amendments Section 122) and SDWA (40 CFR 141).

3.3 Alternatives. The principal existing technology for remediation of TCE-contaminated groundwater is pumping followed by air stripping. Unsaturated sediment contamination can only be remediated by vapor extraction. Neither of these are TCE destruction technologies; in both cases the TCE is either discharged to the atmosphere or captured on activated carbon for subsequent disposal. At the SRS no air emission restrictions are presently in force, and air stripping is being used already. However, the lack of emission restrictions is not the usual case, and may well change at the SRS in the very near future.

Preliminary economic evaluations have shown that while air stripping without emissions control is the least costly technique, biodegradation will be very competitive with air stripping with emissions control.

3.4 Benefits. In situ bioremediation technology is based on biological destruction of the contaminants at the site. Therefore, risks normally associated with handling, transporting, and treating or storing contaminated residuals are avoided. In this sense there is a very significant reduction of risk.

Costs for in situ bioremediation of TCE are not known since this is an emerging technology. However, current in situ bioremediation technologies for other organics (such as gasoline) are nearly always less expensive than alternative technologies that provide destruction of the contaminant (and hence permanent remediation). Cost analysis of methanotrophic bioreactors compared with air stripping combined with carbon adsorption of the air stream and direct carbon adsorption from the water have suggested that for several TCE concentrations and flow rates that the methanotrophic system would save 40-60% over conventional technologies. We expect that these observations will also be the case for in situ bioremediation of TCE alone or in combination with bioreactors.

In situ bioremediation coupled with air stripping is expected to lead to a significant reduction in the time required to complete the remediation because bioremediation provides a second simultaneous pathway for removal (destruction) of the TCE. Furthermore, the stimulated indigenous microorganisms will gain access to TCE in the vadose zone and aquifer matrices that may be very difficult to remove by air stripping. Thus a "cleaner" endpoint should be reached in less time.

The enzymes induced in the microbe by the methane, cometabolically oxidize a host of other organic compounds, including toluene, benzene, etc. Since many contaminated sites also contain these or similar compounds, in situ bioremediation and bioreactor systems also address their degradation. Preliminary laboratory studies have demonstrated the proof of this principle (Phelps et al., 1990)

3.5 Acceptability. Bioremediation technologies enjoy relatively high regulatory acceptability in cases where the technology has been demonstrated to be effective. Regulatory agencies are also showing interest in the addition of specialized microbial cultures to the site. California has already granted permits for demonstration projects that inject nutrients and TCE-degrading bacteria into a contaminated aquifer. California, Texas and Michigan have also allowed field project injection of methane and nutrients for in situ bioremediation of TCE contaminated aquifers. Massachusetts and other states have also allowed methane to be injected into aquifers as a tracer for several years. There is a clear precedence for this type of project in the field. This general environment bodes well for approval to use in situ bioremediation at the SRS.

Bioremediation enjoys relatively favorable societal acceptance, in part because it is perceived to be "natural." Essentially ambient process conditions and the lack of unsightly large equipment also contribute to societal acceptability. Use of genetically engineered organisms is not yet socially acceptable. However, such organisms will not be needed at the SRS (although they may offer process advantages at a later date when the acceptability issue has been resolved).

3.6 Site Description. The Savannah River Site is a 300 square mile facility owned by the U.S. Department of Energy and operated under contract DE-AC09-89R180035 by the Westinghouse Savannah River Company. The site is near Aiken, South Carolina (Figure 3.1). The site has been operated as a nuclear production facility for DOE since 1950. The production processes carried out over the past 40 years have generated considerable waste and waste sites. This waste includes radiological, heavy metals, organic solvents, sanitary landfills and other types of mixed wastes. Many contaminated environments at SRS have been identified including both surface water and soils, subsurface sediment and groundwater. Cleanup of these wastes and waste sites has become a top priority for DOE. Due to the large number of waste sites and large volume contaminants at many of these sites a considerable amount of time and money will be required to complete the required cleanup. Thus, another priority stemming from this cleanup program is to develop and demonstrate new and innovative

technologies that may decrease costs, decrease time, decrease environmental impact and/or result in a cleaner end point.

The 300-M Area operations of SRS fabricated fuel and target elements to be irradiated in SRS reactors (Figure 3.1). During these operations the elements are degreased at several stages in the process. These degreasing operation generated large amounts of metal-degreasing solvent wastes. From 1952 to 1982, M Area used and estimated 13 million pound of chlorinated degreasing solvents (Marine and Bledsoe, 1984). Evaporation alone accounted for 50 to 95% while the remainder went to the M Area process sewer system. Marine and Bledsoe (1984) estimate that as much as 2 million pounds may have been released to the sewer that leads to the M Area settling basin, another 1.5 million pounds went directly to the A-14 outfall at Tims Branch. These discharges consisted primarily of trichloroethylene (TCE: 317 thousand lbs), tetrachloroethylene (PCE: 1,800 thousand lbs), and 1,1,1-trichloroethane (TCA: 19 thousand pounds) (Marine and Bledsoe, 1984). From 1952 until 1962 TCE was used, in 1962 the process in one of the facilities was changed and PCE was substituted for TCE, TCE continued to be used until 1971. In 1979 PCE was replace by TCA. By 1976 all discharges from the area were disposed of via direct release into the M Area seepage basin (Figure 3.1). Solvents were detected in the groundwater below M Area Basin in 1981 and visual inspection of the terra cotta pipe of the process sewer line revealed cracks and root penetration, this pipe was relined in 1984. The solvents discharged into the settling basin spread through the vadose zone and entered the groundwater below the basin. The leaking process sewer line used to convey these wastes to the basin also releasing large quantities of the solvents into the surrounding vadose zone sediments. The process sewer line was abandoned and removed in 1986. The seepage basin was contained via a clay cap closure (RCRA) completed in 1991 (DPSPU 84-11-11). Groundwater and sediment contamination in the area is extensive, however, vadose zone (surface to water table) contamination is confined to the linear source associated with the leaking process sewer line. A conventional groundwater extraction and treatment system (air stripper) has been in operation since 1984 and has removed more than 230,000 pounds of solvents from the groundwater. For detailed descriptions of discharges from the area see Marine and Bledsoe (1984), Christensen and Brendell (1982), and Pickett (1985).

The residual solvents in the vadose zone associated with the abandoned process sewer line continue to leach into the groundwater covering more than 1 square mile. Since the plume in this area was linear horizontal wells were selected as the injection and extraction system that would best remediate the site. The horizontal wells were installed in 1989 (Kaback et al., 1989) and the area has been extensively characterized in terms of its hydrology, geology and ecology, for a complete characterization of the site see Eddy et al. (1991). From July 1990 to December 1990 the site was used to demonstrate in situ air stripping via the horizontal wells, for a complete description of the test see Looney et al. (1991).

Figure 3.1. Integrated Demonstration Project Area Location Map.

4.0 Test Plan

4.1 Criteria for Success. There are four primary criteria by which the overall success of this demonstration will be evaluated:

1. <u>Evidence of biological destruction (biodegradation) of TCE from the contaminated soils and water</u>. Since a major advantage of bioremediation is <u>destruction</u>, it is important and significant to demonstrate that biodegradation is occurring. The evidence is expected to come primarily from comparison of the compositions of the off-gases before and after addition of methane to stimulate biodegradation, and from laboratory studies in soil columns using soil cores from the site. In the latter case we expect to show that radiolabeled TCE is degraded under conditions similar to those in the field.

2. <u>Increased reductions of TCE in soil and water samples from the site during periods of biostimulation</u>. The technology is expected to accelerate the removal of TCE over in situ air stripping alone, which is the focus of the first phase of the integrated demonstration.

3. Reduced cost over comparable conventional technologies. Comparison of costs of air stripping currently in use at the site and cost of in situ air stripping from the first demonstration. Costs of both operations and the bioremediation can be compared to rates of removal and/or degradation to arrive at normalized costs for both processes for the same site.

4. Relatively simple and trouble-free operation. These characteristics contribute to favorable economics. A critical assumption for the successful demonstration is that gases can be successfully injected via the lower horizontal well and recovered via the upper well. This ability has been demonstrated in phase 1 of the integrated demonstration project. The wealth of data from phase 1 can be compared and used as a control for the bioremediation project.

The **principal uncertainties** concern the rate of TCE removal/degradation--how long it will take. The permeability of the soil will influence the delivery of nutrients (gases and potentially liquids) to the bacteria. Slow delivery will mean slow bioactivity. Similarly, heterogeneities in the strata may cause some regions to be bypassed; however, if the contaminants infiltrated these zones, then nutrients will too, but it may occur slowly. We do not believe there will be a danger of plugging the soil around the wells by the growth of biomass. This phenomenon has occurred in the past at other bioremediation sites; however, we now know how to avoid this problem by the proper addition of nutrients.

4.2 Pre-Test Characterization and Monitoring. Continuous cores were collected to a depth of approximately 200 feet from one borehole in each of the ten two well MHT clusters (Figure 4.1). Above the water table, samples were collected using a split spoon sampler with a hollow stem auger. Below the water table, a punch core was used in conjunction with mud rotary drilling to collect the core samples. Geophysical logging of the MHT boreholes included natural gamma ray, sp, resistivity (16" and 64"), density, and neutron logs. The MHT and MHV cores were logged in the field: samples were collected at 5 foot intervals and major lithology changes for VOC analysis; and samples for microbiological analysis were collected every 10 feet. The MHT cores were microscopically examined in the SRL core-logging laboratory. Sand (grains 2 mm - 0.0625 mm), gravel (grains > 2 mm), clay**Figure 4.1. Map of Wells and Boreholes at Integrated Demonstration Project.**

(grains < 0.0625 mm), and carbonate percentages were determined, as were the muscovite, lignite, glauconite and sulfide content of the cores. Selected samples were sieved for grain size analysis. The MHT clusters were completed as four inch monitoring wells and consist of a well screened in the water table (designated with the suffix D) and a well screened with five foot screens in the underlying semiconfined aquifer at elevations ranging from 204 to 214 feet (designated with a suffix C). Ideally, the water table wells were to be screened with twenty foot screens with 5 feet of the screen above the water table and 15 feet below the water table. Since the water table zone is approximately 5 to 10 feet thick, the twenty foot screens were installed with more than 5 feet of the screen above the water table to avoid screening into the underlying semiconfined aquifer. Specific well construction details are given in Eddy et al. (1991). Five borings (designated by the prefix MHV) were cored in order to install piezometer clusters in the vadose zone. MHV4 is located west of the injection and extraction wells, MHV1, MHV3 and MHV5 are located between the vapor extraction and injection wells, and MHV2 is located east the injection and extraction wells (Figure 4.1). These borings were drilled with 6-1/4 inch hollow stem auger and sampled with a split spoon sampler to at least 120 feet. Continuous sediment cores were collected and sampled for VOC analysis. Each of the MHV holes was completed as a multiple piezometer cluster. Three piezometer tubes were installed in each hole: each tube was completed with a one inch tee, one inch ball valve, an access port, and a five foot screen. Specific well construction details are given in Eddy et al. (1991). A HydroPunch sampler was used to collect groundwater samples at discrete depths. Samples collected with the HydroPunch are designated with the prefix MHP and were collected adjacent to the well clusters at MHT2, MHT3, MHT4, MHT5, MHT7, MHT8, MHT9, and MHT10. Each sample was analyzed for VOC content and baseline microbial characteristics. All collection methods were designed to minimize microbial contamination of cores from adjacent depths and drilling fluids. Barrels were steam cleaned between collections.

