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December 6, 1994

94-RF-11971

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FISCAL YEAR 1994 PHASE I AND II REPORT - BIODEGRADATION OF CHLORINATED SOLVENTS - DEPARTMENT OF ENERGY ORDER 4700.1 - WSB-144-94

Action: None

Enclosed is the Fiscal Year 1994 Phase I and II Report - Biodegradation of Chlorinated Solvents for submittal to the Environmental Protection Agency and the Colorado Department of Public Health and Environment.

If you have any questions or comments regarding this report, contact Sandra Spence at extension 5811 or digital page 7409.

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FISCAL YEAR 1994

PHASE I AND PHASE II REPORT  
BIODEGRADATION OF CHLORINATED SOLVENTS

ROCKY FLATS ENVIRONMENTAL TECHNOLOGY SITE

U.S. Department of Energy  
Rocky Flats Field Office  
Golden, Colorado

Environmental Restoration Program

December 1994

## Phase I and II Report

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## List of Acronyms, Abbreviations, and Chemical Formulas

BFB	bromofluorobenzene
° C	degrees celsius
CH <sub>2</sub> Cl <sub>2</sub>	methylene chloride
CH <sub>3</sub> Cl <sub>3</sub>	chloroform
CH <sub>4</sub>	methane
CCl <sub>4</sub>	carbon tetrachloride
CO <sub>2</sub>	carbon dioxide
CMS/FS	Corrective Measures Study/Feasibility Study
DOE	Department of Energy
EPA	Environmental Protection Agency
FY	Fiscal Year
g	gram
GC/MS	gas chromatograph/mass spectrometer
H <sub>2</sub> S	hydrogen sulfide gas
IHSS	Individual Hazardous Substance Site
kg	kilogram
lbs	pounds
mg	milligram
ml	milliliter
mm	millimeter
N <sub>2</sub>	nitrogen gas
O <sub>2</sub>	oxygen
OU	operable unit
PCE	perchloroethylene or tetrachloroethylene
ppm	parts-per-million
psi	pounds per square inch
Site	Rocky Flats Environmental Technology Site
TCA	trichloroethane
TCE	trichloroethylene
TSP	RFETS Environmental Restoration Sitewide Treatability Study Program
μl	microliter

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### ABSTRACT

This report describes the preliminary findings of the remedy-screening level biodegradation study performed at the Rocky Flats Environmental Technology Site (Site) in fiscal year 1994 (FY-94). The biotreatment of chlorinated aliphatic solvents in soil is the focus of this study.

Phase I of this treatability study examined the transformations of solvents occurring when solvents contact sterile and nonsterile soil from OU 2 which was not amended with nutrients or water to stimulate biological activity. Six solvents including perchloroethylene (25 ppm), trichloroethylene (25 ppm), carbon tetrachloride (25 ppm), methylene chloride (25 ppm), 1,1,1-trichloroethane (12.5 ppm), and 1,1,2-trichloroethane (12.5 ppm) were added to sterile and nonsterile soil samples as a mixture. The changes in concentration of the solvents as well as formation of degradation products were measured in soil samples over a 71-day test. Results showed that the concentration of all solvents declined in both the sterile and nonsterile samples in a similar manner. However, no degradation products developed in any sample tested over the 71 day duration. This implies that solvents were lost from the sample vials and not transformed or degraded.

Phase II of this treatability study involves culturing four microbial populations from OU 2 soil, which are known to be important in the degradation of aliphatic solvents, and testing the populations' ability to degrade the solvents described above. This phase consists of Tasks 1, 2, and 3: 1) culturing methanotrophs, denitrifiers, sulfate reducers, and methanogens in OU 2 soil; 2) evaluating and tracking the growth of the populations and producing a growth curve for each; and 3) exposing the populations to chlorinated solvents and evaluating their ability to transform these solvents. Tasks 1 and 2 began in FY-94. Task 3 will occur in FY-95.

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ROCKY FLATS ENVIRONMENTAL TECH. SITE  
ENVIRONMENTAL RESTORATION PROGRAM  
Phase I and Phase II Report for  
Biodegradation of Chlorinated Solvents

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Laboratory enrichment culturing showed successful denitrifier, sulfate reducer, and methanogen growth in OU 2 soil. Growth curves were produced for each population. The presence of methanotrophs in OU 2 soil was suspected but could not be confirmed with the available data from FY-94. Further culturing experiments are planned.

All of these populations have been grown in the laboratory in the presence of chlorinated solvents to promote adaption to and transformations of the solvents of interest for this study. The ability of these populations to transform solvents will be tested in future studies.

8

## 1.0 Introduction

This report describes the preliminary findings of the remedy-screening level biodegradation study performed at the Rocky Flats Environmental Technology Site (Site) during fiscal year 1994 (FY-94). Information presented encompasses the portion of work for the first two phases of experimentation. These phases are part of a four-phase plan for biological treatment of chlorinated solvents in soils at the Site.

### 1.1 Application/Scope

In general, the Site needs economical and effective methods to treat chlorinated solvents in soil and groundwater. Recent studies illustrate that chlorinated solvents can be completely biodegraded to non-regulated compounds under various biological conditions. The technology is relatively new with full-scale and pilot-scale studies under development within the Department of Energy (DOE) complex and in industry. With this in mind, this screening level study provides a relatively quick evaluation of the potential for effectively treating chlorinated solvents in soil at the Site using biological methods only.

This multi-phase Biodegradation Treatability Study directly contributes to the Operable Unit 2 (OU 2) Corrective Measures Study/Feasibility Study (CMS/FS) and is a part of the Environmental Restoration Sitewide Treatability Study Program (TSP). The study supports evaluation of treatment options for solvent contaminated soil in the OU 2 east trenches area; however, the results of this study may be applicable for solvent contaminated media in other OUs.

The OU 2 Biodegradation Treatability Study began with Phase I which examined chemical changes of solvent contaminants caused by the abiotic (nonliving) components of OU 2 soil and by the resident microbial soil population which was not stimulated to grow. This allows an understanding of the underlying changes expected when solvent mixtures contact the Site soil

which has not been altered to enhance biological activity. Phase II involves enhancing the conditions for several mixed microbial populations under aerobic and anaerobic conditions and evaluating their ability to transform six solvents of interest for OU 2. Phase I was completed and Phase II begun in FY-94.

The Site conducted Phases I and II as a laboratory-scale study of the biotreatability of a chlorinated solvent mixture. The solvents found in Individual Hazardous Substance Site (IHSS) 110 within OU 2 include perchloroethylene (PCE) also known as tetrachloroethylene, carbon tetrachloride, methylene chloride, trichloroethylene (TCE), 1,1,1-trichloroethane (TCA), and 1,1,2-TCA.

This treatability study focuses on low concentrations of these solvents on soil in the range of 25 ppm (mg/kg) and below. A wide range of solvent concentrations exist in OU 2 soil from free-product to nondetectable levels. If successful, this technology will be most suited to treat moderate to low concentrations of solvents and will be applicable for free product treatment only if the contaminated media can be mixed with less contaminated soil or water. If free-product treatment is required, ex situ remediation in a bioreactor will most likely be required to control contaminant loading and toxicity. If residual solvent treatment is required in which solvents are at low to moderate concentrations, in situ treatment may be considered. These issues will be examined in more detail during Phase III.

## 1.2 Technology Review

Prior to 1983, chlorinated aliphatic solvents such as TCE were thought to be recalcitrant or unable to be degraded microbially. Since then, numerous studies have shown that important biochemical pathways are available for degradation of TCE, TCA, carbon tetrachloride, methylene chloride, and the other chlorinated solvents, and that degradation or transformations will occur when conditions are appropriate (Egli et al. 1988, Egli et al. 1990, Fogel et al. 1986, Gossett 1985, Nelson et al. 1987, Vogel et al. 1987, Wackett et al. 1989).

### 1.2.1 Anaerobic Treatment

Anaerobic treatments show that microbes are able to reductively dechlorinate solvents such as carbon tetrachloride and PCE as well as other highly chlorinated solvents. For some degradation mechanisms such as the acetyl CoA pathway, the rate of reaction for chlorine removal increases with increasing numbers of chlorine atoms (Hughes and Parkin 1991). For example, carbon tetrachloride ( $\text{CCl}_4$ ) is dechlorinated faster than chloroform ( $\text{CHCl}_3$ ) which, in turn, is dechlorinated faster than methylene chloride ( $\text{CH}_2\text{Cl}_2$ ) under this pathway (Hughes and Parkin 1991). Highly chlorinated solvents such as TCE, TCA, and PCE are good targets for anaerobic treatment.

While highly chlorinated compounds tend to dechlorinate rapidly, the degradation products of dechlorination are themselves less chlorinated compounds which may tend to accumulate and not dechlorinate further under anaerobic conditions (Kästner 1991, Vogel and McCarty 1985). Anaerobic treatment can be used to convert highly chlorinated solvents to less chlorinated compounds which are then susceptible to aerobic biodegradation as discussed below.

### 1.2.2 Aerobic Treatment

Aerobic treatment of chlorinated solvents shows that compounds such as TCE, TCA, methylene chloride, and less chlorinated solvents can be degraded via oxidation of the solvent molecules under methanotrophic and other aerobic conditions (Fogel et al. 1986, Janssen et al. 1988, Little et al. 1988, Oldenhuis et al. 1989). However, highly chlorinated solvents such as PCE and carbon tetrachloride tend to be resistant to methanotrophic treatment (Janssen et al. 1991).

Only a few aliphatic chlorinated solvents have been found to serve as primary growth substrates for microbial populations (Janssen et al. 1991). This means that, typically, chlorinated solvents are not directly oxidized as a food or energy source for microbes. Oxidation of solvents

occurs incidentally to other metabolic processes and the microbes obtain no energy from the transformation. This usually involves the production of nonspecific enzymes which coincidentally oxidize compounds which are not the target energy source. This process is called cometabolism (Brock 1984).

The previous section briefly describes a few of the many pathways available for biotreatment of chlorinated solvents. This study examines several of these pathways for use in treating solvent contaminated soil at the Site. Specifically, one aerobic and three anaerobic pathways are examined.

### 1.3 Study Objectives

The overall objective of a treatability study for remedy screening is to determine whether a particular treatment technology shows promise in its ability to treat particular contaminants to levels which are protective of human health and the environment. Figure 1 shows the logical progression of information which must be obtained in order to meet this objective for the biodegradation treatability study.

#### 1.3.1 Phase I Abiotic/Unamended Soil Study Objectives

Item B in Figure 1 identifies the need to determine whether abiotic transformations of chlorinated solvents in soil are significant. Phase I of this study was designed to address this question.

Phase I evaluated the transformations of solvents in sterile and nonsterile soil which was not amended with water or nutrients that promote microbial activity. The test objective of this Phase was to determine the types of chemical changes which occur when solvent mixtures contact the Site soil which is: 1) no longer biologically active; or 2) biologically active but not nutrient enhanced. The value of this test objective to the overall study is that by knowing the

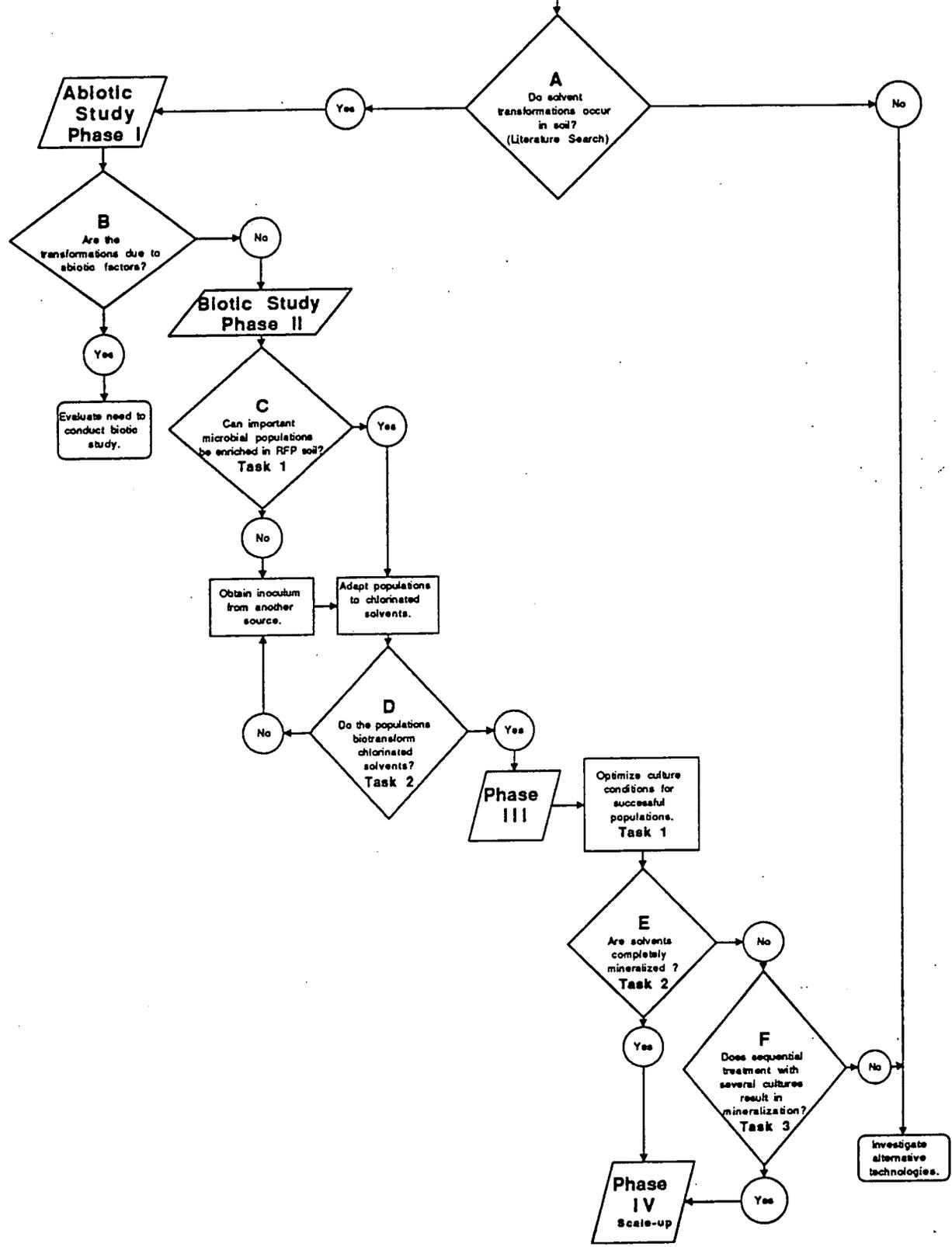


Figure 1. Flow diagram of the Biodegradation Treatability Study.

underlying transformations expected when solvents contact the Site soil, the extent to which enhanced biological activity in the soil alters the types and rate of solvent transformations can be determined in Phase II. This objective was accomplished by exposing sterilized and unamended Site soil to chlorinated solvents and monitoring solvent structure and concentration changes.

A comparison of solvent changes in sterile and nonsterile soil provided insight into:

- 1) chlorinated solvent alterations caused by the nonliving components of the soil (abiotic - sterile soil test); and
- 2) chlorinated solvent alterations caused by the unenhanced resident microbial populations in the soil (nonsterile soil test).

### 1.3.2 Phase II Biotic Study Objectives

After Phase I, the remaining questions in Figure 1 are concerned with the growth of biological populations and their ability to biotransform the solvents of interest. Phase II provides information regarding which populations are of greatest importance for further study in Phase III, thus narrowing the scope of future experiments.

The test objective for the FY-94 portion of Phase II was to determine if four microbial populations, which have shown potential for biotransformation of chlorinated solvents in literature, can be enriched in Site soil. The test objective for FY-94 was accomplished by growing microorganisms from OU 2 soil in laboratory cultures which promote the development of methanotrophic, denitrifying, sulfate reducing, and methanogenic populations.

At the time of preparation of this report, Phase II was not complete. The remaining Phase II objective in FY-95 is to determine if these populations are capable of transforming chlorinated solvents in flask tests, and which of the populations are the most effective at transforming the solvents based on their ability to change the chemical structure of the parent solvents.

Future Phase II activities include: 1) exposing subsamples of each population to a mixture of chlorinated solvents and measuring the change in concentration of solvents and degradation products over time; and 2) comparing the change in concentration of solvents and degradation products formed during testing between the four populations to determine if solvent transformations exist. Information regarding the populations' ability to transform the solvents will be provided in a report at the conclusion of Phase II.

## 2.0 Methods and Materials

### 2.1 Soil Sampling

This treatability study is intended to support evaluation of treatment options for contaminated soil in OU 2 (East Trenches area). Soil was collected from within and around IHSS 110. The samples included soil down to approximately 6 inches. IHSS 110 is an East Trench within OU 2 that was formerly used for burial of contaminated solvents and cutting oils from machining processes at the Site. The IHSS is known to contain free-phase solvents.

Approximately 100 lbs of soil and rock was collected from outside the boundary of the IHSS by removing grasses and using a pick and shovel to collect soil down to 6 inches depth. This "clean" soil was sieved through a 2 mm screen to remove rocks and debris. The soil was stored in an air-dry state prior to use. This soil was used as the nonradioactive/nonsolvent contaminated matrix onto which solvents were spiked for experimentation.

The same soil collection procedure was used to collect a 10 lb sample from within the IHSS. The soil from within the boundary of IHSS 110 was collected as a source of microorganisms which have been exposed to, and may be adapted to, the presence of chlorinated solvents. A portion of this living soil was mixed with the sterile clean soil for biological testing. This soil was stored in a moist condition at -4°C to preserve microbial activity prior to use.

The Building 881 laboratory in which experiments were performed is not approved for handling radioactive material. Subsurface soil from within the IHSS was not used as the bulk matrix for transformation studies because there was the possibility it could contain measurable radioactive or solvent contaminants which could interfere in testing. It was difficult to obtain large quantities of soil from IHSS 110 without collecting some material containing above background radioactivity. The small quantity collected from IHSS 110 as a microbial seed was analyzed for radioactivity prior to shipment to Building 881.

## 2.2 Phase I - Abiotic/Unamended Soil Study

For Phase I, two tests were run simultaneously (referred to as Test 1 and Test 2). Test 1 evaluated the fate of the solvents on sterilized soil (abiotic soil), while Test 2 evaluated the fate of solvents on "living" soil with no attempt to stimulate biological activity (unamended soil). Table 1 shows the number of samples, controls, and standards tested for Phase I.

A total of seventy-two 1.5 g air-dried soil samples were placed in double septa 40 ml purge-and-trap vials. For Test 1, 32 of the soil samples were sterilized by autoclaving at 121° C for 45 minutes to eliminate biological activity. These sterile samples were used to study the effect of the nonliving or abiotic components of OU 2 soil on solvent transformations. For Test 2, 32 of the soil samples were tested in a nonsterile air-dried state. These unsterile samples were used to study the effect of the existing microbial soil populations which were not stimulated by the addition of water or nutrients (unamended) on solvent transformations.

A solvent mixture in a methanol carrier was delivered to the soil in the purge-and-trap vials through the vial septum using a gas-tight syringe. Vials were then stored in the dark at 25° C to eliminate photodegradation until analysis.

Table 1. Number of samples, controls, and standards analyzed for Test 1 and Test 2 of the abiotic/unamended soil study.

Experiment Start Date	Analysis Date	Standard Nonsterile Soil with Solvent	Test 1 Sterile Soil		Test 2 Nonsterile Soil	
			Control Soil without Solvents	Soil with Solvents	Control Soil without Solvent	Soil with Solvents
5/17/94	5/20/94	1	1	3	1	3
5/17/94	5/22/94	1	1	3	1	3
5/17/94	5/26/94	1	1	3	1	3
5/17/94	6/4/94	1	1	3	1	3
5/17/94	6/10/94	1	1	3	1	3
5/17/94	6/23/94	1	1	3	1	3
5/17/94	7/7/94	1	1	3	1	3
5/17/94	7/27/94	1	1	3	1	3

Note: Solvent mixture added to each soil sample created concentrations of PCE (25ppm), TCE (25 ppm), carbon tetrachloride (25 ppm), methylene chloride (25 ppm), 1,1,1-TCA (12.5 ppm), and 1,1,2-TCA (12.5 ppm).

A 75  $\mu$ l addition of solvent mixture to each 1.5 g soil sample vial resulted in the following concentrations: PCE (25 ppm), TCE (25 ppm), carbon tetrachloride (25 ppm), methylene chloride (25 ppm), 1,1,1-TCA (12.5 ppm), and 1,1,2-TCA (12.5 ppm). All test solvents purchased from Supelco<sup>®</sup> were certified solvent standard quality at 99% or greater purity.

Eight analysis events occurred over a two month period for both Test 1 and Test 2 (Table 1). For chlorinated solvent analyses, entire 1.5 g soil samples were sacrificed in triplicate. Analysis was performed for chlorinated solvents using a purge-and-trap system and a Varian Star 3400 CX Saturn 3 gas chromatograph/mass spectrometer (GC/MS). A modified EPA method 8260 was followed as described in volume IB of SW-846, *Test Methods for Analyzing Solid Waste* (EPA 1986). Controls, blanks, external, and internal standards were analyzed with every set of samples.

The external standard consisted of 1.5 g soil samples in the purge-and-trap vials. The same solvent concentrations of PCE (25 ppm), TCE (25 ppm), carbon tetrachloride (25 ppm), methylene chloride (25 ppm), 1,1,1-TCA (12.5 ppm), and 1,1,2-TCA (12.5 ppm) in a methanol carrier were spiked on one soil sample prior to each analysis event. The standard was also analyzed using the GC/MS method.

The control also consisted of a 1.5 g soil sample in the purge-and-trap vials, with no solvent added. The purpose of the control was to determine what solvent background levels appeared due to unknown soil contamination or laboratory contamination.

### 2.3 Phase II - Biotic Study

The FY-94 portion of Phase II included 1) preparing separate enrichment cultures for methanotrophic, denitrifying, sulfate reducing, and methanogenic bacteria in the Site soil and subculturing the populations to maintain adequate growth, and 2) monitoring the growth of the populations with respect to time and establishing a growth curve for each population.

Tasks 1 and 2 dealt with establishing adequate microbial populations for the biotransformation experiments. Enrichment culturing is a microbiological technique used to increase the size of a particular microbial population in a culture by using medium and growth conditions highly selective for that particular microbe (Brock et al. 1984). Growth media added to the soil to stimulate biological activity contained nutrients and energy sources for the microbes.

### 2.3.1 Phase II - Task 1 Enrichment Culturing

Quart-sized canning jars were equipped with two valve inlets in the jar lids to allow the introduction and removal of liquids and gases from the cultures. One hundred fifty grams of clean OU 2 soil were added to each of four culture jars. Media described in Table 2 was prepared for methanotrophs, denitrifiers, sulfate reducers, and methanogens and 150 ml were added to the respective culture jars. The pH of the soil and media mixture was adjusted to achieve the pH requirements of each media shown in Table 2. Soil, culture jars, and media were sterilized in an autoclave at 121° C and 21 psi for 45 minutes on three consecutive days.

For the anaerobic population cultures, media addition and inoculation of the soil cultures were carried out in a glove-bag filled with an argon atmosphere to maintain oxygen-free conditions. For the aerobic methanotrophic culture, media addition and inoculation were carried out under atmospheric conditions.

For the first cultures prepared (first generation), sterile soil was inoculated with 50 grams of IHSS 110 soil utilized as a microbial seed. For second and third generation cultures (subcultures), sterile soil was inoculated with 50 grams of soil taken from the previous generation of cultures. Anaerobic indicator strips were placed in each culture. The cultures were then sealed.

The aerobic methanotrophic culture was then purged with 10 flask volumes of a mixture of 2.5% methane in air to provide methane for growth. The anaerobic cultures were purged with

Table 2. Nutrient media used for enrichment culturing.

Organism	Chemical	Quantity per liter	Final pH at 25°C
Methanotrophs	NH <sub>4</sub> Cl	0.290 g	6.8 to 7.2
	KH <sub>2</sub> PO <sub>4</sub>	0.040 g	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.150 g	
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.0250 g	
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.60 mg	
	CH <sub>4</sub>	2.5% Volume in Headspace	
	O <sub>2</sub>	20.4% Volume in Headspace	
Denitrifiers	NH <sub>4</sub> Cl	0.290 g	6.8 to 7.2
	KH <sub>2</sub> PO <sub>4</sub>	0.040 g	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.150 g	
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.0250 g	
	FeSO <sub>4</sub> ·7H <sub>2</sub> O	0.60 mg	
	KNO <sub>3</sub>	4.040 g	
	Sodium Acetate	5.67 g	
	Sodium Lactate	5 ml of 60% solution	
Sulfate Reducers	NH <sub>4</sub> Cl	0.290 g	7.2 to 7.5
	KH <sub>2</sub> PO <sub>4</sub>	0.040 g	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.150 g	
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.0250 g	
	Ascorbic Acid	0.100 g	
	Na <sub>2</sub> SO <sub>4</sub> ·7H <sub>2</sub> O	2.680 g	
	Sodium Acetate	5.67 g	
	Sodium Lactate	5 ml of 60% solution	
Methanogens	NH <sub>4</sub> Cl	0.290 g	6.8 to 7.2
	KH <sub>2</sub> PO <sub>4</sub>	0.040 g	
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.150 g	
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.0250 g	
	Sodium Acetate	5.67 g	
	Sodium Lactate	5 ml of 60% solution	

10 flask volumes of argon to remove any traces of oxygen. Replacing the jars' headspace with an inert atmosphere created the most oxygen-free environment possible in the jars. The cultures were incubated in the dark at 25° C.