Data reported from this pre-test characterization for the in situ air stripping demonstration provides much of the baseline data for the demonstration site in terms of geology, hydrology and biology (Eddy et al., 1991). Data on the following parameters are included in this report: elevation of geological picks, monitoring well completion details, stratigraphy, geologic cross sections, 3-D mapping, geophysical logs, conceptual description of SRS groundwater system, aquifer characteristics, high and low nutrient heterotrophic plate counts, TCE/PCE concentration, acridine orange direct counts, phospholipid fatty acids, DNA probes, fluorescent antibody probes, plasmid frequency, methanotroph counts and hydropunch samples of water coincident with sediment sampling at the time of boring. Additional analyses not included in this report are currently in progress; sulfate, sulfide, total sulfur, phosphate, total phosphorus, total organic carbon, total Kjeldahl nitrogen, nitrate, nitrite, ammonia, cation exchange, chloride, and iron, for methods see below Section 4.3.4.

The post-test characterization for the in situ air stripping demonstration will also be used to provide the most recent pre-test characterization data for the geology, hydrology and biology data for the in situ bioremediation demonstration. Sampling for the post-test/pre-test characterization began in March 1991 and was completed in June 1991. Eight additional boreholes (MHT-10B, MHT-9B, MHT-11C, MHT-5V, MHT-7T, MHT-3T, MHT-1V, MHT-2T) were drilled and sediment samples taken as described above in this section 4.2. The sample analyses were done as described above. Data analysis for these samples has not yet been completed, but will be added to the characterization data as another report by 1/92. See Eddy et al. (1991) and section 4.3.4 below for all methods and procedures.

Pre-test monitoring from groundwater began 8/15/91. Water samples will be collected using dedicated submersible pumps according to documented SRS well sampling protocols (DPSOP 254). Bulk water parameters including temperature, pH, dissolved oxygen (DO), conductivity, and oxidation-reduction potential (ORP) were measured using a Hydrolab Surveyor model (Hydrolab Inc., Houston, TX). Samples were collected for microbiological studies in a bottle and VOC analysis in a headspace vial. Based upon previous sampling (Looney et al., 1991) the following wells will be sampled every 2 weeks for the duration of the project: MHT-1C, MHT-

2C, MHT-3C, MHT-4C, MHT-5C, MHT-6C, MHT-7C, MHT-8C, MHT-9C, MHT-11C, MHT-9B, MHT-10B. All sampling and analysis are as described below in section 4.3.3.

4.3 Test Description.

4.3.1 Injection Protocol. For site plan layout see Figure 4.2. Extraction of the upper horizontal well (AMH2) in the vadose zone, will begin first at a rate of 240 SCFM for 2 weeks. This initial extraction-only operation should encourage flow of air through the vadose zone. After 2 weeks 100% air will be injected at 200 SCFM (100 psig) for 2 weeks into the lower horizontal well (AMH1) in the saturated zone. This is as per requirements of the SRS-IDP Monitoring Technical Support Group, to establish baseline injection data for in situ flow sensors, etc. After 2 weeks of 100% air, the injection will be changed to 4% methane in air at 100 psig injected at the rate of 200 SCFM. Initially 4% will be used to provide a greatest quantity of methane possible and a margin of safety of 20% with the lower explosive limit of 5%. Should lower injection or extraction rates be required for any reason, both will be adjusted so that the extraction rate is no more than 20% higher than the injection rate. This strategy is used to insure that we are preventing plume spreading from injection strategy but minimizing in situ air stripping. The rates and pressures were determined from the previous in situ air injection test with the same wells (Looney et al., 1991). The in situ air stripping test demonstrated that injection rates of 170 SCFM stimulated bacteria density increases in the groundwater, the lower rate of 65 SCFM had no effect and 270 SCFM stimulated bacteria only marginally in some parts of the formation over the medium rate (Looney et al., 1991). The gaseous residence times varyed from several hours to several weeks. Given the long residence times and apparently tortuous paths that the air can follow in this subsurface site the Air/Methane mixture will be injected continuously at concentrations of 4% of the air flow. Thus methane will not reach explosive concentrations, and aerobic conditions will be maintained within the subsurface sediments. After three months of operation, if biodegradation rates are significantly increased and methane does not appear to be limiting then a pulsing regime may be initiated. Operation protocol will be reviewed every 3 months by the SRS-IDP Bioremediation Technical Support Group to determine if any changes are necessary to better accomplish the demonstration objectives and criteria for success. Pulsing of the methane flow could have two advantageous effects; an increase in degradative efficiency by decreasing competition for TCE and methane at enzyme sites and then provision of methane for growth and cellular maintenance; and eliminating the constant availability of methane near the injection well to expand the breadth of the biomass enriched zone.

Another possibility is if monitoring of inorganic compounds during the initial operating campaign suggests that one or more of these compounds is limiting microbial degradation of TCE. Then the later part of the operating campaign may also employ supplementation of limiting nutrients, eg. nitrogen. **Figure 4.2. SRS Integrated Demonstration Project Site Plan.**

Ammonia could be added as a source of nitrogen. Delivery of ammonia will be at low levels (less than 0.1%) and it will be pulsed counter to methane so that zones around the injection well will not see an abundance of nitrogen and energy source simultaneously, thereby increasing the zone of influence of the biomass stimulation. Another advantage to ammonia additions could be the alkaline buffering capacity. Waters in the vicinity are slightly acidic pH 5-6.5, so the addition of ammonia will assist in maintaining a pH more suitable to methanotrophic bioremediation (pH = 5-7.5). However process control experiments are currently underway to determine if nitrous oxide may have more advantages over ammonia.

Other parameters which can be tested in the gaseous substrate injection test include changes in influent flow and pressure, alteration in extraction vacuum, and enrichment with propane. In previous studies TCE degraders from nearby subsurface sediments are greatly stimulated in microcosm studies after the addition of propane. Propane supplements at the level of 5-30% of the methane additions (>2% total flow) may be tested based upon results of laboratory process control experiments (Section 4.3.6). Input from the results of these Process Control Studies will be critical in deciding what alterations will be made over the course of test.

The equipment, maintenance and operations of the compressor, vacuum blower, methane blending systems, and offgas treatment system will be handled by a subcontractor as specified in the Scope of Work, Appendix C.

4.3.2 Tracer Studies. Every month 3 standard cylinders of helium (250 cu ft) will be added to the injected air over a 24 hour period using a regulator and flow meter. All of the identifiable potential exit points for gas to leave the system will be sampled. The procedure for these studies is as described by Looney et al. (1991). Helium analysis methods are described below in section 4.3.5. By comparing the times and rates of recovery of helium from the extraction well (AMH2) and monitoring wells, changes in geohydrological structure that could effect flow rate can be detected and/or confirmed. *SRL and subcontractor.*

4.3.3 Groundwater Monitoring. Water samples will be collected every two weeks from MHT-1C, MHT-2C, MHT-3C, MHT-4C, MHT-5C, MHT-6C, MHT-7C, MHT-8C, MHT-9C, MHT-11C, MHT-9B, MHT-10B using dedicated submersible pumps according to documented SRS well sampling protocols (DPSOP 254). Bulk water parameters including temperature, pH, dissolved oxygen (DO), conductivity, and oxidation-reduction potential (ORP) were measured using a Hydrolab Surveyor model (Hydrolab Inc., Houston, TX). Samples were collected for microbiological studies in 4 sterile 250 ml capped, disposable Erlenmeyer flasks (Corning Inc.) and VOC analysis in a headspace vial (Hewlett-Packard Inc.). The 2 of the flask for microbiological analysis were fixed in the field with 1 ml of formalin (37% vol/vol), these samples were to be used for direct counts. The remaining two flasks are to be used for viable counts. PLFA, etc. analysis. Water is filtered in the field with charged microporous cartridges (Virosorb, Cuno Inc.). These filters are transported in sterile bags for extraction of nucleic acids in the laboratory (Hazen et al., 1990). All samples shipped off site for analysis will have a chain of custody. *SRL*

4.3.3.1 Analysis of VOC. TCE, PCE and all of the potential daughter products (c-DCE, t-DCE, VC, CO_2 and CH_4) will be measured. VOC analyses will be performed on a Hewlett-Packard 5890 Gas Chromatograph with an electron capture detector, an HP 19395A Headspace Sampler, an HP 3392A Networking Integrator, computer controlled data control and acquisition via Chemstation software, and a 60 m x 0.75 mm ID Supelco VOCOL wide bore capillary column coated with a 1.5 mm film. The instrument is calibrated using samples spiked with standard solution. Within the headspace sampler, the teflon-lined vials are punctured, and the gases are released into the gas chromatograph. The gases

are analyzed in the gas chromatograph, and the analysis is printed out (EPA Method 524.2). *SRL*

4.3.3.2 Acridine Orange Direct Counts (AODC). AODC will provide a direct estimate of the total number of bacteria in the environment, regardless of ability to grow on any media that might be used. Samples fixed with formalin in the field are concentrated by continuous centrifugation (Serval, E. I. duPont de Nemours Company) at 6000 RPM from a initial volume of 500 ml to 10 ml final volume. Ten microliters of supernatant is spotted onto each well of a toxoplasmosis microscope slide (Celline Inc.), stained 2 min with 0.01% acridine orange (Difco, Detroit, MI), then rinsed with distilled water. The number of cells stained with acridine orange are counted by epifluorescent microscopy (Hazen et al., 1991; Sinclair and Ghiorse, 1989). *SRL*

4.3.3.3 Aerobic Heterotrophic Plate Count. This method will provide an estimate of the total number of viable aerobic and facultatively anaerobic bacteria in the groundwater. Low and high nutrient concentrations of a medium will be used to suggest differences in bacteria adapted to oligotrophic and eutrophic conditions. Unfixed samples of 1, 10 and 100 ml are filtered through 0.45 µm pore size, 47 mm diameter membrane filters (HAWG, Millipore Co., Bedford, MA). Media 1% and full strength formulation of peptone trypticase yeast extract (PTYG) with 0.1% cycloheximide to inhibit fungal growth will be used (Balkwill, 1989). Plates are incubated at room temperature (25°C) for at least two weeks prior to counting. Bacterial colonies are counted with the aid of low power magnification. *SRL*

4.3.3.4 Methane Enrichment Plate Count. This method will provide an estimate of the total number of viable aerobic and facultatively anaerobic bacteria capable of living in an enriched methane groundwater. Differences between low nutrient heterotrophic plate counts and methane enriched heterotrophic plate counts will indicate the proportion of methanotrophs in the total heterotrophic community. Successful bioremediation of TCE/PCE can also be in terms of increased microbial activity, increased biomass; particularly biomass which contains TCE degrading machinery, increased biomass capable of consuming methane as evidence of stimulation by treatments. Unfixed samples of 1, 10 and 100 ml are filtered through 0.45 µm pore size, 47 mm diameter membrane filters (HAWG, Millipore Co., Bedford, MA). Media 1% formulation of peptone trypticase yeast extract (PTYG) are used (Balkwill, 1989). Plates are incubated at room temperature (25°C) in a 4% methane/air atmosphere in Gas-Paks (BBL, Baltimore, MD) for at least two weeks prior to counting. Bacterial colonies are counted with the aid of low power magnification. *SRL, ORNL, UT*

4.3.3.5 Anaerobic Plate Count. This method will provide an estimate of the total number of anaerobic viable bacteria in the groundwater. Anaerobic reductive dechlorination of PCE will be essential for its destruction. Unfixed samples of 1, 10, and 100 ml are filtered through 0.45 µm pore size, 47 mm diameter membrane filters (HAWG, Millipore Co., Bedford, MA). Media ,1% formulation of peptone trypticase yeast extract (PTYG) are used (Balkwill, 1989). Plates are incubated at room temperature (25°C) in a 5% CO_2 atmosphere in a Gas-Pak (BBL, Baltimore, MD) for at least two weeks prior to counting. Bacterial colonies are counted with the aid of low power magnification. *SRL, ORNL, UT*