For the second generation cultures, 4 ppm of TCE was added after three days of incubation via the lid valves. This provided an environment in which the developing microbes could adapt to the presence of a chlorinated solvent.

### 2.3.2 Phase II - Task 2 Growth Curve Preparation

Headspace gases in the quart jars were analyzed for the first of generation cultures over a period of 2 months. During this time, the mass spectrometer used for gas analysis experienced plugging at the gas inlet due to condensed water and only limited data could be gathered (3 headspace analyses). The populations were then subcultured into fresh media following the culturing procedure described above using 50 grams of the previous culture as the microbial seed. Headspace gases in second generation cultures were monitored to determine the growth rate of each population for 36 days. Cultures were grown and maintained in the dark at 25° C. Headspace samples were removed from the cultures via the lid valves for gas analysis approximately every day or every other day (16 to 18 samples analyzed for each culture). Headspace gases were analyzed using mass spectrometry with an improved inlet system to determine:

- 1) CH<sub>4</sub> removal from the headspace of the methanotrophic culture;
- 2) N<sub>2</sub> generation in the denitrification culture;
- 3) CH<sub>4</sub> generation in the methanogenic culture; and
- 4) CO<sub>2</sub> generation in all of the cultures.

The plot of the natural log-millimoles-headspace gas produced with respect to time showed the growth of the populations.

#### 2.4 Deviations From the Work Plan

The methods described in this section deviate from those described in the Bioremediation Treatability Study Work Plan (1994) in several minor instances.

The Work Plan stated that the Abiotic Study would be carried out over an estimated 64-day duration. The actual Abiotic Study lasted 71 days. Review of data from the early sampling events during experimentation revealed that solvent concentrations were not changing quickly in soil samples so additional time was added to the study.

For Phase II, the Work Plan stated that headspace gas analyses would be carried out once a week until detection of key headspace gases occurred. At that time, subculturing would occur followed by sampling at intervals such as 2 hours, 4 hours, 8 hours, etc. The Work Plan also stated that subculturing would be carried out every two weeks once growth occurred. These estimates were based on the extremely quick growth rates of heterotrophic bacteria grown in pure culture liquid media. These subculturing schedules and analysis schedules were not practical since these were relatively slow growing organisms. Subculturing was carried out only when headspace analysis indicated that microbial activity was declining in the culture. Also, headspace analysis required approximately 4 hours for each sampling event so only one sampling event was scheduled per day. Methods to shorten headspace analysis times are being considered for future studies.

### 3.0 RESULTS AND DISCUSSION

#### 3.1 Phase I - Abiotic/Unamended Soil Study

Phase I provided data which revealed whether unamended OU 2 soil transformed PCE, TCE, carbon tetrachloride, methylene chloride, 1,1,1-TCA, and 1,1,2-TCA; and, if transformed, what the resulting degradation products were. This information will be used for further solvent transformation comparisons in biologically active soil studies in Phase II.

For the sterile soil of Test 1, soil collected just outside of IHSS 110 was air-dried and sterilized in an autoclave at 121° C for 45 minutes, cooled, and spiked with the solvent mixture as described in the Materials and Methods Section. For the nonsterile soil of Test 2, the same type of soil was air-dried with no autoclaving and spiked with the solvent mixture. Test 1 and Test 2 each included solvent spiked triplicate samples for each analysis event (32 samples for each test) (Table 1). Preparation and spiking of all samples occurred 5/17/94 with weekly or biweekly analyses continuing over 71 days.

One control, containing only soil with no added solvents, was prepared for each analysis event (8 sterile and 8 nonsterile controls). Controls were maintained in the same environment and conditions as all the samples. Solvent concentrations in the controls illustrate background solvent concentrations already existing in the OU 2 soil samples or laboratory contamination. Prior to analysis, a solvent standard was prepared on nonsterile OU 2 soil and analyzed to illustrate GC/MS accuracy (8 standards).

In summary, for every analysis date shown on Table 3, one standard, one sterile control, three sterile solvent-spiked samples, one nonsterile control, and three nonsterile solvent-spiked samples were analyzed.

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Table 3. Solvent concentrations for the standard, sterile soil and nonsterile soil samples as measured by a Varian Star 3400CX Saturn 3 GC/MS equipped with a purge-and-trap system.

Solvent	Analysis Date	Standard Nonsterile Sol with Solvent	Sterile Soil with Solvent			Nonsterile Soil with Solvent		
			Control without Solvent	Triplicate Mean	Triplicate Std. Dev.	Control without Solvent	Triplicate Mean	Triplicate Std. Dev.
Tetrachloroethylene	5/20/94	38.695	0.630	21.391	9.081	0.503	15.857	4.682
Tetrachloroethylene	5/22/94	(1)	0.063	14.860	5.136	0.011	18.126	1.524
Tetrachloroethylene	5/26/94	29.183	0.067	13.572	4.367	0.009	16.257	0.544
Tetrachloroethylene	8/4/94	28.358	0.092	11.439	3.849	0.011	11.663	0.200
Tetrachloroethylene	8/10/94	38.057	0.018	10.128	3.221	0.008	12.180	1.045
Tetrachloroethylene	8/23/94	14.878	0.008	2.001	0.251	0.010	2.047	0.170
Tetrachloroethylene	7/7/94	21.130	0.008	0.538	0.530	0.003	0.885	0.075
Tetrachloroethylene	7/27/94	26.504	0.010	0.433	0.102	0.004	0.255	0.242
Trichloroethylene	5/20/94	29.125	0.037	18.234	4.314	0.011	28.611	3.364
Trichloroethylene	5/22/94	(1)	0.033	13.990	6.792	0.010	18.865	2.864
Trichloroethylene	5/26/94	23.584	0.035	11.078	4.766	0.006	14.433	1.044
Trichloroethylene	8/4/94	32.753	0.052	8.570	3.948	0.010	9.538	0.263
Trichloroethylene	8/10/94	70.768	0.015	6.537	2.592	0.006	8.429	0.708
Trichloroethylene	8/23/94	28.315	0.009	1.538	0.260	0.008	1.620	0.152
Trichloroethylene	7/7/94	8.054	0.018	0.310	0.394	0.003	0.533	0.056
Trichloroethylene	7/27/94	25.365	0.011	0.185	0.075	0.004	0.099	0.125
Carbon Tetrachloride	5/20/94	27.874	0.017	18.328	6.727	0.007	27.388	0.932
Carbon Tetrachloride	5/22/94	(1)	0.013	12.452	6.575	0.005	17.855	5.199
Carbon Tetrachloride	5/26/94	18.879	0.015	15.695	8.014	0.004	19.158	2.248
Carbon Tetrachloride	8/4/94	24.968	0.022	18.802	9.464	0.005	22.909	1.743
Carbon Tetrachloride	8/10/94	21.541	0.009	24.813	13.461	0.003	37.996	6.155
Carbon Tetrachloride	8/23/94	7.341	0.004	7.877	1.370	0.002	10.175	0.653
Carbon Tetrachloride	7/7/94	26.901	0.006	4.983	6.941	0.001	7.082	1.168
Carbon Tetrachloride	7/27/94	26.522	0.004	4.416	2.977	0.002	2.224	3.113
Methylene chloride	5/20/94	30.230	0.085	18.580	2.321	0.013	24.312	2.247
Methylene chloride	5/22/94	(1)	1.595	18.970	11.712	0.377	7.468	3.546
Methylene chloride	5/26/94	43.103	0.523	5.746	2.476	0.364	7.685	3.277
Methylene chloride	8/4/94	25.291	0.538	6.490	3.262	0.465	7.178	0.544
Methylene chloride	8/10/94	94.740	0.289	21.289	13.494	0.256	29.251	2.227
Methylene chloride	8/23/94	8.087	0.026	3.085	0.312	0.150	2.717	0.241
Methylene chloride	7/7/94	56.898	0.292	1.058	1.235	0.213	1.928	0.399
Methylene chloride	7/27/94	10.538	0.014	1.122	0.601	0.200	0.677	0.705
1,1,1-trichloroethane	5/20/94	17.069	0.009	9.632	3.289	0.004	15.233	0.924
1,1,1-trichloroethane	5/22/94	(1)	0.007	7.280	3.890	0.003	6.334	4.641
1,1,1-trichloroethane	5/26/94	11.004	0.011	10.221	5.379	0.003	12.730	1.552
1,1,1-trichloroethane	8/4/94	10.848	0.015	11.589	5.966	0.004	14.765	0.845
1,1,1-trichloroethane	8/10/94	12.642	0.005	11.596	5.928	0.002	19.200	3.060
1,1,1-trichloroethane	8/23/94	7.419	0.005	7.798	1.176	0.003	10.212	0.323
1,1,1-trichloroethane	7/7/94	12.900	0.009	4.267	5.883	0.002	8.285	1.659
1,1,1-trichloroethane	7/27/94	16.284	0.006	4.974	2.839	0.002	3.289	4.571
1,1,2-trichloroethane	5/20/94	13.236	0.030	11.295	0.506	0.004	14.039	1.359
1,1,2-trichloroethane	5/22/94	(1)	0.031	10.258	1.776	0.006	12.037	0.303
1,1,2-trichloroethane	5/26/94	12.622	0.034	10.194	1.726	0.002	11.971	0.220
1,1,2-trichloroethane	8/4/94	14.868	0.045	10.597	1.699	0.004	12.571	0.203
1,1,2-trichloroethane	8/10/94	13.466	0.007	12.431	2.894	0.002	18.169	0.676
1,1,2-trichloroethane	8/23/94	5.801	0.017	3.160	0.211	0.011	4.373	0.205
1,1,2-trichloroethane	7/7/94	6.081	0.043	3.341	0.389	0.003	3.548	0.385
1,1,2-trichloroethane	7/27/94	6.001	0.017	1.631	0.540	0.003	3.058	0.449

Note : (1) Analysis of the Standard on 5/22/94 was not possible due to a purge-and-trap automation problem.

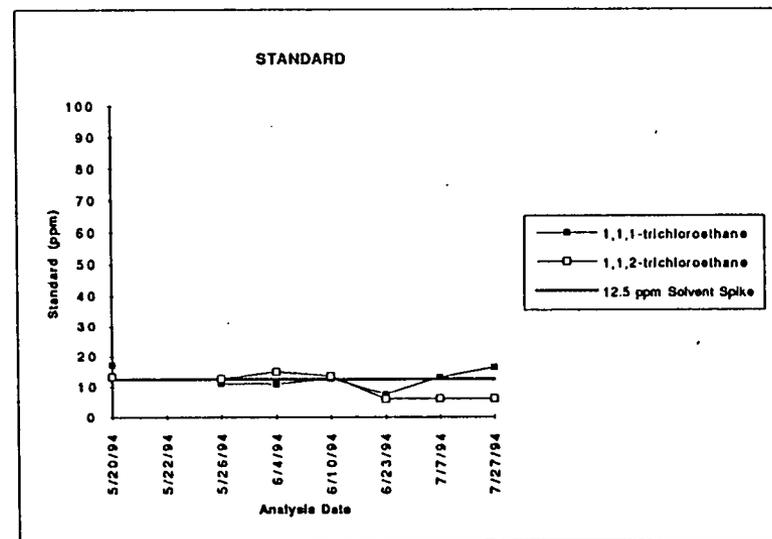
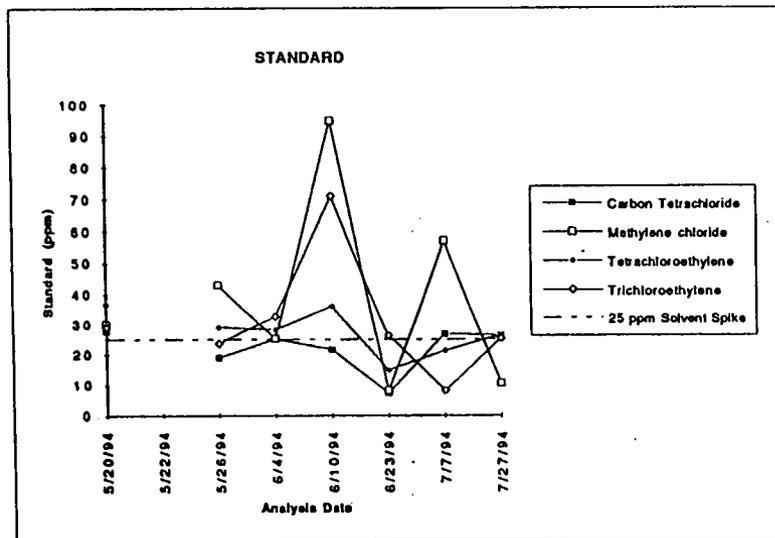
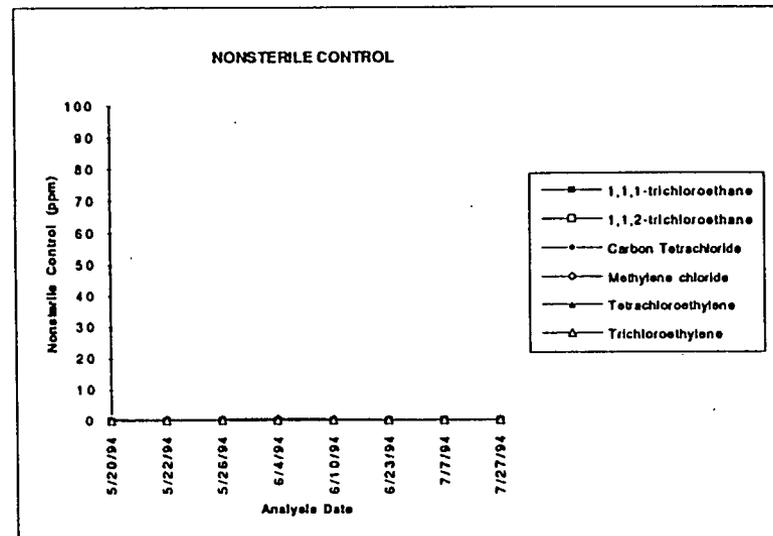
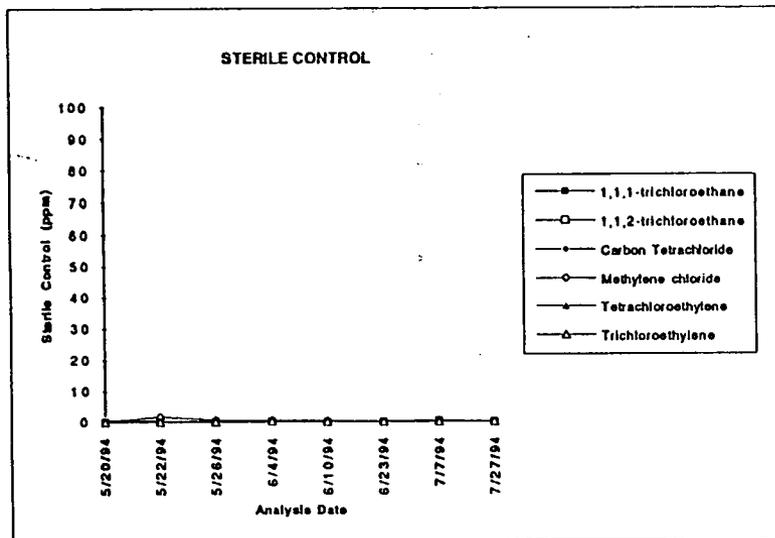
### 3.1.1 Soil Control

Over the 71-day duration of the test, a total of eight sterile controls and eight nonsterile controls were analyzed. Background solvent concentrations measured in the soil controls were minimal (Table 3). Concentrations for five of the solvents ranged from 0.004 to 0.630 ppm while methylene chloride ranged from 0.014 to 1.595 ppm. The low levels of methylene chloride and other solvents seen in the controls in which no solvents were added were most likely caused by the presence of these solvents in the laboratory in which the samples were being tested. These low solvent concentrations existing in the OU 2 soil did not interfere with the 12.5 and 25 ppm solvents tested on the sterile and nonsterile soils (Figure 2).

### 3.1.2 Solvent Standards

One standard was prepared prior to each analysis event by injecting carbon tetrachloride, methylene chloride, PCE, and TCE resulting in 25 ppm each, and 1,1,1- and 1,1,2-TCA resulting in 12.5 ppm each into a purge-and-trap vial containing 1.5 g of nonsterile soil. The standard data points were rarely measured at the respective 25 or 12.5 ppm concentrations at which they were injected. Assuming a maximum instrument error of 25%, 43% of the standard data points were outside of the instrument error interval. Hence, these outliers could not be explained simply as instrument error. Alternatively, solvent concentrations spiked into the sterile and nonsterile soil samples in triplicate showed good precision over the eight analysis events. If the standard fluctuations were caused by instrument error, sterile and nonsterile soil sample data would fluctuate similarly, which is not the case (Figure 3).

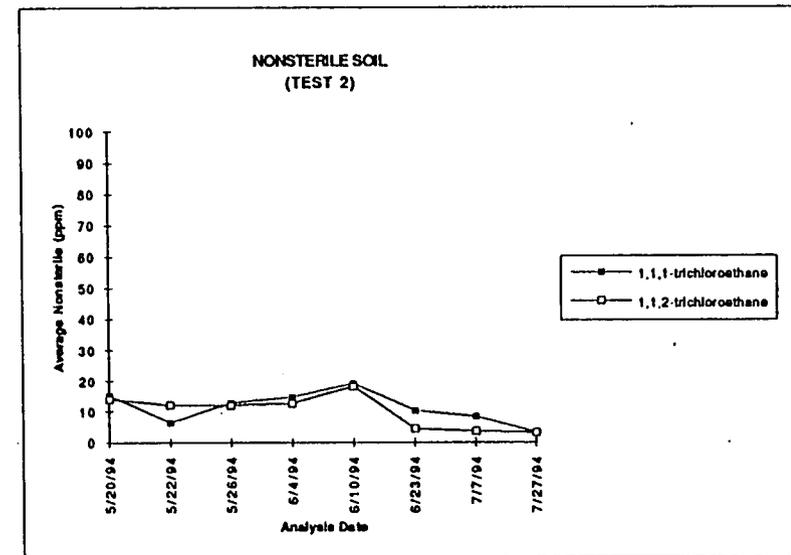
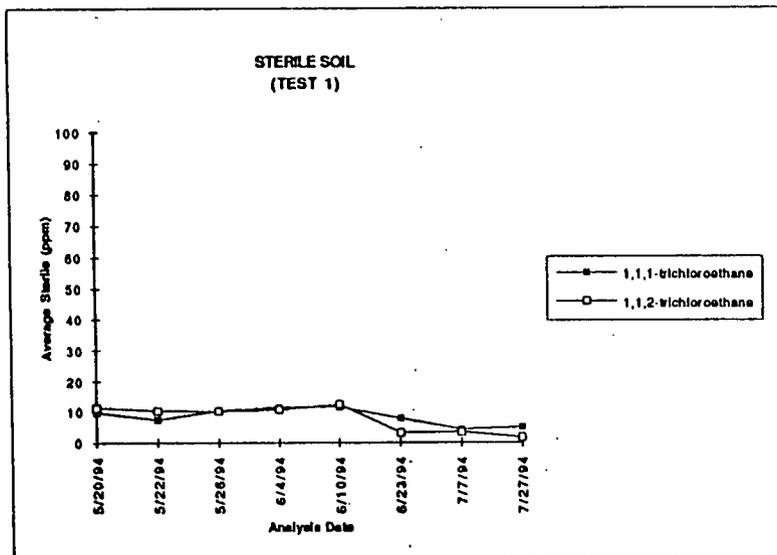
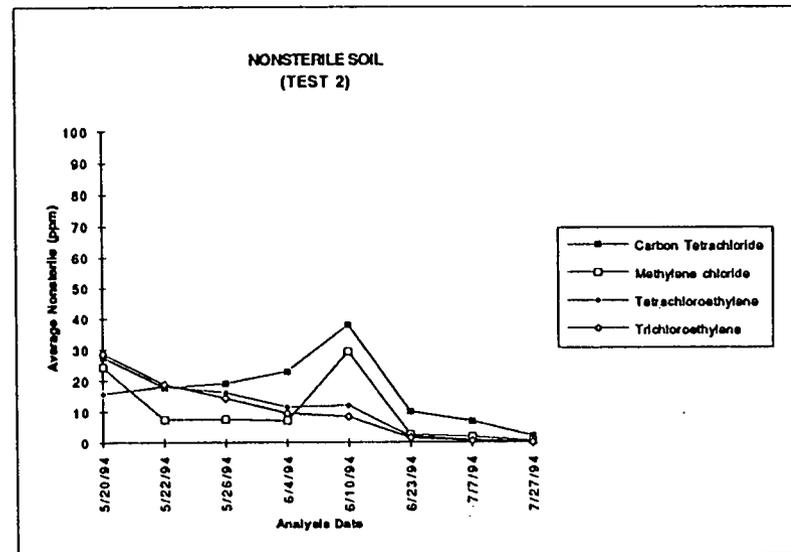
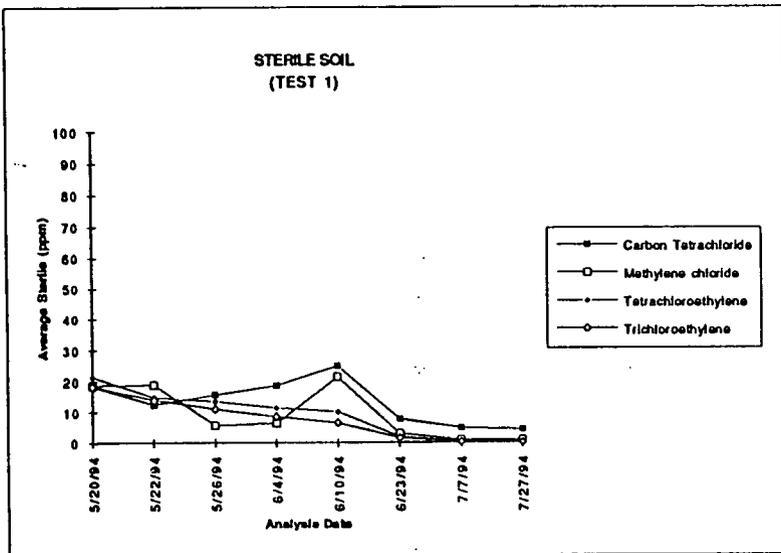
One theory for the inconsistencies in the data for the standards includes the potential effect of using a soil matrix for standard preparation, solvent injection onto the matrix, and immediate solvent analysis prior to the solvent equilibrating in the soil. Though it was assumed that all of



- Notes: (1) The Sterile Control tests background levels of the 8 solvents in soil collected outside of OU2 IHSS 110.  
 (2) The Standard tests 25 ppm (CT, MC, TCE, and PCE) and 12.5 ppm (1,1,1- and 1,1,2-TCA) injections into Nonsterile Soil samples collected outside of OU2 IHSS 110.  
 (3) Analysis by purge-and-trap system and Varian Star 3400 CX Saturn 3 GC/MS.

Figure 2. Sterile control, nonsterile control, and standard solvent concentrations in samples from May 20 to July 27, 1994.

9c



- Notes: (1) The instrument error based on calibration curve EPA method SW-846 method 8260 is within 25% of the illustrated value.  
 (2) CT, MC, PCE, and TCE are illustrated separately from 1,1,1- and 1,1,2-TCA for clarity.  
 (3) Analysis by purge-and-trap system and Varian Star 3400 CX Saturn 3 GC/MS.

Figure 3. Sterile soil and nonsterile soil solvent concentrations in samples from May 20 to July 27, 1994.

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the solvents would be effectively volatilized by the helium purge gas, soil heterogeneity may have prevented complete removal. Water may be a more acceptable matrix than soil for future standards of this type since dispersion of solvents in water is more rapid.

A second theory is that the test solvents used for the standards were taken from the same two vendor vials which were opened repeatedly over the 71-day test. Once a vial is open, the solvents may volatilize and be lost at different rates, changing their concentration ratios, and affecting the concentration pipetted. To eliminate this problem in future studies, new solvent vials will be used each time a new standard is prepared.

Though the error in the solvent standards is a concern, other calibration tests were performed on the GC/MS daily to ensure accuracy. First, a bromofluorobenzene (BFB) standard was run every eight hours of GC/MS operation to ensure the mass spectrometer was operating correctly. The instrument passed all BFB tests prior to analysis for this study specified in the EPA national standard criteria (SW-846 Method 8260, EPA 1986). This ensures that the measurements taken from this instrument match other instruments across the nation.

Second, three internal standards (bromochloromethane, 1,4-difluorobenzene, and chlorobenzene-d5) were added at 1 ppm to each vial analyzed and were used to quantify solvent concentrations in the samples.