4.3.3.6 Community Diversity/Functionality. Changes in in relative community structure may be important in determining: 1. the overall stability of the biological community, 2. the potential for producing unwanted effects, and 3. the relative changes in the functional capability of the community related to nutrient input and contaminant degradation. Community diversity will be determined via colony morphology and biochemical/physiological

characterization. Every bacterial colony type is noted, counted, and cataloged for calculation of diversity indices (Shannon) and measurement of structural diversity. Representatives of these isolates are grown in pure culture and frozen for future biochemical studies and measurement of functional diversity. Biochemical/physiological traits will be catalogued by inoculating pure cultures of bacteria into a 96 well microtiter screening plate (MT and GN type Biolog Inc.) Similarity and cluster analysis will be used to compare groups of random isolates overtime, by wells. *SRL, USC*

4.3.3.7 Fluorescent Antibody Direct Counts. Since nitrogen is believed to be limiting these probes will direct estimates to suggest if certain types of nitrogen transformers are changing. It has been found that these bacteria are critical to activity in the soil (Dommergues et al., 1978). It will also provide direct measurements of a TCE degrader isolated from the site. A reference pathogen will also be analyzed (Fliermans et al., 1979). Samples are prepared as for AODC described above in section 4.3.3.2. Samples fixed on slides are stained by incubation with fluorescien isothiocyanate labeled antibodies (specific for a particular bacteria, eg. TCE-degrading bacteria isolated from M area sediment) for 1 hour and then excess stain was washed away with buffer. The stained slides are then examined with a fluorescent microscope and the number of yellow/green fluorescing cells enumerated as with AODC. Fluorescent antibodies for several nitrogen transforming organisms are also being tested: Nitrosomonas eurpoea, Nitrobacter agilis and winogradsky combined, Ferrobacillus ferrooxidans, Nitrosolobus sp (AV), Azotobacter chroococum, and Beijerinckia japonicum; a pathogen.Legionella pneumophila Sero 1; a SRL-TCE degrader, and a methanotroph. All antibodies were prepared and supplied by E. L. Schmidt, University of Minnesota. For details on preparation of antibodies and staining technique see Fliermans et al. (1974) and Bohlool and Schmidt (1980). *SRL*

4.3.3.8 Signature Biomarkers: Phospholipid Fatty Acid Analysis and Other Physiological Analyses. Culturing techniques are severely limited in determining the overall community structure, microbial biomass and nutritional status, since these techniques rely upon a general media an incubation conditions that are totally unlike anything that microbial community may have been exposed to before. Signature biomarker compounds over come many of these limitations by allowing direct determination of sub-femtomolar quantities of compound used by microbes for energy storage, metabolic intermediaries and enzymes (White et al., 1990). One such group of compounds is the PLFA. Ester-linked phospholipid fatty acids (PLFA) are extracted from filtered samples via inverse serial extraction, fractionated and methylated by microtechnique. Identifications are made by comparison of retention times to standards after extracting specific ions from a total ion chromatogram obtained with electron impact GC/MS. These techniques minimized the input of contaminants while maximizing sample input.

Additional techniques could be used to nutritional status and metabolic activity. Water samples, 10 ml will be incubated with ^{14}C-acetate or ^{14}C-thymidine for 24 hours at in situ temperatures. The samples will then be fixed with chloroform-methanol and filtered through 0.2 μm pore size filters. The acetate incubated samples will be extracted with chloroform-methanol, dried, resuspended in 2.0 ml chloroform and aliquots counted by liquid scintillation counting to determine the amount of radioactivity incorporated into microbial lipids. Thymidine incubated samples will be filtered and fixed by freezing at -20°C. The frozen sample is thawed lysed with sodium hydroxide containing 1% sodium dodecyl sulfate, 10 mM thymidine and 1% humic acid. After heating at 110°C for 4 h, the supernatants are collected an dialyzed. The radioactivity incorporated into DNA is determined using liquid scintillation counting. See Phelps et al. (1989, 1991) for details. *UT*

4.3.3.9 Nucleic Acid Analysis. Recent techniques for probing environmental samples with nucleic acid probes have allowed for the first time truly synecological studies (Hazen and Jiménez, 1989). The section of genomic structure that codes for enzymes involved in biodegradation, regardless of species can finally be assayed. These probes allow a nearly direct estimate of the functional capability of the environment being tested. Direct extraction of the DNA from filtered water allows direct determination of the presence and amount of certain conserved nucleic acid sequences that code for the enzymes involved in contaminate degradation. These probes should allow direct assessment of the amount of methanotrophs and other groups of organisms capable of degrading TCE/PCE and/or providing essential conditions, eg. nitrogen, pH for optimal in situ bioremediation. By filtering large quantities of DNA (Section 4.3.3) a number of probes can be tested simultaneously.

Total DNA will be extracted from filters by placing the sample into a solution of 2.5% Sodium Dodecyl Sulfate (SDS) in (0.1 M) sodium phosphate buffer, pH 8.0 for 1 hour to lyse the cells. After a 1 hour incubation at 70°C proteins and cells debris are separated from the DNA by the addition of 0.5 volume of sodium acetate or ammonium acetate. The sample was incubated then for 30 minutes at -20°C. After incubation the mixture was centrifuged at 12,000 x g for 15 minutes. The supernatants are pooled and transferred to another container and 2 volumes of 95% ethanol are added, then DNA was precipitated overnight at room temperature. Samples are centrifuged at 12,000 x g for 30 minutes to recover the DNA. Buoyant density centrifugation in Cesium Chloride-Ethidium Bromide Gradients was performed as described elsewhere (Maniatis et al., 1987). DNA was extracted and purified from the gradients as described by Maniatis et al. (1987). Concentration of DNA and purity was determined by absorbance at 260 nm and 280 nm. If the ratio of 260/280 was lower than 1.8 the solution was purified by a cesium chloride-ethidium bromide gradient. DNA concentration per gram sediment was then calculated from the initial dry weight used. Slot blots are used to further purify genomic fragments. The resultant purified DNA is then hybridized under stringent conditions with specific DNA probes. For DNA probes with known primers, polymerase chain reaction (PCR) will be used to amplify samples with low concentrations. Total DNA is also being subjected to thermal melting point determinations via a melting point spectrophotometer and subsequent calculation of mol% G+C for diversity estimates. RNA will be extracted in a similar fashion (Sayler et al., 1989). *ORNL, UT*

The following probes have been chosen as being important and readily available:

1. A TCE-degrading type I methanotroph (68-1) probe. The probe is DNA fragment that encodes a putative gamma subunit of methane monooxygenase and 16S rRNA. *ORNL, UT, UM*
2. A type II B gene methanotroph 16S rRNA probe. *UM*
3. A potentially TCE-degrading Tod(C_2C_1BA) toluene dioxygenase complex, Pseudomonas putida F1. *ORNL, UT*
4. A potentially TCE-degrading nahA Naphthalene dioxygenase Pseudomonas putida NAH7. *ORNL, UT*
5. A potentially TCE-degrading TOL upper pathway xylene oxidase, Pseudomonas putida mt2, pWWO. *ORNL, UT*
6. 16S rRNA sequences from SRS subsurface bacteria. *UI*
7. Acetogen specific DNA and RNA probes from Clostridium thermoaceticum. *USCC*
8. A nitrogen fixing, aromatic degrading nifH fragment from Klebsiella pneumoniae. *USCC*
9. A aromatic degrading catechol dioxygenase fragment from Rhizobium leguminosarum. *USCC*

10. A potentially TCE-degrading TOL plasmid probes. *USCA*
11. A TCE-degrading probe from an SRS bacteria that has a toluene dioxygenase. *PNL*
12. A TCE-degrading probe (oxidative and reductive) from an SRS bacteria cytochrome P45cam. *PNL*
13. A TCE-degrading probe from a broad specificity oxygenase xylMA involved in xylene/toluene degradation. *PNL*

4.3.3.10 TCE/PCE Mineralization Analysis. The greatest measure of success would be demonstration of ^{14}C-TCE disappearance in microcosms within hours of collection of water samples and continuing for days, as compared to controls. Second best measure of success would be substantial loss of TCE in enrichments as compared to controls and background samples. ^{14}C-labelled TCE and PCE is injected into sealed tubes with 10 ml of groundwater sample and incubated at in situ temperature for 48 h. The nonradioactive and radioactive carbon dioxide concentration in the sample is determined with gas chromatography-gas proportional counting as described by Phelps et al. (1989). *SRL, ORNL, UT*

4.3.3.11 Toxicity Analysis. Disappearance of target compounds, eg. TCE/PCE, may not have to correlate with decrease in health hazards associated with the treated material (Mueller et al., 1991). In situ bioremediation involves manipulation of an extremely complex milieu. Various biodegradation products and substances transformed by the changes caused in the physical/chemical environment of the contaminated soil and groundwater could go undetected by the standard analytical procedures employed. In order to monitor changes in the health hazard of the groundwater a microbial bioassay will be employed. Water samples will be inoculated into a Microtox model 500 toxicity autoanalyzer (Microbics Carp., Carlsbad, CA). This assay evaluated the toxicity by measuring the change in light level of viable luminescent bacteria upon their exposure to test substrates. *SRL, ORNL, UT*

4.3.3.12 Physical/Chemical Analysis. The physical and chemical nature of the environment is critical to understanding biological phenomena, eg. degradation rates. In addition, some of these parameters have implication on nutrient requirements (P, N, S, Fe), effects that the biomass may be having on the environments, eg. pH, conductivity, TOC. These measurements could be critical to a thorough understanding of the in situ bioremediation process and the potential for controlling degradation rates, destruction efficiency and adverse phenomena. All methods will be EPA approved and/or in Standard Methods (APHA, 1989). Temperature, dissolved oxygen, pH, conductivity, oxidation-reduction potential, and salinity will be determined at the well head by specific probes on a Hydrolab Surveyor II equipped with a data logger and flow through cell (Hydrolab Inc., Austin, TX). The pH and dissolved oxygen probe are calibrated daily, and the remaining probes calibrated monthly. The remainder of the assays will be performed by a subcontractor with EPA approved methods in an EPA certified laboratory. Iron will be determined by inductively coupled plasma-atomic emission spectroscopy with pre acid digestion (EPA SW-846). Total Organic Carbon (TOC) will be determined by the ultraviolet oxidation method (EPA 415.1). Samples will acidified and stored at 4°C prior to analysis. Ortho Phosphate concentrations will be measured by the ascorbic acid colorimetric determination method (EPA 365.2). Total Phosphorus will be determined by the persulfate digestion and ascorbic acid colorimetric determination (EPA 365.2). Total Kjeldahl Nitrogen (TKN), which includes free-ammonia plus organic nitrogen will be determined by the colorimetric, following digestion, distillation and Nesslerization method (EPA 351.3). Ammonia as distilled ammonia nitrogen will be determined by the colorimetric, following

distillation and Nesslerization method (EPA 350.2). Chloride, Nitrate, Nitrite, and Sulfate will be determined by the ion chromatography method (EPA 300.0). *SRL and subcontractor*

4.3.3.13 Fungal Analysis (Optional). Large increases in biomass during remediation projects may also cause increases in fungal biomass. Some yeast have been implicated in TCE degradation (Wackett et al. 1989). The importance of fungi in contaminated environments has largely gone unstudied. Water samples will be enumerated with acidified mycological agar and mycological agar with chloramphenicol. Enrichment cultures would also be done by supplementation with methane and TCE/PCE. Isolates will then be identified and further characterized in terms of ability to degrade TCE/PCE. This project is in the proposal stage and needs further consideration. *GSU*

4.3.4 Sediment Monitoring. Every three months two bore holes will be drilled and sampled from the surface to 200 ft. in the area of expected remediation influence (see DSOP 254 and Eddy et al., 1991 for methods and procedures). An additional two bore holes will be drilled in adjacent areas not expected to be influenced as a control. Sediment samples will be collected at ten foot intervals and, in addition, at all significant lithologic changes in the core. All collection methods are designed to minimize microbial contamination of cores from adjacent depths and drilling fluids. Barrels were steam cleaned between collections. Samples will be collected using a modified syringe tube and plunger. This technique results in the collection of a consistent volume of sediment. Immediately after collection, the sediment sample was placed in a headspace vial. Five milliliters of solution, comprised of 10 grams of sodium sulfate and 0.3 milliliters of phosphoric acid (0.15%) added to 200 milliliters of distilled water, is added to the vial. The vials are sealed with crimped aluminum rings over teflon-lined septa. Samples are placed in a cooler on ice. The samples are collected daily and refrigerated in the lab. Prior to sample analysis, the samples are weighed in order to determine the mass of the sample. Samples are then placed in the sonic dismembrator for fifteen minutes in order to disaggregate the sediment.