Third, a calibration curve made up of five dilutions was created for each of the test solvents prior to this study. The internal standard was added to each of the five calibration curve dilutions. The calibration curves for each of the six solvents had linear regressions with fits greater than 0.995 (with 1.000 being a perfect fit).

Based on the results of the BFB tests, the use of three internal standards for each sample and control, and the favorable linear response of the calibration curve standards, the inaccuracy of the solvent/soil standards was apparently due to the preparation process for the standards and

not instrument error. Therefore, the GC/MS results presented for Phase I sterile and nonsterile soil samples are of acceptable accuracy. When it is necessary to use a similar solvent/soil standard in the future, the preparation will be reexamined.

### 3.1.3 Solvent Tests on Sterile Soil versus Nonsterile Soil

Twenty-four sterile (abiotic) and 24 nonsterile (unamended) soil samples from OU 2 were spiked with a mixture of 6 solvents on 5/17/94 and placed in a dark incubator at 25° C until analysis. For each analysis date shown on Table 3, three solvent-spiked sterile soil samples and three solvent-spiked nonsterile soil samples were sacrificed for analysis.

Table 3 shows the average value of each solvent based on triplicate samples analyzed in sterile and nonsterile samples for each analysis event. For example on analysis date 5/20/94, the solvent-spiked sterile soil sample had average values of 21.39 ppm PCE, 18.23 ppm TCE, 18.33 ppm carbon tetrachloride, 18.59 ppm methylene chloride, 9.63 ppm 1,1,1-TCA, and 11.30 ppm 1,1,2-TCA.

Figure 3 illustrates solvent concentrations for the sterile soil and nonsterile soil samples spiked with carbon tetrachloride, methylene chloride, PCE, and TCE (25 ppm each) illustrated separately from the 1,1,1- and 1,1,2-TCA (12.5 ppm each) for clarity though all six solvents were added to the soil as a mixture. Solvent concentrations in the sterile and non-sterile soil purge-and-trap vials diminished over the 71-day test. Both sterile and nonsterile soils followed similarly decreasing trends from the original concentration down to less than 5 ppm for each solvent.

All the solvents decreased in concentration over the 71-day test in both the sterile and nonsterile soils, except for the analysis on 6/10/94. The data from 6/10/94 is not consistent with the data prior to or after this date and is suspect.

A paired t-Test defined the relationship between solvent concentration changes of the sterilized versus non-sterilized soil samples. This tests whether the means of two sample sets are the same, so it compares the average of each sample set. Each solvent concentration in the sterilized soil was compared to that solvent concentration in the non-sterilized soil (Table 4).

Of the six solvents tested, the paired t-Test showed there was no difference between the concentration of PCE and methylene chloride in sterile and nonsterile soil samples for the entire experiment. This indicates that these two solvents were lost to the same extent in sterile and nonsterile samples.

However, the paired t-Test showed that there was a difference in concentrations of TCE, 1,1,1-TCA, 1,1,2-TCA, and carbon tetrachloride in sterile and nonsterile soil samples. The unsterilized soil vials had higher concentrations of these solvents than the sterile soil vials. Hence, the sterile sample vials lost more solvent than unsterile sample vials. First, if the decrease of the solvents is due to physical loss from the sample vials, sterilization of soil and sample vials may have reduced the integrity of the vials resulting in greater solvent loss. Sterilization occurs at 121° C and 21 psi, which could affect the vial septa and cap. Second, soil sterilization may have modified soil absorbance properties, resulting in more solvent loss from the sterile soil than the nonsterile soil.

Though the concentration of the solvents decreased over the duration of the test, no volatile daughter products were detected in any sample. Abiotic dechlorination of solvents has been reported (Lyman et al. 1990, Howard 1990); however, the degradation products of dechlorination are volatile and are detectable by the GC/MS. There is the possibility that nonvolatile degradation products were formed and were not detected by GC/MS. However, the fact that all the solvents tended to decline consistently and at similar rates over the 71 days

Table 4. Paired t-Test of solvent concentration changes for Sterile and Nonsterile soil samples.

**Tetrachloroethylene Sterile with Nonsterile Soil Paired t-Test**

	<i>Sterile</i>	<i>Nonsterile</i>
Mean	9.2950	9.6587
Variance	58.4761	55.3393
Observations	8	8
H1: Difference = 0		
H2: Less solvent in Sterile than Nonsterile		
df	7	
t	-0.3779	
P	0.3584	
T one-tail table value	1.8946	
Is $p < 0.05$ ?		
0.3584 < 0.05	No	No Difference

**1,1,1-Trichloroethane Sterile with Nonsterile Soil Paired t-Test**

	<i>Sterile</i>	<i>Nonsterile</i>
Mean	8.4198	11.2560
Variance	7.9495	27.2884
Observations	8	8
H1: Difference = 0		
H2: Less solvent in Sterile than Nonsterile		
df	7	
t	-2.5945	
P	0.0179	
T one-tail table value	1.8946	
Is $p < 0.05$ ?		
0.0179 < 0.05	Yes	Accept H2

**Methylene Chloride Sterile with Nonsterile Soil Paired t-Test**

	<i>Sterile</i>	<i>Nonsterile</i>
Mean	9.5437	10.1519
Variance	73.8637	114.3045
Observations	8	8
H1: Difference = 0		
H2: Less solvent in Sterile than Nonsterile		
df	7	
t	-0.2996	
P	0.3866	
T one-tail table value	1.8946	
Is $p < 0.05$ ?		
0.3866 < 0.05	No	No Difference

**Carbon Tetrachloride Sterile with Nonsterile Soil Paired t-Test**

	<i>Sterile</i>	<i>Nonsterile</i>
Mean	13.3957	18.0983
Variance	52.8302	134.6624
Observations	8	8
H1: Difference = 0		
H2: Less solvent in Sterile than Nonsterile		
df	7	
t	-2.8433	
P	0.0125	
T one-tail table value	1.8946	
Is $p < 0.05$ ?		
0.0125 < 0.05	Yes	Accept H2

**Trichloroethylene Sterile with Nonsterile Soil Paired t-Test**

	<i>Sterile</i>	<i>Nonsterile</i>
Mean	7.5549	10.2659
Variance	44.7022	100.6569
Observations	8	8
H1: Difference = 0		
H2: Less solvent in Sterile than Nonsterile		
df	7	
t	-2.1564	
P	0.0340	
T one-tail table value	1.8946	
Is $p < 0.05$ ?		
0.0340 < 0.05	Yes	Accept H2

**1,1,2-Trichloroethane Sterile with Nonsterile Soil Paired t-Test**

	<i>Sterile</i>	<i>Nonsterile</i>
Mean	7.8633	9.9705
Variance	18.9562	31.3126
Observations	8	8
H1: Difference = 0		
H2: Less solvent in Sterile than Nonsterile		
df	7	
t	-3.6464	
P(T<=t) one-tail (Calculated T)	0.0041	
t Critical one-tail (Table Value)	1.8946	
Is $p < 0.05$ ?		
0.0041 < 0.05	Yes	Accept H2

suggests that the solvents were being lost from the sample vials. If chemical reactions were occurring which were changing the concentration of the solvents, it would be expected that these reactions would have different rates for some of the solvents and may not have occurred at all for others.

The most likely explanation for solvent concentration decline in the vials over time is the migration of the solvents through the sample vials' teflon septa or through the vial cap. The teflon septum was penetrated with a gas-tight syringe when solvents were added, and may have allowed for solvent volatilization through the remaining hole over 71 days.

Vapor pressures of the solvents range from 40.7° C to 120.8° C at 760 mm of mercury. The most volatile is methylene chloride and the least volatile PCE, but vapor pressures of the six solvents do not trend with the decreasing concentrations in the sterile or nonsterile soil samples.

In summary, the unamended OU 2 soil did not transform the six test solvents, and vial/soil autoclaving as a sterilization method requires further evaluation.

### 3.2 Phase II - Growth of Microorganisms

The goal of the enrichment portion of Phase II was to determine whether four microbial populations (one aerobe and three anaerobes) which have shown potential for biotransformation of chlorinated solvents in literature could be cultured from OU 2 surface soil.

The first generation cultures were grown from an OU 2 soil inocula. The second generation cultures were inoculated with first generation culture material. A third generation was inoculated with culture material from the second generation cultures; however, this culturing experiment is still underway so data will not be presented at this time.

This report provides data collected from second generation cultures inoculated on 8/15/94 and monitored through 9/20/94 (36 days of growth). During first generation culture growth, the mass spectrometer inlet used for gas analysis experienced plugging due to water condensation so data could not be consistently collected. For subsequent generations, the inlet system was improved to eliminate plugging.

To prepare a growth curve, the natural log of some measurable product of microbial growth is plotted versus time. The microbial products which may be quantified for this purpose include cell bodies (direct counts), biomass, carbon dioxide generation, utilization of a substrate, generation of a byproduct, and many others. Because this study was conducted in soil with a mixed culture, products such as direct cell counts and biomass could not be used to measure growth due to interference of the soil matrix and competing populations. For this study, a byproduct of microbial activity unique to the microbial population of interest and unencumbered by the soil matrix had to be quantified to follow growth. Production or utilization of key headspace gases by methanotrophs, denitrifiers, sulfate reducers, and methanogens was chosen to quantify microbial growth for this study. The gases of interest for each population are discussed in the sections below.

The headspace data is presented in two forms for each population. First, the cumulative millimoles of each relevant headspace gas for each culture are plotted versus time to show how the gases increased or decreased in relation to one another within the culture headspace. It is important to note that the aerobic culture began with a headspace similar to ambient air plus 2.5% CH<sub>4</sub> (volume %). The moles of headspace gases produced by this culture were compared to the moles of gases in ambient air containing 2.5% CH<sub>4</sub>. The anaerobic cultures began with a headspace containing only argon and trace amounts of O<sub>2</sub>.

The second way in which the data is presented is a plot of the natural log of the key gas (specifically generated or used by the population of interest) versus time to show the shape and phases of growth. When plotting a logarithmic growth curve for a batch culture, three distinct

phases of growth are expected (Figure 4). The first is lag phase which occurs during the time in which the microbial population is acclimating to the new culture environment and growth is slow or nonexistent. Secondly, once the population has acclimated, the lag phase transitions into the exponential phase. During the exponential phase, microbial population growth is the most active. Finally, stationary phase or death phase occurs when some component of the culture environment such as an energy source, electron acceptor, or space becomes limiting and growth slows or stops. Such a growth curve was prepared for each of the four populations cultured in this study.

### 3.2.1 Aerobic/Methanotrophic Culture

Methanotrophs are aerobes which use  $\text{CH}_4$  as an energy source (electron donor) resulting in the oxidation of  $\text{CH}_4$  to  $\text{CO}_2$  and the reduction of  $\text{O}_2$  (electron acceptor) (Burrows et al. 1984, Fogel et al. 1986, Janssen et al. 1988, Little et al. 1988, Norris et al. 1994, Oldenhuis et al. 1989). An increase in  $\text{CO}_2$ , a decrease in  $\text{O}_2$ , and a decrease in  $\text{CH}_4$  in the headspace of a culture indicates that aerobic methanotrophic metabolism is occurring.

It appears that near the end of the second generation culturing experiment, methanotrophic bacteria were enriched from OU 2 soil. Though growth was monitored for 36 days, methane removal was not apparent until 25 days into the experiment. This limited the amount of data gathered on the methanotrophs to eleven days prior to discontinuation of the experiment.

Figures 5a, 5b, and 5c show the moles of  $\text{CO}_2$ ,  $\text{O}_2$ , and  $\text{CH}_4$  in the headspace of the methanotrophic culture over 36 days. Figure 5a shows that over time, the  $\text{CO}_2$  concentration increased to levels which were approximately four orders of magnitude greater than the concentration expected in air ( $10^{-2}$  moles in the culture versus  $10^{-6}$  moles in air at pressure=630 Torr, temp=25° C, headspace volume=0.7415 liters). This increase in  $\text{CO}_2$  indicates that biological metabolism was occurring in the culture. The culture had to be purged with the 2.5%  $\text{CH}_4$  in air mixture

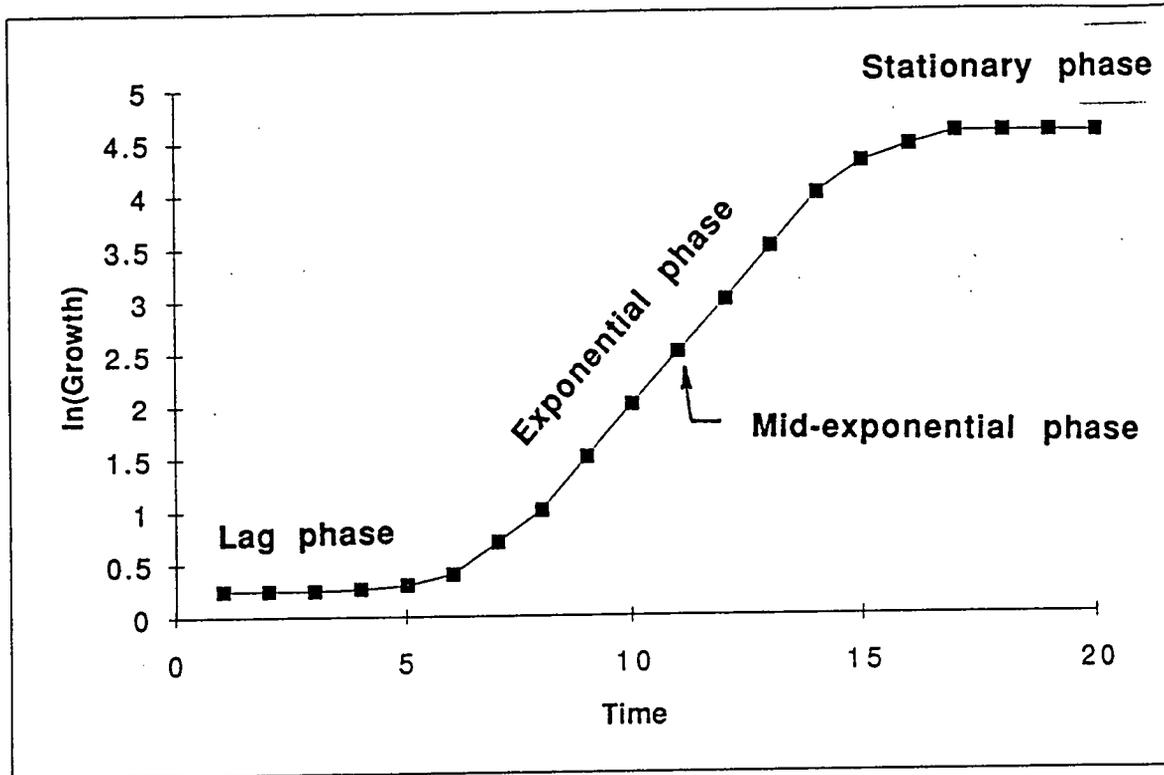
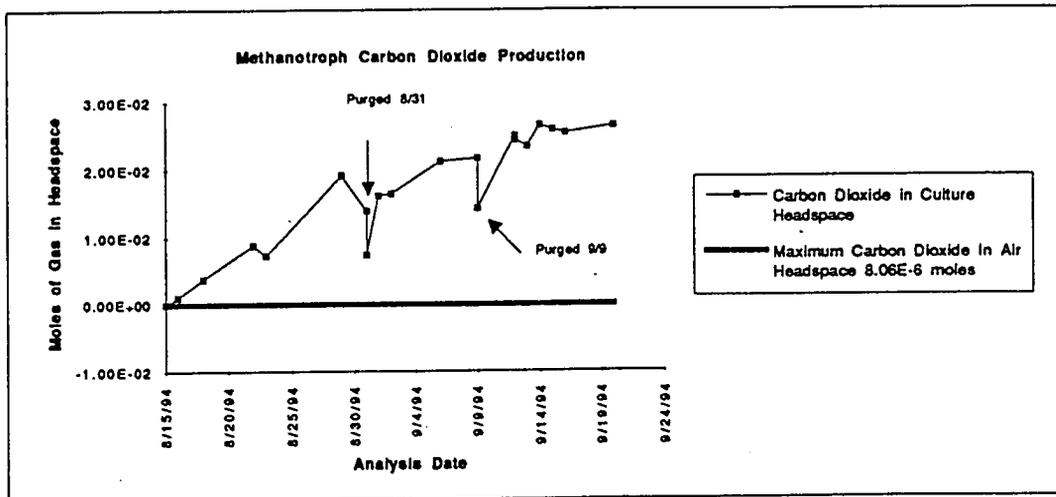
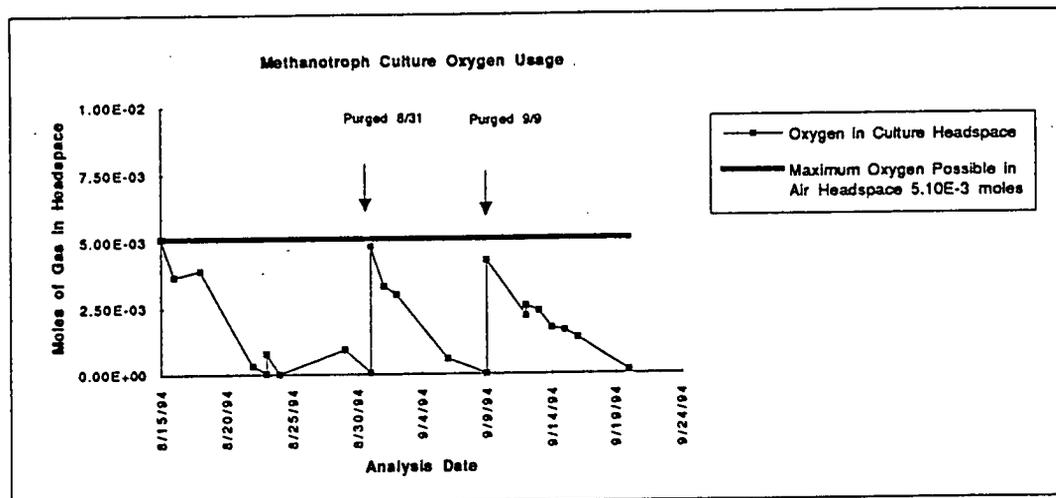


Figure 4. Idealized growth curve for microorganisms.

a)



b)



c)

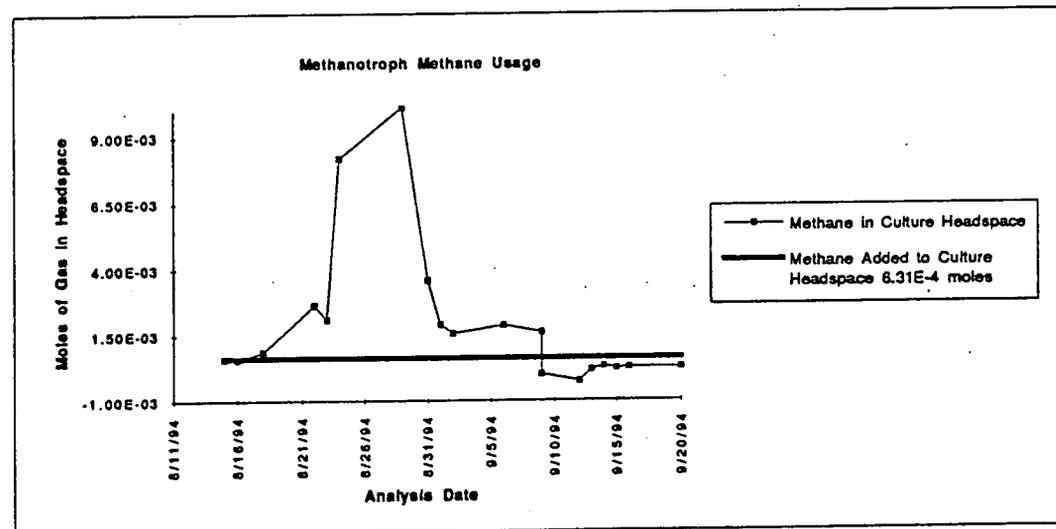


Figure 5 a, b, and c: a) moles of carbon dioxide in methanotroph headspace compared to moles of carbon dioxide in a headspace filled with air; b) moles of oxygen in methanotroph headspace compared to moles of oxygen in a headspace filled with air; c) moles of methane in methanotroph headspace compared to moles of methane added to this headspace on 8/15/94 and during purging with 2.5% methane in air gas mixture.

several times over the month of study to resupply O<sub>2</sub> to the organisms. The purging reduced the amount of CO<sub>2</sub> in the headspace as seen in the data from 8/31/94 and 9/9/94. The cultures quickly regained the previous CO<sub>2</sub> levels within days after purging.

Figure 5b shows the moles of O<sub>2</sub> present in the culture headspace over time versus the amount expected in ambient air containing 2.5% CH<sub>4</sub> (pressure=630 Torr, temp=25° C, headspace volume=0.7415 liters). By 8/23/94 (day 8 of growth), the methanotrophic culture had decreased the O<sub>2</sub> content of the headspace to anaerobic conditions. From the period of 8/22/94 to 9/1/94 the culture was essentially anaerobic. The culture was purged with the 2.5% CH<sub>4</sub> in air gas mix to replenish the O<sub>2</sub>. Purging was successful at increasing the O<sub>2</sub> content of the headspace to near ambient O<sub>2</sub> levels. Again, after each purge the O<sub>2</sub> was quickly removed by the growing cultures.

Figure 5c shows the moles of CH<sub>4</sub> present in the culture headspace over time versus the amount originally added to the culture (2.5% or 6.0 x 10<sup>-4</sup> moles of CH<sub>4</sub> at pressure =630 Torr, temp=25° C, headspace volume=0.7415 liters). From 8/15/94 to 9/9/94, the culture did not appear to use CH<sub>4</sub> as an energy source since the CH<sub>4</sub> concentration increased or remained close to the original level. A large increase in the CH<sub>4</sub> concentration occurred from 8/22/94 to 8/29/94. This was the period of time when the culture was anaerobic (See Figure 5b). Anaerobic methanogenic metabolism, which will be discussed below, can result in the conversion of CO<sub>2</sub> to CH<sub>4</sub> which increases the amount of CH<sub>4</sub> in the headspace. Methanogenic metabolism may have been occurring in the culture during this anaerobic period.

On 8/31/94, the culture was purged, which brought the CH<sub>4</sub> concentration back down to ~6.0 x 10<sup>-4</sup> moles and supplied the microorganisms with O<sub>2</sub> for aerobic metabolism. After the second purge on 9/9/94, the CH<sub>4</sub> concentration in the culture began to decrease. This removal

of CH<sub>4</sub> with increasing CO<sub>2</sub> in the presence of O<sub>2</sub> may indicate the activity of methanotrophic bacteria. Unfortunately, the long time prior to the onset of CH<sub>4</sub> removal only allowed eleven days of growth monitoring. Future culturing will allow longer growth times to verify the repeated disappearance of CH<sub>4</sub> added over time.

Figure 6 shows the natural log of CO<sub>2</sub> generation plotted versus time (CO<sub>2</sub> concentration after purging events are not shown). This figure shows the expected shape of a microbial growth curve with the period from 8/15/94 to 8/18/94 being the time of exponential growth and the period from 8/15/94 to 9/20/94 being the stationary or death phase.

No lag phase is apparent from this data. The lag phase is the period of time in which the microbial population is adjusting to the environment and has not begun to grow. This phase was most likely very short and was missed with sampling only once a day. However, it is sufficient to know that the lag phase of this culture is less than one day and that mid-exponential phase is reached between day 0 and day 3 of growth. Since exponential phase is the time of most active growth, it will be useful to conduct future solvent degradation studies during this phase so that degradation potential is at its maximum. It may also be useful to conduct solvent degradation studies throughout the exponential and stationary phases to see how degradation rates change with growth rates. This is true for all the populations being studied.

It should be mentioned that the removal of CH<sub>4</sub> from this culture's headspace was intended to be used as the indicator of methanotroph growth and not CO<sub>2</sub> generation. This is because CO<sub>2</sub> is a gas released by multitudes of organisms and indicates general microbial activity but does not indicate the type of population generating the CO<sub>2</sub>. Because CH<sub>4</sub> removal was delayed in this experiment, the data was difficult to interpret and was not adequate for use in generating a growth curve.