Core specimens for microbial analysis are obtained directly from the split spoon or barrel. Cores were sectioned into 3 inch lengths with sterile spatulas and the outermost layer (about 1/4 the diameter of the core) was scraped off using a sterile scoopula. The sample is then placed in a sterile Whirl-Pak bag (Ft. Wilkinson, WI) for immediate transport to the laboratory for analysis. All samples shipped off site for analysis will have a chain of custody. *SRL*

4.3.4.1 Analysis of VOC. TCE, PCE and all of the potential daughter products (c-DCE, t-DCE, VC, CO2 and CH4) will be measured. VOC analyses will be performed on a Hewlett-Packard 5890 Gas Chromatograph with an electron capture detector, an HP 19395A Headspace Sampler, an HP 3392A Networking Integrator, Computer controlled data control and acquisition via Chemstation software, and a 60 m x 0.75 mm ID Supelco VOCOL wide bore capillary column coated with a 1.5 mm film. The instrument is calibrated using samples spiked with standard solution. Within the headspace sampler, the teflon-lined vials are punctured, and the gases are released into the gas chromatograph. The gases are analyzed in the gas chromatograph, and the analysis is printed out (EPA Method 524.2; Sims et al., 1991). *SRL*

4.3.4.2 Acridine Orange Direct Counts (AODC). AODC will provide a direct estimate of the total number of bacteria in the environment, regardless of ability to grow on any media that might be used. Samples are preserved in phosphate buffered formalin. Samples (1 to 3 grams) are extracted three times with a non-ionic homogenizing detergent to remove bacteria from the sediment particles. Homogenates are cleared by low speed centrifugation and the supernatants were pooled. Ten microliters of supernatant is spotted onto each well of a

toxoplasmosis microscope slide, stained with 0.01% acridine orange, then rinsed with distilled water. The number of cells stained with acridine orange are counted by epifluorescence microscopy. The number of cells per sample is normalized by dividing by the dry weight of the sediment. Counts are reported as cells per gram (Sinclair and Ghiorse, 1989). *SRL*

4.3.4.3 Aerobic Heterotrophic Plate Count. This method will provide an estimate of the total number of viable aerobic and facultatively anaerobic bacteria in the groundwater. Low and high nutrient concentrations of a medium will be used to suggest differences in bacteria adapted to oligotrophic and eutrophic conditions. Samples (1 to 3 grams) are weighed directly into 15 ml conical centrifuge tubes containing 9 ml of pyrophosphate buffer. Subsequent serial dilutions are made in phosphate buffered saline. 0.1 ml of each appropriate dilution was inoculated onto a corresponding plate of appropriate medium. For this study, 1% and full strength formulation of peptone trypticase yeast extract (PTYG) are used (Balkwill, 1989). A glass rake and turntable are used to spread the inoculum evenly over the entire surface of the agar. Plates are incubated at room temperature for at least two weeks prior to counting. Bacterial colonies are counted with the aid of low power magnification. Counts are normalized to sediment dry weights and reported as colony forming units (CFU) per gram. *SRL*

4.3.4.4 Methane Enrichment Plate Count. This method will provide an estimate of the total number of viable aerobic and facultatively anaerobic bacteria capable of living in an enriched methane sediment. Differences between low nutrient heterotrophic plate counts and methane enriched heterotrophic plate counts will indicate the proportion of methanotrophs in the total heterotrophic community. Successful bioremediation of TCE/PCE can also be in terms of increased microbial activity, increased biomass; particularly biomass which contains TCE degrading machinery, increased biomass capable of consuming methane as evidence of stimulation by treatments. Samples (1 to 3 grams) are weighed directly into 15 ml conical centrifuge tubes containing 9 ml of pyrophosphate buffer. Subsequent serial dilutions are made in phosphate buffered saline. 0.1 ml of each appropriate dilution was inoculated onto a corresponding plate of appropriate medium. Media 1% and full strength formulation of peptone trypticase yeast extract (PTYG) are used (Balkwill, 1989). Plates are incubated at room temperature (25°C) in a 4% methane/air atmosphere for at least two weeks prior to counting. Bacterial colonies are counted with the aid of low power magnification. *SRL, ORNL, UT*

4.3.4.5 Anaerobic Plate Count. This method will provide an estimate of the total number of anaerobic viable bacteria in the sediment. Anaerobic reductive dechlorination of PCE will be essential for its destruction. Samples (1 to 3 grams) are weighed directly into 15 ml conical centrifuge tubes containing 9 ml of pyrophosphate buffer. Subsequent serial dilutions are made in phosphate buffered saline. 0.1 ml of each appropriate dilution was inoculated onto a corresponding plate of appropriate medium. Medium, 1% formulation of peptone trypticase yeast extract (PTYG) are used (Balkwill, 1989). Plates are incubated at room temperature (25°C) in a 5% CO_2 atmosphere in a Gas-Pak (BBL, Baltimore, MD) for at least two weeks prior to counting. Bacterial colonies are counted with the aid of low power magnification. *SRL, ORNL, UT*

4.3.4.6 Community Diversity/Functionality. Changes in in relative community structure may be important in determining: 1. the overall stability of the biological community, 2. the potential for producing unwanted effects, and 3. the relative changes in the functional capability of the community related to nutrient input and contaminant degradation. Community diversity will be determined via colony morphology and biochemical/physiological

characterization. Every bacterial colony type is noted, counted, and cataloged for calculation of diversity indices (Shannon) and measurement of structural diversity. Representatives of these isolates are grown in pure culture and frozen for future biochemical studies and measurement of functional diversity. Biochemical/physiological traits will be catalogued by inoculating pure cultures of bacteria into a 96 well microtiter screening plate (MT and GN type Biolog Inc.) Similarity and cluster analysis will be used to compare groups of random isolates overtime by wells. *SRL, USC*

4.3.4.7 Fluorescent Antibody Direct Counts. Since nitrogen is believed to be limiting these probes will direct estimates to suggest if certain types of nitrogen transformers are changing. It has been found that these bacteria are critical to activity in the soil (Dommergues et al., 1978). It will also provide direct measurements of a TCE degrader isolated from the site. A reference pathogen will also be analyzed (Fliermans et al., 1979). Samples are prepared as for AODC described above in Section 4.3.4.2. Samples fixed on slides are stained by incubation with fluorescien isothiocyanate labeled antibodies (specific for a particular bacteria, eg. TCE-degrading bacteria isolated from M area sediment) for 1 hour and then excess stain was washed away with buffer. The stained slides are then examined with a fluorescent microscope and the number of yellow/green fluorescing cells enumerated as with AODC. Fluorescent antibodies for several nitrogen transforming organisms are also being tested: Nitrosomonas eurpoea, Nitrobacter agilis and winogradsky combined, Ferrobacillus ferrooxidans, Nitrosolobus sp (AV), Azotobacter chroococum, and Beijerinckia japonicum; a pathogen.Legionella pneumophila Sero 1; a SRL-TCE degrader, and a methanotroph. All antibodies were prepared and supplied by E. L. Schmidt, University of Minnesota. For details on preparation of antibodies and staining technique see Fliermans et al. (1974) and Bohlool and Schmidt (1980). *SRL*

4.3.4.8 Phospholipid Fatty Acid Analysis and Other Physiological Measurements. Culturing techniques are severely limited in determining the overall community structure, microbial biomass and nutritional status, since these techniques rely upon a general media an incubation conditions that are totally unlike anything that microbial community may have been exposed to before. Signature biomarker compounds over come many of these limitations by allowing direct determination of sub-femtomolar quantities of compound used by microbes for energy storage, metabolic intermediaries and enzymes (White et al., 1990). One such group of compounds is the PLFA. Ester-linked phospholipid fatty acids (PLFA) are extracted from filtered samples via inverse serial extraction, fractionated and methylated by microtechnique. Identifications are made by comparison of retention times to standards after extracting specific ions from a total ion chromatogram obtained with electron impact GC/MS. These techniques minimized the input of contaminants while maximizing sample input.

Additional techniques could be used to nutritional status and metabolic activity. Sediment samples, 10 ml will be incubated with ^{14}C-acetate or ^{14}C-thymidine for 24 hours at in situ temperatures. The samples would then be fixed with chloroform-methanol and filtered through 0.2 µm pore size filters. The acetate incubated samples will be extracted with chloroform-methanol, dried, resuspended in 2.0 ml chloroform and aliquots counted by liquid scintillation counting to determine the amount of radioactivity incorporated into microbial lipids. Thymidine incubated samples will be filtered and fixed by freezing at -20°C. The frozen sample is thawed lysed with sodium hydroxide containing 1% sodium dodecyl sulfate, 10 mM thymidine and 1% humic acid. After heating at 110°C for 4 h, the supernatants are collected an dialyzed. The radioactivity incorporated into DNA is determined using liquid scintillation counting. See Phelps et al. (1989, 1991) for details. *UT*

4.3.4.9 Nucleic Acid Analysis. Recent techniques for probing environmental samples with nucleic acid probes have allowed for the first time truly synecological studies(Hazen and Jiménez, 1989). The section of genomic structure that codes for enzymes involved in biodegradation, regardless of species can finally be assayed. These probes allow a nearly direct estimate of the functional capability of the environment being tested. Direct extraction of the DNA from filtered water allows direct determination of the presence and amount of certain conserved nucleic acid sequences that code for the enzymes involved in contaminate degradation. These probes should allow direct assessment of the amount of methanotrophs and other groups of organisms capable of degrading TCE/PCE and/or providing essential conditions, eg. nitrogen, pH for optimal in situ bioremediation.

Total DNA will be extracted from sediment samples by placing the sample into a solution of 2.5% Sodium Dodecyl Sulfate (SDS) in (0.1 M) sodium phosphate buffer, pH 8.0 for 1 hour to lyse the cells. After a 1 hour incubation at 70°C proteins and cells debris are separated from the DNA by the addition of 0.5 volume of sodium acetate or ammonium acetate. The sample was incubated then for 30 minutes at -20°C. After incubation the mixture was centrifuged at 12,000 x g for 15 minutes. The supernatants are pooled and transferred to another container and 2 volumes of 95% ethanol are added, then DNA was precipitated overnight at room temperature. Samples are centrifuged at 12,000 x g for 30 minutes to recover the DNA. Buoyant density centrifugation in Cesium Chloride-Ethidium Bromide Gradients was performed as described elsewhere (Maniatis et al., 1987). DNA was extracted and purified from the gradients as described by Maniatis et al. (1987). Concentration of DNA and purity was determined by absorbance at 260 nm and 280 nm. If the ratio of 260/280 was lower than 1.8 the solution was purified by a cesium chloride-ethidium bromide gradient. DNA concentration per gram sediment was then calculated from the initial dry weight used. Slot blots are used to further purify genomic fragments. The resultant purified DNA is then hybridized under stringent conditions with specific DNA probes. For DNA probes with known primers, polymerase chain reaction (PCR) will be used to amplify samples with low concentrations. Total DNA is also being subjected to thermal melting point determinations via a melting point spectrophotometer and subsequent calculation of mol% G+C for diversity estimates. RNA will be extracted in a similar fashion (Sayler et al., 1989). *ORNL, UT*

The following probes have been chosen as being important and readily available:

1. A TCE-degrading type I methanotroph (68-1) probe. The probe is DNA fragment that encodes a putative gamma subunit of methane monooxygenase and 16S rRNA. *ORNL, UT, UM*
2. A type II B gene methanotroph 16S rRNA probe. *UM*
3. A potentially TCE-degrading Tod(C_2C_1BA) toluene dioxygenase complex, Pseudomonas putida F1. *ORNL, UT*
4. A potentially TCE-degrading nahA Naphthalene dioxygenase Pseudomonas putida NAH7. *ORNL, UT*
5. A potentially TCE-degrading TOL upper pathway xylene oxidase, Pseudomonas putida mt2, pWWO. *ORNL, UT*
6. 16S rRNA sequences from SRS subsurface bacteria. *UI*
7. Acetogen specific DNA and RNA probes from Clostridium thermoaceticum. *USCC*
8. A nitrogen fixing, aromatic degrading nifH fragment from Klebsiella pneumoniae. *USCC*
9. A aromatic degrading catechol dioxygenase fragment from Rhizobium leguminosarum. *USCC*
10. A potentially TCE-degrading TOL plasmid probes. *USCA*

11. A TCE-degrading probe from an SRS bacteria that has a toluene dioxygenase. *PNL*
12. A TCE-degrading probe (oxidative and reductive) from an SRS bacteria cytochrome P45cam. *PNL*
13. A TCE-degrading probe from a broad specificity oxygenase xylMA involved in xylene/toluene degradation. *PNL*

4.3.4.10 TCE/PCE Mineralization Analysis. The greatest measure of success would be demonstration of ^{14}C–TCE disappearance in microcosms within hours of collection of sediment samples and continuing for days, as compared to controls. Second best measure of success would be substantial loss of TCE in enrichments as compared to controls and background samples. ^{14}C–labelled TCE and PCE is injected into sealed tubes with 1-3 g of sediment sample in 10 ml distilled water and incubated at in situ temperature for 48 h. The nonradioactive and radioactive carbon dioxide concentration in the sample is determined with gas chromatography-gas proportional counting as described by Phelps et al. (1989). *SRL, ORNL, UT*

4.3.4.11 Toxicity Analysis. Disappearance of target compounds, eg. TCE/PCE, may not have to correlate with decrease in health hazards associated with the treated material (Mueller et al., 1991). In situ bioremediation involves manipulation of an extremely complex milieu. Various biodegradation products and substances transformed by the changes caused in the physical/chemical environment of the contaminated soil and groundwater could go undetected by the standard analytical procedures employed. In order to monitor changes in the health hazard of the sediment a microbial bioassay will be employed. Sediment samples will be inoculated into a Microtox model 500 toxicity autoanalyzer (Microbics Carp., Carlsbad, CA). This assay evaluates the toxicity of a sample by measuring the change in light level of viable luminescent bacteria upon their exposure to test substrates. *SRL, ORNL, UT*

4.3.4.12 Physical/Chemical Analysis. The physical and chemical nature of the environment is critical to understanding biological phenomena, eg. degradation rates. In addition, some of these parameters have implication on nutrient requirements (P, N, S, Fe), effects that the biomass may be having on the environments, eg. pH, conductivity, TOC. These measurements could be critical to a thorough understanding of in situ bioremediation process and the potential for controlling degradation rates, destruction efficiency and adverse phenomena. All methods will be EPA approved and/or in Standard Methods (APHA, 1989). The following assays will be performed by a subcontractor with EPA approved methods in an EPA certified laboratory. Iron will be determined by inductively coupled plasma-atomic emission spectroscopy with pre acid digestion (EPA SW-846). Total Organic Carbon (TOC) will be determined by the ultraviolet oxidation method (EPA 415.1). Samples will acidified and stored at 4°C prior to analysis. Ortho Phosphate concentrations will be measured by the ascorbic acid colorimetric determination method (EPA 365.2). Total Phosphorus will be determined by the persulfate digestion and ascorbic acid colorimetric determination (EPA 365.2). Total Kjeldahl Nitrogen (TKN), which includes free-ammonia plus organic nitrogen will be determined by the colorimetric, following digestion, distillation and Nesslerization method (EPA 351.3). Ammonia as distilled ammonia nitrogen will be determined by the colorimetric, following distillation and Nesslerization method (EPA 350.2). Chloride, Nitrate, Nitrite, and Sulfate will be determined by the ion chromatography method (EPA 300.0). *SRL and subcontractor*

4.3.4.13 Protozoan Analysis. Recent work has that small number so protozoa commonly inhabit subsurface soils at pristine sites at various

geographical locations. The ubiquitous distribution of protozoa in the subsurface have important implications in bioremediaton operations. When nutrients are added to increase bacteria biomass concomitant increases in protozoan populations occur. These protozoa could be important in removing bacterial biomass and cycling of contaminants that were only adsorbed and not degraded, they also could be important in maintaining hydraulic conductivity and insuring proper flow of nutrients into contaminated zones. Protozoa also may be important in maintaining balanced growth thus facilitating greater metabolic efficiency. Samples will be diluted plated inside plastic rings imbedded in non-nutrient agar base. One milliliter of water will be added to each ring, and replenished as needed. Non-growing cells of Enterobacter aerogenes will be supplied as a food source. Cultures will be checked between 3 days and 2 months by making a wet mount and examining the slides with phase microscopy. Protozoan counts will be expressed as counts per gram dry weight. Basic identification of representative protozoa will also be done. *ManTech Environmental Technology Inc., EPA*

4.3.4.14 Fungal and Actinomycete Analysis (Optional). Large increases in biomass during remediation projects may also cause increases in fungal biomass. Some yeast have been implicated in TCE degradation (Wackett et al., 1989). The importance of fungi in contaminated environments has largely gone unstudied. Sediment samples will be enumerated with acidified mycological agar and mycological agar with chloramphenicol. Enrichment cultures would also be done by supplementation with methane and TCE/PCE. Isolates will then be identified and further characterized in terms of ability to degrade TCE/PCE. This project is in the proposal stage and needs further consideration. The actinomycete analysis method has not been received. *GSU, PNL*

4.3.5 Offgas Monitoring. Offgas from the extraction well (AMH2) and from the vadose zone piezometers will be collected and analyzed daily by the operations subcontractor as specified in the scope of work for this contract (see Appendix C). The subcontractor shall collect pressure and vacuum data from approximately 20 vadose zone piezometers and 22 groundwater wells, as well as from the vacuum and pressure well heads and 6 downhole tubes in each horizontal well. The subcontractor shall provide the gas concentration measurements for gases collected from the approximately 20 vadose zone piezometers, gases collected from the 6 downhole tubes in the vacuum horizontal well (AMH-2), and for the vacuum well head. Up to 10 samples will be collected each day for chemical analysis of trichloroethylene (TCE), tetrachloroethylene (PCE), cis- and trans- dichloroethylene (DCE), vinyl chloride (VC), methylene chloride (MC), methane (CH_4) and carbon dioxide (CO_2). Detection limits of less than or equal to 5 ppmV for chlorinated compounds and less than or equal to 0.1 % for methane and carbon dioxide are required.

WSRC personnel will collect and analyze helium from the approximately 20 vadose zone piezometers, gases collected from the 6 downhole tubes in the vacuum horizontal well (AMH-2), and for the vacuum well head. The procedure to be used is as described previously by Looney et al. (1991) Samples are collected using a 50 ml disposable syringe and the samples are placed in 30 ml preevacuated serum vials. These vials are analyzed using a helium mass spectrometer that has been modified to sample the serum vials at a constant rate. The mass spectrometer is calibrated in two steps. First, the mass spectrometer is tuned and the sensitivity adjusted to an internal calibrated leak (diffusion) standard in units of standard ml of He per second; after this step, gas standards prepared in the serum vials are used to convert the instrument reading to ppm (volume) and check the stability of the tuning.

4.3.6 Laboratory Process Control Studies. These studies are done using soil columns with sediments from the subsurface and liquid and gas phase bioreactors to recommend injection protocol, feeding regimens, and test treatments that would

provide faster and/or more complete biodegradation of more recalcitrant species like PCE. Concentrations, flows, vacuum, and pulsing will be varied to examine the impact on TCE and PCE degradation in laboratory soil reactors. Limitations with respect to moisture, phosphate, nitrogen will also be examined. Success of these studies will be provision of data for modeling efforts and determination of factors limiting TCE/PCE degradation, and identification of treatment regimes that could compromise continued operation of the wells. The operation of the bioreactors will yield an evaluation of the potential effects of alterations on TCE degradation rate. Treatment regimens investigated in the lab soil reactors and found to be potentially more successful than current field operating conditions could than be tried in the field and thus significantly improve the rates of bioremediation. The data could also be used in subsurface models by identifying factors controlling the degradation rate. The soil columns and bioreactors may be critical in identifying regimens which could lead to plugging the sediments. In addition, optimization of the bioreactors and soil columns will provide ex-situ treatments that could be used in later demonstrations and process design information for still other demonstrations.

4.3.6.1 SRL. Studies at SRL will utilize two pilot-scale (1 gpm) methanotrophic trickle filter bioreactors for treatment of TCE/PCE contaminated groundwater. These systems will operate with contaminated water from SRS wells and suggest what groundwater parameters are important to optimal degradation by SRS microbes. Later in the year a pilot-scale (2-3 gpm) fluidized expanded bed bioreactor will be tested with the same water in cooperation with GRI (Radian, MBI and Envirex. Another major task will focus on stimulation of TCE/PCE degradation in surface soils by vegetation. Field plots at SRS will provide evidence of the effectiveness of this treatment and potential to couple this with methane injection. TTP No. SR 0308-AA. *SRL, GRI, USAF, UT, MBI, Radian, Envirex, ORNL, Stanford University*

4.3.6.2 ORNL. Studies at ORNL will utilize a pilot-scale (1 gpm) methanotrophic trickle filter bioreactor to treat TCE/PCE contaminated groundwater at ORNL that also has high concentrations of other organics and metals. A steam stripper will be used to pretreat the groundwater and provide better control over reactor operating conditions (TTP No.: OR 0369-ABD). An additional bioreactor will be set up for soil column testing using SRS contaminated soils. This system will specifically test the strategies for biodegradation of PCE under methanotrophic conditions. This project will provide vital information on methods for effecting simultaneous degradation of TCE and PCE in the vadose zone and water. (TTP No.: OR 0368-AL). *ORNL, UT*

4.3.6.3 INEL. Studies at INEL will use the differential soil bioreactor (DSBR) to develop microbial activity (growth and contaminant degradation) to be followed with time under realistic subsurface conditions. The realism includes the use of samples of subsurface material and groundwater form SRS, sot that indigenous microbial activity is studied and all of the many geochemical variable that affect microbial metabolism are correct. (TTP No.: ID 0566-AA) *INEL*

Some of the parameters which can be tested in the gaseous substrate injection test in the above bioreactors include changes in influent flow and pressure, alteration in extraction vacuum, and enrichment with propane. In previous studies TCE degraders from nearby subsurface sediments are greatly stimulated in microcosm studies after the addition of propane. Propane supplements at the level of 5-30% of the methane additions (>2% total flow) may be tested based upon results of laboratory process control experiments. Input from the results of these studies will be critical in deciding what alterations will be made over the course of test.

4.3.7 Ancillary IDP Monitoring Activities. These activities include in situ flow sensors, EM tomography and other activities covered in TTP No.: SR 0566-2. *SNL, ORNL, LLNL, BNL*

4.3.8 Meteorological Data. SRS is the Southeastern Emergency Weather Station. The weather station is physically less than one (1) mile from the demonstration site. Data is available on rainfall, temperature, barometric pressure, humidity, etc on a daily basis. This station is manned by the Environmental Technology Section of the Savannah River Laboratory.