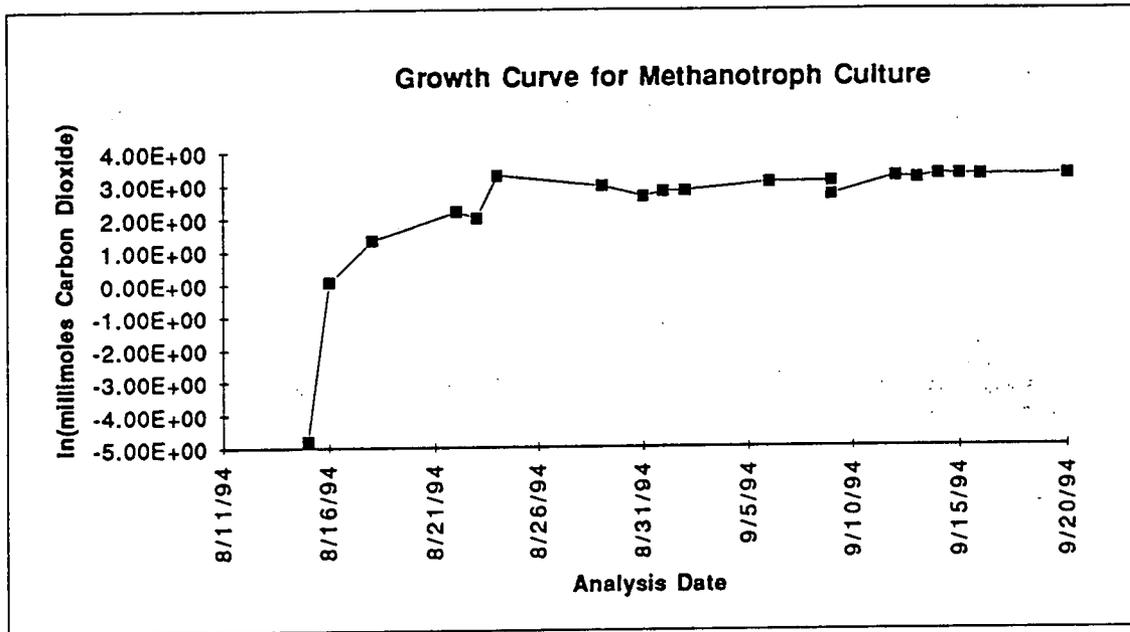


Figure 6. Natural log methanotroph growth curve based on carbon dioxide generation.

This experiment showed that, in general, microbial activity was vigorous in the culture and removal of methane from the culture in the later days of study could have indicated emergence of a methanotrophic population. Additional study is required to verify the growth of methanotrophs and longer culturing times will be employed.

### 3.2.2 Anaerobic/Denitrifying Culture

Denitrifiers are anaerobes which use carbohydrates as energy sources and use nitrate in the place of  $O_2$  as an electron acceptor (Brock et al. 1984). Denitrifiers oxidize their energy source to  $CO_2$  and reduce nitrate to  $N_2$  gas. If denitrifiers are operating in an environment,  $CO_2$  and  $N_2$  gas should increase in the headspace when  $O_2$  is not present.

Headspace data from the denitrifier culture showed that more than one anaerobic population could be enriched in the same culture flask. Both, denitrifiers and methanogens were grown using a media specific for denitrification. Sulfate producer presence was suspected, but could not be verified in this culture. Denitrifiers were relatively easy to grow in the laboratory and were readily enriched.

Figure 7 shows the moles of  $O_2$ ,  $CO_2$ ,  $CH_4$ , and  $N_2$  in the headspace of the denitrifier culture. The figure shows that in a very short period of time (several days), the trace amount of  $O_2$  in the argon headspace had been eliminated and  $N_2$  gas and  $CO_2$  levels began to rise. The  $N_2$  gas concentration began to level out after 18 days of growth (9/2/94) at which time  $CH_4$  was increasing.

Denitrifier growth was plotted using the natural log of millimoles of  $N_2$  gas in the headspace versus time (Figure 8). This data shows that denitrifiers emerged in a short period of time in

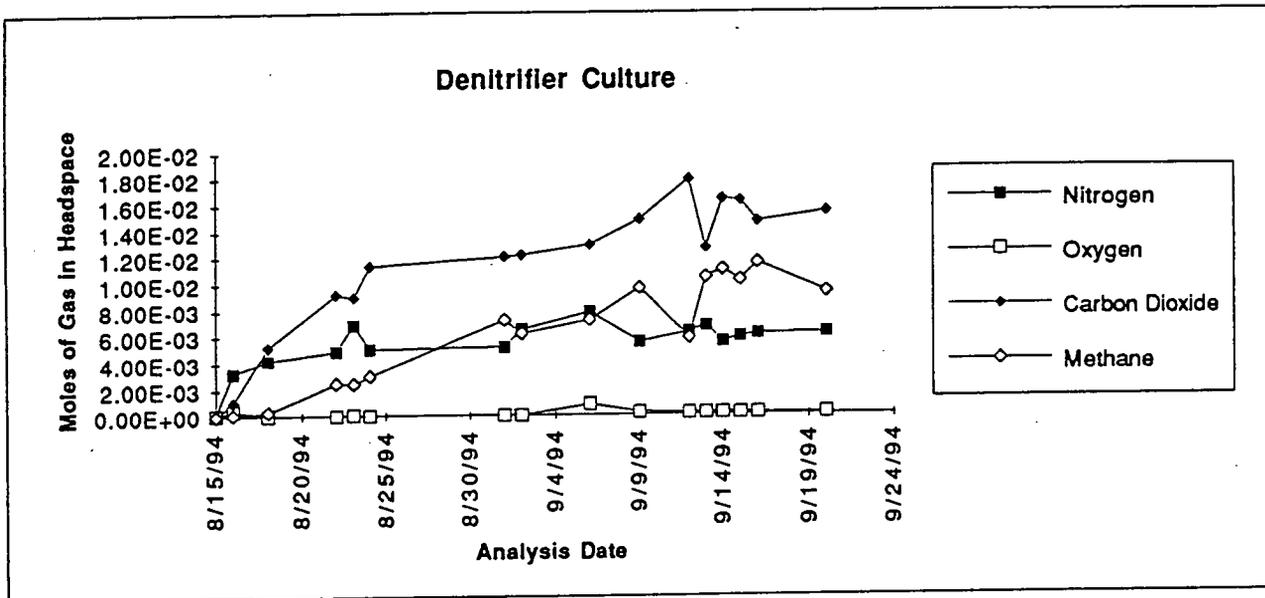


Figure 7. Headspace analysis of denitrifier culture. Culture headspace initially purged with argon to remove all gases. Production of nitrogen gas, carbon dioxide, and methane was measured over 36 days.

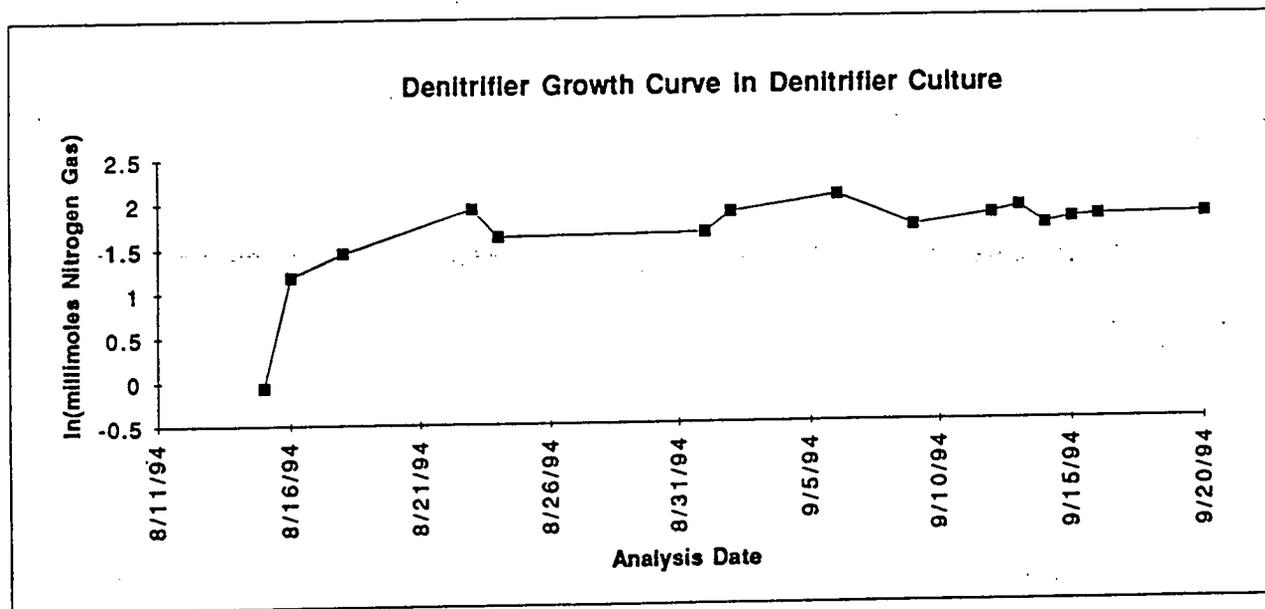


Figure 8. Natural Log growth curve of denitrifiers in the denitrifier culture based on nitrogen gas production.

the OU 2 enrichment culture. Again, there was no apparent lag phase prior to the onset of exponential growth. This suggests that denitrifiers should be relatively easy to enrich for further experiments or treatments of OU 2 soil, and that mid-exponential phase is reached between day 0 and day 3 for this population.

When  $O_2$  is not present in an environment, organisms will use alternate electron acceptors such as nitrate, sulfate, and  $CO_2$ . Nitrate has the greatest affinity for electrons followed by sulfate and finally by  $CO_2$  which has the lowest affinity (Norris et al. 1994). Therefore, nitrate will be consumed first until it is depleted, sulfate consumption will follow, and  $CO_2$  consumption will occur last.

Nitrogen gas will be produced when nitrate is the electron acceptor (Norris et al. 1994). When sulfate is used as an electron acceptor, either hydrogen sulfide gas or ferric sulfide precipitate will form (Norris et al. 1994). Unfortunately, the mass spectrometer used to measure headspace gases is relatively insensitive to hydrogen sulfide so this compound was not detected in the cultures. However, ferric sulfide is a black precipitate and is readily visible when present. When  $CO_2$  is used as an electron acceptor,  $CH_4$  is produced (Norris et al. 1994).

Figure 7 shows that once the concentration of  $N_2$  was leveling out,  $CH_4$  formation was increasing. This indicates that the denitrifying population most likely utilized the majority of the available nitrate as an electron acceptor and were succeeded by organisms which use  $CO_2$  as an electron acceptor and which produce  $CH_4$  (methanogens). This is the expected successional order. One would expect that if nitrate, sulfate, and  $CO_2$  are available in an anaerobic system, denitrifiers will emerge first, followed by sulfate reducers and then methanogens. In the experimental

denitrifier culture, there was evidence of denitrification and methanogenesis but not sulfate reduction. Trace amounts of hydrogen sulfide may have been generated but were not detected, or trace amounts of ferric sulfide precipitate formed but could not be seen visually in the soil matrix.

Figure 9 shows the growth curve of the methanogens which were growing in the denitrifier culture. The rate of growth for the methanogens is slower than for the denitrifiers based on the time required to reach stationary phase. These methanogens reach mid-exponential phase between day 0 and day 8 of growth.

The lack of abundant sulfate reduction seen in the denitrifier culture most likely indicates that sulfate was limited or unavailable. The nutrient solution provided to this culture was specific for denitrification and provided no sulfate to the culture. The only available sulfate would have come from the OU 2 soil matrix. A nutrient analysis of the OU 2 soil is in process.

### 3.2.3 Anaerobic/Sulfate Reducing Culture:

Sulfate reducers are anaerobes which use carbohydrates as energy sources and use sulfate in the place of  $O_2$  as an electron acceptor (Brock et al. 1984). Sulfate reducers oxidize their energy source to  $CO_2$  and reduce sulfate to sulfide, which is released as hydrogen sulfide gas or precipitates as ferric sulfide if ferric iron is available. If sulfate reducers are operating in an environment,  $CO_2$  and hydrogen sulfide gas and/or ferric sulfide should increase in the culture when  $O_2$  is not present.

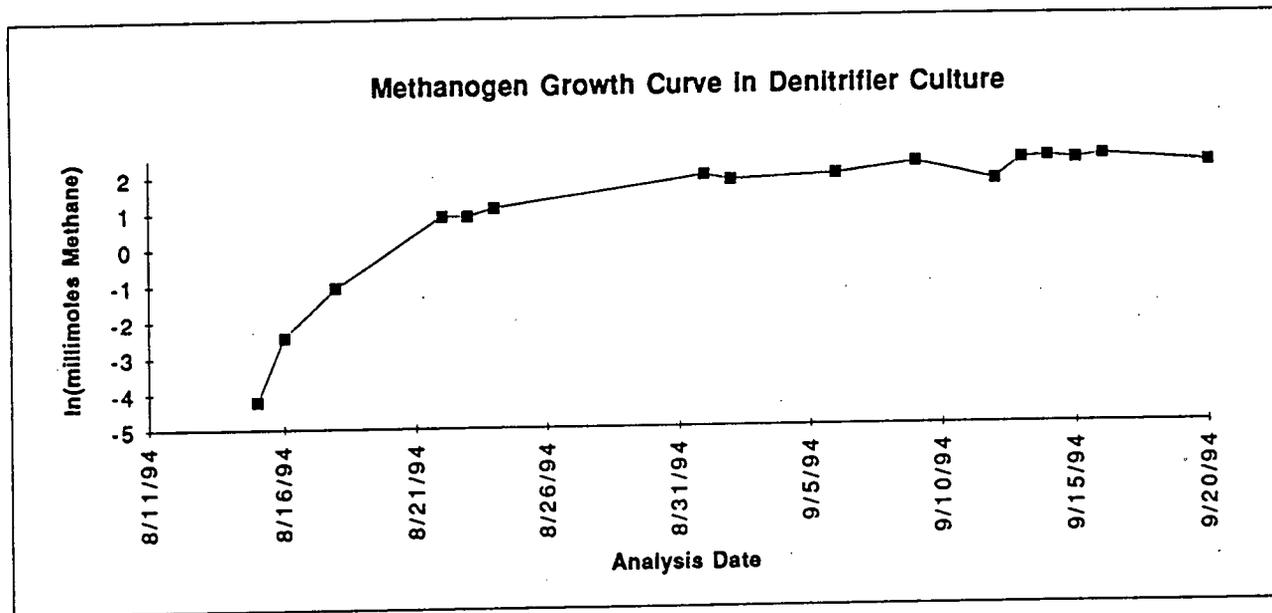


Figure 9. Natural log growth curve of methanogens in the denitrifier culture based on methane production.