4.3.9 Ancillary Groundwater Data. Water is collected quarterly (SRS Quarterly Groundwater Monitoring Report) from more than 50 wells within 1 mile of the demonstration site and hydrogeological summaries (Bledsoe 1984, 1986, 1988). Parameters include basic physical/chemical parameters, VOC's (including TCE/PCE), radionuclides, and heavy metals. In addition, data is also available on operation of the M-Area above ground air stripper system which is part of the groundwater corrective action plan for M-area. This data includes operating costs, VOC removals and amounts stripped (Christensen and Gordon, 1983).

4.4 Post-Test Characterization and Monitoring. The objective of these measurements is to determine how rapidly the environment returns to pre-stimulation conditions and/or how long contaminant changes last. This monitoring will also allow monitoring for adverse changes in the environment, eg. toxic daughter products, anaerobic conditions, acidic conditions.

4.4.1 Groundwater Monitoring. All parameters (Section 4.3.3) will be measured every two weeks as during the test for two (2) months. The sampling interval will than be changed to monthly for two (2) more months. All methods used will be as described above in Section 4.3.3.

4.4.2 Sediment Characterization. Ten boreholes will be drilled from the surface to 200 ft., one borehole adjacent to each of the 10 existing clusters. All techniques and methods will be as those described above in Section 4.3.4 and Section 4.2.

4.5 Modelling and data interpretation including hydrological modeling, modeling of the degradative processes and evaluation of the data. The objective is to compare results with theoretical models, interpret data, and facilitate communications between investigators. Models will be developed for methane dispersion, TCE loss, bioremediation, air, water and gas flows with pressure, impact of bioremediation. This work is a continuation of the modeling activity initiated in the In Situ Air Stripping Demonstration (Looney et al., 1991). *SRL, ORNL, LLNL, INEL*

4.6 Schedule of Events and Reports.

4.6.1 Project Planning and Management. January 1990. The Bioremediation Technical Support Group (BTSG) will be selected. They will be presented with the characterization data and discuss appropriate biotechnologies. A draft Technical Task Plan (TTP) will be prepared by the BTSG and reviewed. A final meeting will be held to discuss the TTP and look for fatal flaws. Based on recommendations of the BTSG sampling and research programs for various laboratories will be scoped, prepared and submitted as TTPs or requisitioned as contracts. In addition appropriate personnel from SRS and from participating institutions will be contacted and asked to prepare the essential documentation associated with the detailed test plan, quality assurance, operational and worker safety, site access and security, sample handling/analysis procedures, and waste handling disposal. This task was begun in July 1990 and is on going.

4.6.2 Process Design and Modeling. June 1990. This task will entail a review of the existing air stripping system including the physical arrangement of the piping and other

hardware, horizontal wells, site hydrology, soil permeability, etc., so that the bioremediation system and sampling requirements can be effectively integrated with the existing physical equipment with minimal effort. Based on site characteristics, initial modeling studies will be carried out to estimate potentially affected zones surrounding the primary injection and recovery points to assist planning of the sampling campaign. Operational parameters deemed necessary to promote biodegradation yet compatible with site characteristics will be selected.

4.6.3 Operations Contract. May 1991. Operation scope of work (see appendix C) prepared 5/91, submitted 6/91, sent out for bid by procurement 7/91, proposals received 8/91, technical evaluation 8/91, contract currently in negotiation by procurement.

4.6.4 Permitting. November 1990. Appropriate personnel at SRS were contacted and permits prepared for Underground Injection submitted 5/91 approved 7/91, NEPA approved 8/91, Air Permit submitted with new information 9/91, appropriate site use, clearance, etc. completed 9/91.

4.6.5 Analytical Facilities. January 1990. An on-site analytical and laboratory facility will be established. A trailer (climate controlled for instrument operation) will be obtained and equipped with a gas chromatograph for monitoring chlorinated alkenes, methane, oxygen and carbon dioxide in the water and influent and effluent gas phases, a pH meter, dissolved oxygen probe and specific ion electrode(s) for determining water quality, and miscellaneous small equipment (e.g. bench-top centrifuge) for sample handling and preparation. The SRL Mobile Microbial Ecology Laboratory (MMEL) is satisfactorily equipped and was used for the duration of the phase 1 task. Access to two additional gas chromatographs in a nearby support laboratory will also be required to handle the extensive volatile organic analysis needed for both water and off-gas monitoring during operating campaigns. Additional support trailer has been obtained and an additional GC has been purchased and is operational as of 9/91.

4.6.6 Pre-Test Characterization. March 1991. Completed July 1991, analysis still in progress.

4.6.7 Finalization of Test Plan by BTSG. October 1991.

4.6.8 Monitoring. September 1991. In Progress.

4.6.9 QA and Safety Report. November 1991. Must be submitted and approved prior to mobilization (appendix C).

4.6.10 Mobilization. November 1991. Dependent on operations contract, bids received August 1991, Contract in negotiation.

4.6.11 Test Schedule. November 1991. Dependent on operations contract, bids received August 1991, Contract in negotiation. Operations contractor submits weekly operations report. Monthly reports submitted by all investigators to Principal Investigators. Monthly reports submitted by Principal Investigators to WSRC Procurement, SRS-ER, DOE-OTD and SCDHEC. Quarterly reports submitted by Principal Investigators to Technical Support Group. If the technology is determined to be sound 3-5 months before the end of the project a recommendation will be sent to ER to begin permitting and contracting so that full-scale use of the technology can be started at the end of the project.

- **11/91** Start Extraction. 2 weeks
- **11/91** Start Air Injection. 2 weeks
- **12/91** Start Air/Methane Injection. 3 months
- **2/92** First Quarterly Sediment Sampling.
- **2/92** First BTSG Quarterly Review.

2/92 First Change Injection and Continue Monitoring.
5/92 Second Quarterly Sediment Sampling.
5/92 Second BTSG Quarterly Review.
5/92 Second Change Injection, Continue Monitoring.
8/92 Third Quarterly Sediment Sampling.
8/92 Third BTSG Quarterly Review.
8/92 Third Change Injection, Continue Monitoring.
10/92 Stop Methane injection.
11/92 Stop Air Injection.
12/92 Stop Extraction.

4.6.12 Post-Test Monitoring. December 1992.

4.6.13 Post-Test Characterization. December 1992.

4.6.14 Demobilization. December 1992.

4.6.15 Final Operations Report. January 1993.

4.6.16 BTSG Review of Test and Final Test Report. February 1993.

4.6.17 Post-Test Characterization Report. March 1993.

4.6.18 BTSG Review and Recommendations. March 1993.

4.6.19 Final Technology Report. June 1993.

4.7 Communications and Technology Transfer.

4.7.1 WIN. All members of the Bioremediation Technical Support Group (BTSG) and investigators will be given a WIN account to allow direct electronic mail communications of meetings, reports, reviews and support information.

4.7.2 Reports and Sample Shipments. All monthly and quarterly reports will be sent to all BTSG members and Investigators. Investigators will be notified by WIN and/or telephone when a sample has been shipped. All sample shipments will be by overnight mail scheduled to arrive on weekdays. Should weekend arrival be necessary then the investigator will be notified by phone and WIN 48 h in advance of when the sample will arrive.

4.7.3 Bioremediation Technical Support Group. The BTSG will meet at a minimum every 3 months to review progress of the test and make recommendations on new courses of action and future directions.

4.7.4 In Situ Bioremediation Demonstration Project Symposium. At the end of the project all investigators and the BTSG will give presentations and publish a proceedings of project work. The Symposium will be internationally advertised to the academic, government and industrial communities.

4.7.5 Publications and Presentations. All investigators will be encouraged and assisted in presenting and publishing investigations conducted during the project. In addition, press releases will be sent periodically to radio, TV, and newspapers throughout the tenure of the project.

5.0 Organizational Structure and Funding. For more details of the Organization Structure of the Integrated Demonstration Program see " Integrated Demonstration for Cleanup of Organics in Soils and Groundwater at Non-Arid Sites Project Management Plan (IDP-0566). For details on funding see individual Technical Task Plans (OTD). For WSRC organizational structure see WSRC Management Policies, WSRC-1-01

5.1 U.S. Department of Energy. As per direction of the Secretary of Energy as outlined in the Environmental Restoration and Waste Management Five-Year Plan (1989) the U.S. Department of Energy is striving to implement initiatives for environmental protection and waste management at DOE facilities. The **Office of Technology Development** (0TD) through the Division of Demonstration, Testing, and Evaluation (DT&E) provides programmatic direction, and overview of the Integrated Demonstration Program (IDP). Funding and DOE Oversight of the individual tasks associated with IDP will be through Technical Program Officers (TPO) at the respective operations offices, eg. **Savannah River Operations**. The prime contractor at the DOE office, eg. **Westinghouse Savannah River Company,** will appoint a Technical Program Manager (TPM) for the site to manage the tasks at that site

5.2 Westinghouse Savannah River Company. WSRC has designated the TPM to be in the **Savannah River Laboratory** division of the company. The TPM acts as manager for all Technical Task Plans for WSRC. The TPM with the concurrence of the TPO selects a Integrated Demonstration Coordinator.

5.3 Integrated Demonstration Project. The Integrated Demonstration Planning Group is responsible for acting as a steering committee for the program and advising the Project Coordinator Manager. The Planning Group is also responsible for selecting Technical Support Groups (TSG) and their chairpersons. The Program has TSGs for Analysis and Evaluation, Monitoring, Characterization, Drilling, Regulatory and Remediation. The Remediation TSG is further subdivided into Bioremediation and Physical Chemical. The TSGs provide technical guidance to the Planning Group and Technical Support for the program in their area of expertise.

5.4 Bioremediation Technical Support Group. The BTSG was established in January 1990. The following are currently members: Terry C. Hazen, SRL (chairman); Fred Brockman, PNL; Carl Fliermans, SRL; John Wilson, USEPA; Jim Spain, USAF; Rashalee Levine, USDOE; Graham Andrews, INEL; Perry McCarty, Stanford U.; John Knezovich, LLNL; Gary Sayler, U. Tennessee; Tom Phelps, U. Tennessee; Carl Gehrs, ORNL; Tony Palumbo, ORNL; Frank Chappelle, USGS; Brian Looney, SRL; Terry Donaldson, ORNL; Tom Hayes, GRI; Paul Wichlacz, INEL; and Tom Brouns, PNL

5.5 Primary and Ancillary Technical Task Plans and Funding. Only those ancillary TTPs that have been identified with Bioremediation Demonstrations at the SRS Integrated Demonstration are listed. Other ancillary TTPs associated with these and other laboratories have been identified with the other TSG of the program. Only requested FY92 budgets are presented.

5.5.1 Savannah River Site

5.5.1.1 SR 0566-01. SRS Integrated Demo: Remediation Tasks. $2,090K. PI: T. C. Hazen and B. B. Looney.

5.5.1.2 SR 0566-02. SRS Integrated Demonstration Directional Drilling & Characterization. $2,400K. PI: C. A. Eddy and D. S. Kaback

5.5.1.3 SR 0566-03. Integrated Demonstration for Cleanup of Soils and Groundwater at Non-Arid Site: Off-Gas Treatment. $1,100K. PI: J. Haselow and B. B. Looney.

5.5.1.4 SR 0308-AA. TCE Biodegradation Demonstration. $1,150K. PI: T. C. Hazen.

5.5.2 Oak Ridge National Laboratory

5.5.2.1 OR 0369-ABD. Demonstration of Co-Metabolic Technology. $320K. PI: A. Palumbo and S. Herbes.

5.5.2.2 OR 0368-AL. Bioremediation of Groundwater (PCE). $250K. PI: S. Herbes.

5.5.2.3 OR 0369-AH. TCE Degradation Demo Support. $625K. PI: A. Palumbo.

5.5.2.4 OR 0566 AC. Vegetation Enhancement. $400K. PI: B. Walton and N. Edwards.

5.5.3 Idaho National Engineering Laboratory

5.5.3.1 ID 0533-RD. Biodegradation Screening of Microbes. $345K. PI: F. Colwell.

5.5.3.2 ID 0566-AA. Soil Bioreactor Studies. $100K. PI: G. Andrews.

5.5.4 Pacific Northwest Laboratory

5.5.4.1 RL 0566-AB. Biomolecular Probe Analysis. $70K. PI: F. Brockman.

5.6 Participants: Government, Industry, Academic

5.6.1 Government: Department of Energy, Environmental Protection Agency, Geological Survey, Air Force, Army Corps of Engineers, South Carolina Department of Health and Environmental Control.

5.6.2 Academia: Stanford University, University of South Carolina, University of Illinois, University of Washington, Utah State University, Georgia State University, University of Minnesota, University of Cincinnati.

5.6.3 Industry: Gas Research Institute, Radian Corp., Eastman Christiansen, Westinghouse, duPont, Michigan Biotech Institute, Envirex Inc., Bechtel Inc., Graves, Obrien and Gerrs, Monitoring Testing Service, General Engineering Lab, Tren Fuels, South Carolina Electric and Gas Co., Terra-Vac

6.0 Permits, Patents, Licences and Contracts.

6.1 Permits

6.1.1 National Environmental Policy Act NEPA Environmental Evaluation Checklist completed 4/8/91. DOE-SR approved as a categorical exclusion SR CX9105008, 8/8/91.

6.1.2 Underground Injection Control Permit from South Carolina Department of Health and Environmental Control. Modification of UIC Permit #103 (WSRC-RP-91-354) prepared and submitted SCDHEC 4/91. Approved by SCDHEC 6/91.

6.1.3 Air Permit from South Carolina Department of Health and Environmental Control. Required to meet Clean Air Act Regulations. Prepared and submitted 9/91 (WSRC-RP-91-????), approved ?????

6.1.4 US Department of Transportation Certification. Required for transporting methane from the filling station to field site for both the vehicle and drivers. SRS obtained.

6.1.5 DOE SRS Site Use, Site Clearance, and Work Clearance Permits. Obtained from WSRC and US DOE Savannah River Operations. See WSRC Engineering and

Engineered Services Procedure Manual (1E) Procedure 3.02 for obtaining these permits and clearances. SRS obtained.

6.2 Patents and Licences: Patent search on 9/6/91 reveals only 4 patents that could have any relationship to demonstration being done.

6.2.1 Patent US 4660639 issued 4/28/87, Removal of Volatile contaminants from the Vadose Zone of Contaminated Ground. The vapor extraction from the upper horizontal well is covered by this patent and WSRC has a paid-up one time license with the assignee; The UpJohn Company, for use of this process with the horizontal wells.

6.2.2 Patent US 4832122, issued 5/23/89, In-Situ Remediation System and Method for Contaminated Groundwater The project will also use In Situ Air Stripping. This patent is assigned to WSRC/DOE.

6.2.3 Patent US 4713343, issued 12/15/87, Biodegradation of halogenated aliphatic hydrocarbons; water purification using microorganism capable of aerobic degradation of low molecular weight alkanes. The demonstration will also use this process but since the assignee for this patent is US EPA no licence in necessary since DOE is also a US government agency.

6.2.4 Patent US 5006250, issued 4/9/91, Pulsing of electron donor and electron acceptor for enhanced biotransformation of chemicals. One of the supplemental injection strategies to be used could be pulsing; however, the patent specifically covers only pulsing of electron donors in "aliquots of water". It does not specifically cover pulsing of electron donors in air, thus no licences should be necessary. Prior to initiating a pulsing regimen of we will have the legal department examine this patent and if necessary obtain a licence from the assignee: Stanford, Leland Jr University Trustees.

6.2.5 Catalytic Destruction of Offgas Contaminants. It shall be the responsibility of the suppliers of this treatment unit to obtain licences for the process used as appropriate.

6.3 Contracts and Agreements This does not include the specific tasks covered by joint participants TTP's at ORNL, PNL and INEL, see Section 5.5 above.

6.3.1 Operations Contract: Injection/Extraction and Offgas Treatment Equipment. A single competitively bid contract will cover procurement, maintenance and operation of the following equipment: air compressor, vacuum blower, air/methane blending system, field engineered manifold, and offgas catalytic treatment. The successful bider will provide 24 h/day coverage of field operations including monitoring of pressure and offgas chemical analysis of trichloroethylene (TCE), tetrachloroethylene (PCE), cis- and trans- dichloroethylene (DCE), vinyl chloride (VC), methylene chloride (MC), methane (CH_4) and carbon dioxide (CO_2). Detection limits of less than or equal to 5 ppmV for chlorinated compounds and less than or equal to 0.1 % for methane and carbon dioxide are required. See Appendix C for the scope of work for this contract.

6.3.2 Methane Supply and Storage. Gas Research Institute has agreed to specify in their contract with Radian Corp. (Houston, TX) that Radian contract Tren Fuels Inc. (Denver, Co.) to fabricate a trailer with a tank capable of suppling compressed natural gas for the demonstration. The two trailers containing CNG cylinders will be DOT approved and certified. Each trailer will have a filled capacity of 12,480 std. cu ft at 3000 psi. Radian Corp. will also contract South Carolina Electric and Gas Company for a fill station to be located on SCE&G property in Jackson, South Carolina. The fill station will have a fill rate of ??? psi/hr, enabling a fill time of ??? hours. WSRC will provide vehicles and certified drivers for refilling the tank trailer at the filling station when required.

6.3.3 Chemical Analysis of Water and Soil. Samples to be analyzed for sulfur, nitrogen, nitrate+nitrite, sulfate, sulfide, dissolved organic carbon, ammonia, phosphorus, phosphate, chloride, iron, and cation exchange capacity will be done under a WSRC task order contract (AX?????) to General Engineering Laboratory (Charleston, SC). Procedures to be used are all EPA approved or recommended.

6.3.4 Stanford University. Scope of Work for Kinetics of Methanotrophic Reactions, Principal Investigator: Dr. Perry McCarty, AA46349T, October 1, 1991 - September 31, 1992. The objective of this investigation is to determine the kinetics of methanotrophic transformations of trichloroethylene (TCE) and tetrachloroethylene (PCE). This will allow the determination of the important coefficients for growth and substrate utilization by methanotrophs, and will also permit determining the important coefficients for substrate (methane) and inhibitor (TCE) utilization.

6.3.5 ManTech Environmental Technology Inc. - USEPA. All protozoan analysis from sediments will be done by sole source contract (C11591) to Dr. James Sinclair, ManTech Environmental Technology Inc. - USEPA, Kerr Research Lab, Ada, OK. The requisition has been placed and is waiting final contract award.

6.3.6 SCRUEF - University of South Carolina. Task orders for three specific projects have been started through the WSRC contract with the South Carolina Research and Undergraduate Education Foundation.

6.3.6.1 Dr. James Yates. Development of procedures for identification of organisms capable of degrading trichloroethylene in the environment, Started 8/90, AA00900T task 12, Currently in renewal. Development of DNA probes specific for chromosomal sequences present in the host bacterium (designated T1) from which TOL-1 was isolated. Development of a procedure to introduce DNA into T1. Detection of TOL-1 cells after entrapment on filters. The current scope of work also calls for analysis of isolates using Biolog plates.

6.3.6.2 Dr. Charles Lovell. Development of Functional Group Probes: Acetogens, Nitrogen Fixers and Aromatic Degraders, Started 6/90, AA00900T task 10, Currently in renewal. The objective of this investigation is to develop DNA probes for quantitative measurement of acetogen, nitrogen-fixing, and aromatic degrader bacterial populations and genes involved in these activities. To evaluate the sensitivity and specificity of nucleic acid probes for measuring these functional bacterial populations in mixed microbial culture and to correlate these measurements with rates of degradation and/or fixation. To determine these functional bacterial population dynamics in contaminated and uncontaminated environments.

6.3.6.3 Dr. John Morse. Experimental Bioreactor for Treatment of TCE and PCE-Contaminated SRS Groundwater, Started 6/90, AA00900T task 8, Currently in renewal. The objective of this investigation is to evaluate the use of a bench-scale, fluidized expanded-bed, bioreactor for the degradation of trichloroethylene (TCE) and tetrachloroethylene (PCE) in SRS groundwaters. The ultimate goal of the investigation is to demonstrate feasibility of biological remediation of TCE and PCE contaminated groundwater at the Savannah River Site, Aiken, South Carolina.

6.3.7 Georgia State University (Optional) All fungal analysis and identification if done will be by sole source contract to Dr. Donald G. Ahearn, Georgia State University.

7.0 Safety, Quality Assurance and Security.

7.1 Safety.

7.1.1 Savannah River Site. General safety rules for the Savannah River Site are documented in the Savannah River Site (SRS) Safety Manual (8Q) and in compliance with DOE order 5483.1A.

7.1.2 Savannah River Laboratory. Savannah River Laboratory Safety Practices and Procedures Manual (8Q8) documents safety procedures for all activities for SRL Employees, SRL visitors, and Vendors/Subcontractors.

7.1.3 Process Hazards Review. As defined in Savannah River Site (SRS) Safety Manual (8Q) in Procedure 10-1. To be performed on site with cognizant functional personnel required.

7.1.4 Other Safety Information. Other sources of safety information include: SRP Industrial Hygiene (DPSOP 158 Series), SRP Engineering Standards and Specifications (DPSOP 208-1), SRL Occupational Health Control Procedures (DPSTP-R), and SRL Engineering Practices (DPSTOM-51).

7.1.5 Subcontractor. While on SRS, the Successful offerer will be responsible for adhering to the safety regulations of "WSRC Safety Guidelines for Subcontractors". All members of the successful offerer project staff must attend a site safety and security orientation (approximately 8 hours) prior to beginning work at SRS. The orientation includes information about handling chemicals on site, emergency signals, and security.

7.2 Quality Assurance/Quality Control: All activities at SRS are governed by WSRC Quality Assurance Program as outlined in WSRC Management Policies, WSRC-1-01 MP 4.2. Specific Quality Assurance Procedures are documented by organization as required.

7.2.1 Westinghouse Savannah River Company. WSRC Quality Assurance is documented in WSRC Quality Assurance Manual (1Q).

7.2.2 Chemical Processes and Environmental Technology Department. Quality Assurance implementation procedures for the CP&ET Department are documented in CP&ET Quality Assurance Implementation Procedures (1Q31).

7.2.3 Environmental Sciences Section. Quality Assurance implementation procedures for the section are found in ESS Quality Assurance Implementation Procedures (1Q31-1). Operating procedures for the section are documented in ESS Operating Procedures Manual (WSRC-L-14-1).

7.2.4 Subcontractor. All subcontractors will adhere to WSRC Quality Assurance program and submit all document and records in the Quality Assurance Report and in the Final Report.

7.3 Security. Westinghouse Savannah River Company security requirements and procedures are documented in the WSRC Security Manual (7Q). These procedures are as required by Federal Laws and applicable DOE Orders, eg. DOE Order 5631.1A.

8.0 REFERENCES CITED

---------. 1985. Closure Plan for the M-Area Settling Basin and Vicinity at The Savannah River Plant. DPSPU 84-11-11 Rev. 11/85. E. I. du Pont de Nemours and Company, Savannah River Laboratory, Aiken, SC.

---------. 1989. United States Department of Energy Environmental Restoration and Waste Management Five-Year Plan. DOE/S-0070. NTIS, Springfield, VA.

---------. 1991. DPSOP 254. Hydrogeologic Data Collection Methods, Procedures, and Specifications. Westinghouse Savannah River Company, Aiken, SC.

---------. 1991. Modification of UIC Permit #103. WSRC-RP-91-354. Westinghouse Savannah River Company, Aiken, SC.

---------. 1991. Integrated Demonstration Project: Project Management Plan, 3/91. IDP-0566. Westinghouse Savannah River Company, Aiken, SC.

APHA. 1989. Standard Methods for the Examination of Water and Wastewater. 17th ed. American Public Health Association, Washington, DC.

Balkwill, D. L. 1989. Numbers, diversity, and morphological characteristics of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiol. J. 7:33-52.

Bohlool, B. B., and E. L. Schmidt. 1980. The immunofluorescence approach in Microbial Ecology. Adv. Microb. Ecol. 4:203-241.

Bledsoe, H. W. 1984. SRP Baseline Hydrogeologic Investigation - Phase I. DPST-84-833. E. I. du Pont de Nemours and Company, Savannah River Laboratory, Aiken, SC.

Bledsoe, H. W. 1986. SRP Baseline Hydrogeologic Investigation - Phase II. DPST-86-674. E. I. du Pont De Nemours and Company, Savannah River Laboratory, Aiken, SC.

Bledsoe, H. W. 1988. SRP Baseline Hydrogeologic Investigation - Phase III. DPST-88-627. E. I. du Pont de Nemours and Company, Savannah River Laboratory, Aiken, SC.

Christensen, E. J., and C. K. Brendell. 1982. Chlorinated Solvent Releases to M-Area Process Sewers. DPST-81-968. E. I. du Pont de Nemours and Company, Savannah River Laboratory, Aiken, SC.

Christensen, E. J., and D. E. Gordon. 1983. Technical Summary of Groundwater Quality Protection Program at Savannah River Plant. DPST-83-829 volume 1. E. I. du Pont de Nemours and Company, Savannah River Laboratory, Aiken, SC.

Chaudhry, G. R., and S. Chapalamadugu. 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. 55:59-79.

Craun, G. F. 1986. Waterborne Diseases in the United States. 295 pp. CRC Press, Boca Raton, FL.

Dommergues, Y. R., L. W. Belser, and E. L. Schmidt. 1978. Limiting factors for microbial growth and activity in soil. Adv. Microb. Ecol. 2:49-104.

Donaldson, T. L., G. W. Strandberg, et al. 1988. Degradation of trichloroethylene and trans-1,2-dichloroethylene by a methanotrophic consortia in a trickle-type bioreactor. Proceedings DOE Model Conference, Oak Ridge, Tennessee, October 3-7, 1988.

Eddy, C. A., B. B. Looney, J. M. Dougherty, T. C. Hazen, and D. S. Kaback. 1991. Characterization of the Geology, Geochemistry, Hydrology and Microbiology of the In-Situ Air Stripping Demonstration Site at the Savannah River Site. WSRC-RD-91-21. Westinghouse Savannah River Company, Aiken, SC.

Fliermans, C. B., B. B. Bohlool, and E. L. Schmidt. 1974. Autecological study of the chemautotroph Nitrobacter by immunofluorescence. Appl. Microbiol. 27:124-129.

Fliermans, C. B., W. B. Cherry, L. H. Orrison, and L. Thicker. 1979. Isolation of Legionella pneumophila from nonepidemic related aquatic habitats. Appl. Environ. Microbiol. 37:1239-1242.

Fliermans, C. B., and T. C. Hazen (eds.). 1991. Proceedings of the First International Symposium on Microbiology of the Deep Subsurface. January 15-19, 1990. WSRC Information Services, Aiken, SC.

Fliermans, C. B., T. J. Phelps, D. Ringelberg, A. T. Mikell, and D. C. White. 1988. Mineralization of trichloroethylene by heterotrophic enrichment cultures. Appl Environ Microbiol 54:1709-1714.

Fogel, M. M., A. R. Taddeo, and S. Fogel. 1986. Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl Environ Microbiol 51:720-724.

Garabedian, S. P. 1990. A comparison of results from small- and large-scale tracer test in a heterogeneous aquifer, Cape Code, Massachusetts. Proceedings of the First International Symposium on Microbiology of the Deep Subsurface. January 15-19, 1990. WSRC Information Services, Aiken, SC.

Harvey, R. W., and L. H. George. 1987. Growth determinations for unattached bacteria in a contaminated aquifer. Appl Environ Microbiol 53:2992-2996.

Hazen, T. C. 1990. Deep subsurface bacterial responses to contaminants. C. B. Fliermans and T. C. Hazen (eds.), Proceedings of the First International Symposium on Deep Terrestrial Subsurface Microbiology. WSRC Information Services, Aiken, SC.

Hazen, T. C., and L. Jiménez. 1988. Enumeration and identification of bacteria from environmental samples using nucleic acid probes. Microbiol. Sci. 5:340-343.

Hazen, T. C., L. Jiménez, G. López de Victoria, and C. B. Fliermans. 1991. Comparison of bacteria density and activity in groundwater and adjacent sediments. Microb. Ecol. (in press)

Jiménez, L., G. López de Victoria, J. Wear, C. B. Fliermans and T. C. Hazen. 1990. Molecular analysis of deep subsurface bacteria. C. B. Fliermans and T. C. Hazen (eds.), Proceedings of the First International Symposium on Deep Terrestrial Subsurface Microbiology. WSRC Information Services, Aiken, SC.

Hazen, T. C., L. Jiménez, and S. Pfiffner. 1990. Isolation of microbial DNA from groundwater environments. C. B. Fliermans and T. C. Hazen (eds.), Proceedings of the First International Symposium on Deep Terrestrial Subsurface Microbiology. WSRC Information Services, Aiken, SC.

Kaback, D. S., B. B. Looney, J. C. Corey, and L. M. Wright, III. 1989. Well completion report on installation of horizontal wells for in-situ remediation tests. WSRC-RP-89-784. Westinghouse Savannah River Company, Aiken, SC.

Kaback, D. S., B. B. Looney, J. C. Corey, L. M. Wright, III, and J. L. Steele. 1989. Horizontal Wells for In-Situ remediation of Groundwater and Soils. pp. 121-135. Proceedings of the Third Outdoor Action conference, Association of Groundwater Scientists and Engineers, Las Vegas, NV.

Kaback, D. S., B. B. Looney, C. A. Eddy, and T. C. Hazen. In Press. Innovative ground water and soil remediation: in situ air stripping using horizontal wells. National Water Well Association Outdoor Action Conference.

Little, C. D., A. V. Palumbo, S. E. Herbes, M. E. Lidstrom, R. L. Tyndall, and P. J. Gilmer. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl Environ Microbiol 54:951-956.

Looney, B. B. 1991. In situ air stripping final UIC report (07/27/90 to 12/18/90). Memo: SRL-ESS-91-0220. Westinghouse Savannah River Company, Aiken, SC.

Looney, B. B., H. W. Bledsoe, V. Price Jr., J. S. Haselow, D. S. Kaback, R. L. Nichols, D. E. Stephenson, R. K. Aadland, A. L. Stieve, C. A. Eddy, and T. C. Hazen. 1990. Recent measurements of Groundwater elevations in water bearing zones underlying SRS, 1988. WSRC-TR-90-5. Westinghouse Savannah River Company, Aiken, SC.

Looney, B. B., T. C. Hazen, D. S. Kaback, and C. A. Eddy. 1991. Full Scale Field Test of the In Situ Air Stripping Process at the Savannah River Integrated Demonstration Test Site. WSRC-RD-91-22. Westinghouse Savannah River Company, Aiken, SC.

Looney, B. B., and D. S. Kaback. 1991. Field demonstration of in situ air stripping using horizontal wells. Proceedings of Waste Management '91. Cosponsored by the American Nuclear Society, American Society of Mechanical Engineers, U.S. Department of Energy, and the University of Arizona, volume 1, pp. 527-535.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1987. Molecular Cloning. Cold Spring Harbor Laboratory Publication. Cold Spring Harbor, New York.

Marine, I. W., and H. W. Bledsoe. 1984. Supplemental Technical Data Summary: M Area Groundwater Investigation. DPSTD-84-112. E. I. du Pont De Nemours and Company, Savannah River Laboratory, Aiken, SC.

Mayer, K. P., D. Grbic-Galic, L. Semprini, and P. L. McCarty. 1988. Degradation of trichloroethylene by methanotrophic bacteria in a laboratory column of saturated aquifer material. Water Sci. Tech. 20:175-178.

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

Patrick, R., E. Ford, and J. Luarles. 1983. Groundwater contamination in the Unites States. 2nd ed. University of Pennsylvania Press, Philadelphia, PA.

Phelps, T. J., D. Ringelberg, D. Hedrick, J. Davis, C. B. Fliermans, and D. C. White. 1989. Microbial biomass and activities associated with subsurface environments contaminated with chlorinated hydrocarbons. Geomicrobiol J 6:157-170.

Phelps, T. J., J. J. Niedzielski, R. M. Schram, S. E. Herbes, and D. C. White. 1990. Biodegradation of trichloroethylene in continuous-recycle expanded-bed bioreactors. Appl Environ Microbiol 56:1702-1709.

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.

Pickett, J. B. 1985. Extended Characterization of M-Area Settling Basin and Vicinity. DPSTD-85-121. E. I. du Pont De Nemours and Company, Savannah River Laboratory, Aiken, SC.

Sayler, G. S., J. Fleming, B. Applegate, C. Werner, and K. Nikbakht. 1989. Microbial community analysis using environmental nucleic acid extracts, p 658-662. In T. Hattori, Y. Ishida, Y. Maruyama, R. Y. Morita, A Uchida (eds). Recent Advances in Microbial Ecology, Proceedings of the 5th International Symposium on Microbial Ecology, Nagaya, Japan.

Semprini, L. S. U., P. V. Roberts, G. D. Hopkins, and D. M. Mackay. 1988. A field evaluation of in-situ biodegradation for aquifer restoration,. E PA Report #:EPA/600/S2-87/096

Smith, R. L., B. L. Howes, and S. P. Garabedian. 1991. In situ measurement of methane oxidation in groundwater by using natural-gradient tracer tests. Appl Environ Microbiol 57:1997-2004.

Sims, W. R., B. B. Looney and C. A. Eddy, 1991. Evaluation of a Rapid Headspace Analysis Method for Analysis of Volatile Constituents in Soils and Sediments. Proceedings of the Fifth Outdoor Action Conference, Association of Groundwater Scientists and Engineers, Las Vegas, NV, May 1991.

Sinclair, J. L., and W. C. Ghiorse. 1989. Distribution of aerobic bacteria, protozoa, algae, and fungi in deep subsurface sediments. Geomicrobiol. J. 7:15-32.

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.

Vogel, T. M., and P. L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl-chloride, and carbon-dioxide under methanogenic conditions. Appl Environ Microbiol 49:1080-1083.

Wackett, L. P., G. A. Brusseau, S. R. Householder, and R. S. Hansen. 1989. Survey of microbial oxygenases: trichloroethylene degreadatio by propane oxidizing bacteria. Appl. Environ. Microbiol. 55:2960-2964.

White, D. C., D. B. Ringelberg, J. B. Guckert, and T. J. Phelps. 1990. Biochemical markers for in situ microbial community structure. C. B. Fliermans and T. C. Hazen (eds.), Proceedings of the First International Symposium on Deep Terrestrial Subsurface Microbiology. WSRC Information Services, Aiken, SC.

Wickramanayake, G. B., H. Nack, E. H. Hagen, D. T. Palmer, and C. L. Criner. 1990. Aerobic biotreatment of trichloroethylene-contaminated groundwater. Proc. Haz. Wastes Haz. Mat. (HMCRI) 7:359-362.

Wilson, J. T., and B. H. Wilson. 1985. Biotransformation of trichloroethylene in soil. Appl Environ Microbiol 49:242-243.

Wilson, B. H., G. B. Smith, and J. F. Rees. 1986. Biotransformations of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material a microcosm study. Environ Sci Technol 20:997-1002.

Wilson, J. T. 1988. Degradation of halogenated hydrocarbons,. Biosensors And Environmental Biotechnology (Gustav Fisher)

Appendix A. Lists of Analytical Parameters.

Appendix B. Technical Task Plan.

Appendix C. Scope of Work.