Denitrifiers, sulfate reducers, and methanogens were all grown from OU 2 soil using a media specific for sulfate reduction. All three populations were relatively easy to enrich using this method. The extent of denitrification and methanogenesis was very similar between the sulfate reducing and denitrifying cultures. Sulfur nutrient addition along with denitrifier and methanogen activity verified the presence of sulfate reducers.

Figure 10 shows the moles of  $N_2$ ,  $O_2$ ,  $CO_2$ , and  $CH_4$  in the headspace of the sulfate reducer culture. Carbon dioxide increased over time in the culture, indicating biological metabolism. Although the presence of hydrogen sulfide gas could not be verified using the mass spectrometer technique, a hydrogen sulfide aroma was evident during gas sampling and analysis of this culture. Also, the presence of ferric sulfide was obvious in the soil material of the culture. A distinct one-inch layer of black soil formed during the culturing process. This evidence showed that sulfate reduction was occurring in the culture.

Since  $H_2S$  was not at detectable levels in the culture headspace but sulfate reduction was evident,  $CO_2$  generation was used to evaluate the growth of the sulfate reducers. Since these organisms were growing and the culture was expressly designed to support this one population, it was assumed that the majority of  $CO_2$  produced in the culture was from the activity of sulfate reducers with other populations contributing  $CO_2$  to a lesser extent. Figure 11 shows the natural log of the millimoles of  $CO_2$  in the culture headspace versus time. This figure shows that sulfate reducers reached mid-exponential phase between day 0 and day 3 of growth.

Figure 10 shows that  $N_2$  gas and  $CH_4$  were also formed in the culture over time. This is similar to the situation in the denitrifier culture in which there was evidence of growth of more than one population. Likewise, the figure shows production of  $N_2$  gas preceding appearance of  $CH_4$ . Again, this is the expected order of electron acceptor use.

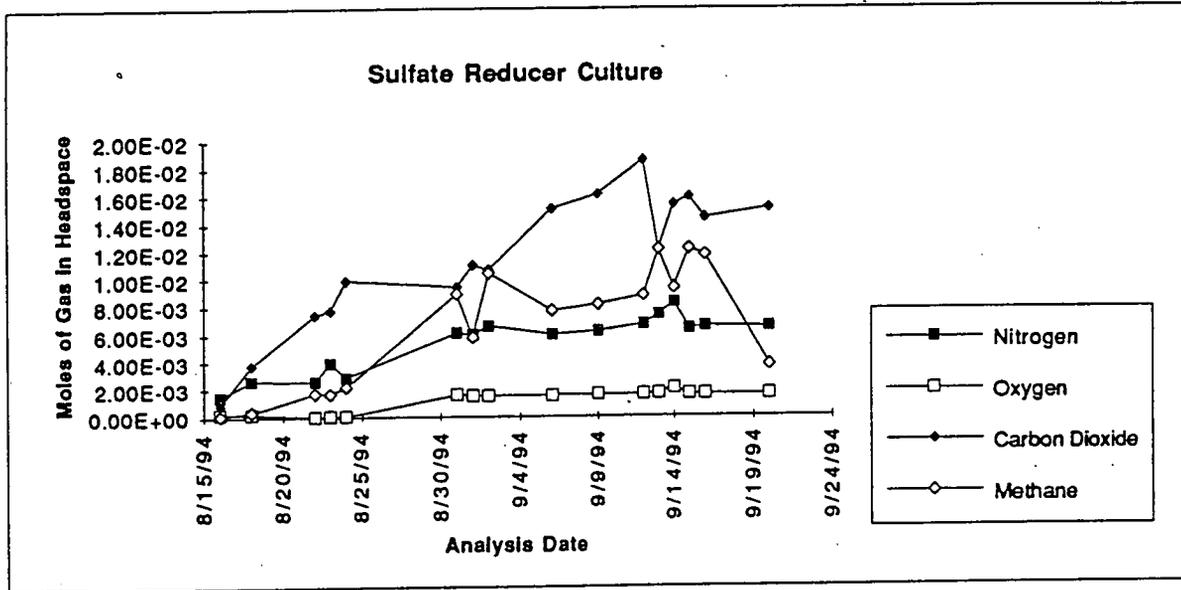


Figure 10. Headspace analysis of sulfate reducer culture. Culture headspace initially purged with argon to remove gases. Production of nitrogen gas, carbon dioxide, and methane was measured over 36 days.

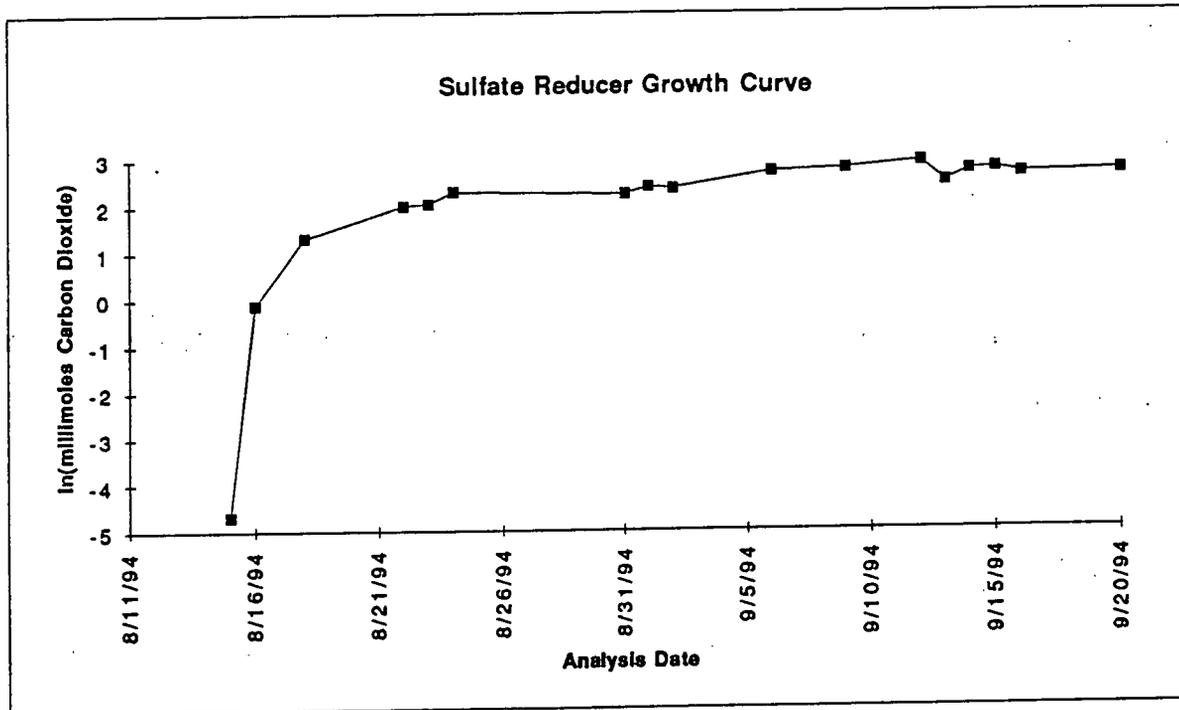


Figure 11. Natural log sulfate reducer growth curve in the sulfate reducer culture based on carbon dioxide production.

Though the nutrient media used for this culture contained no nitrate, the OU 2 soil matrix most likely contained nitrogen sources which supplied this electron acceptor for denitrification. A nutrient analysis of the OU 2 soil is in process.

The quantity of  $N_2$  gas generated in the sulfate reducing culture by 8/22/94 was approximately half the amount generated in the denitrifier culture in which nitrate was specifically added in the media ( $\sim 2.5 \times 10^{-4}$  moles in the sulfate reducing culture and  $\sim 5 \times 10^{-4}$  moles in the denitrifier culture). Eventually, both the denitrifier and sulfate reducer cultures generated similar total quantities of  $N_2$  gas in the range of  $6 \times 10^{-3}$  to  $7 \times 10^{-3}$  moles. Figure 12 shows the growth curve of the denitrifiers in the sulfate reducer culture. The denitrifiers in this culture reached mid-exponential phase between day 0 and day 8 of growth. This is a slower growth rate than the denitrifiers grown in the culture specifically designed for denitrification which reached mid-exponential phase between day 0 and day 3 of growth (See Figure 8).

For both the sulfate reducer and denitrifier cultures,  $CH_4$  generation (methanogenic activity) began to significantly increase between 8/24/94 and 8/31/94. The initial generation rate of  $CH_4$  in the sulfate reducer culture was more rapid than in the denitrifier culture (reaching  $\sim 8 \times 10^{-3}$  moles in 16 days and  $\sim 7 \times 10^{-3}$  moles in 16 days respectively). However, both cultures reached a maximum  $CH_4$  concentration near  $1.1 \times 10^{-2}$  moles. Figure 13 shows the growth curve of the methanogens in the sulfate reducer culture. The methanogens in this culture reached mid-exponential phase between day 0 and day 16. This is a slower growth rate than the methanogens in the denitrifier culture which reached mid-exponential phase between day 0 and day 8 of growth (See Figure 9).

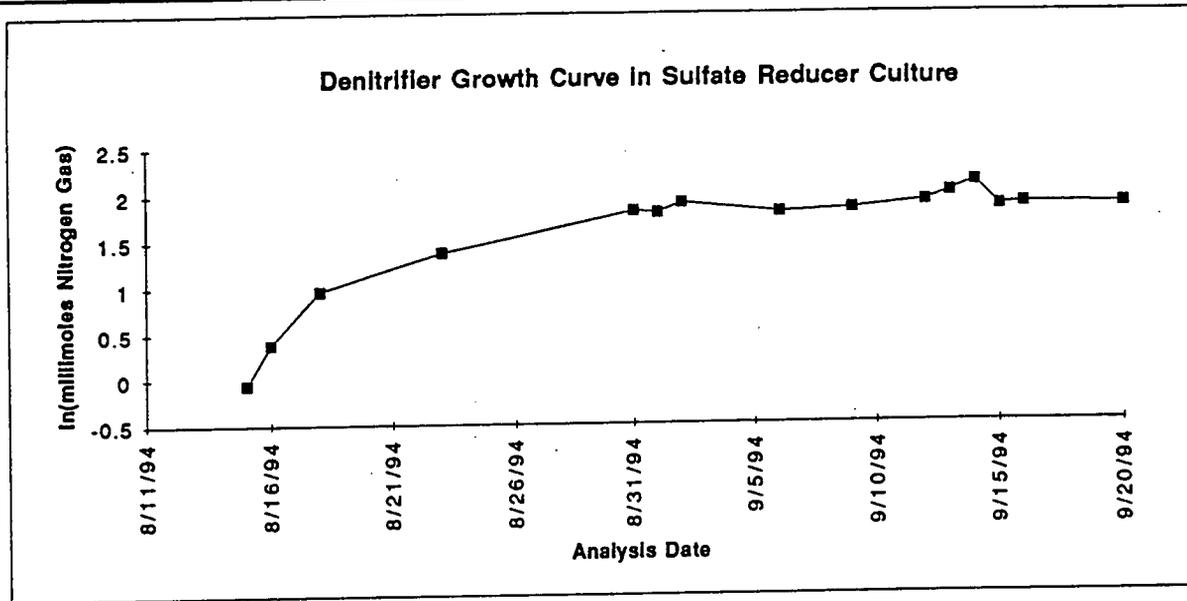


Figure 12. Natural log denitrifier growth curve in sulfate reducer culture based on nitrogen gas production.

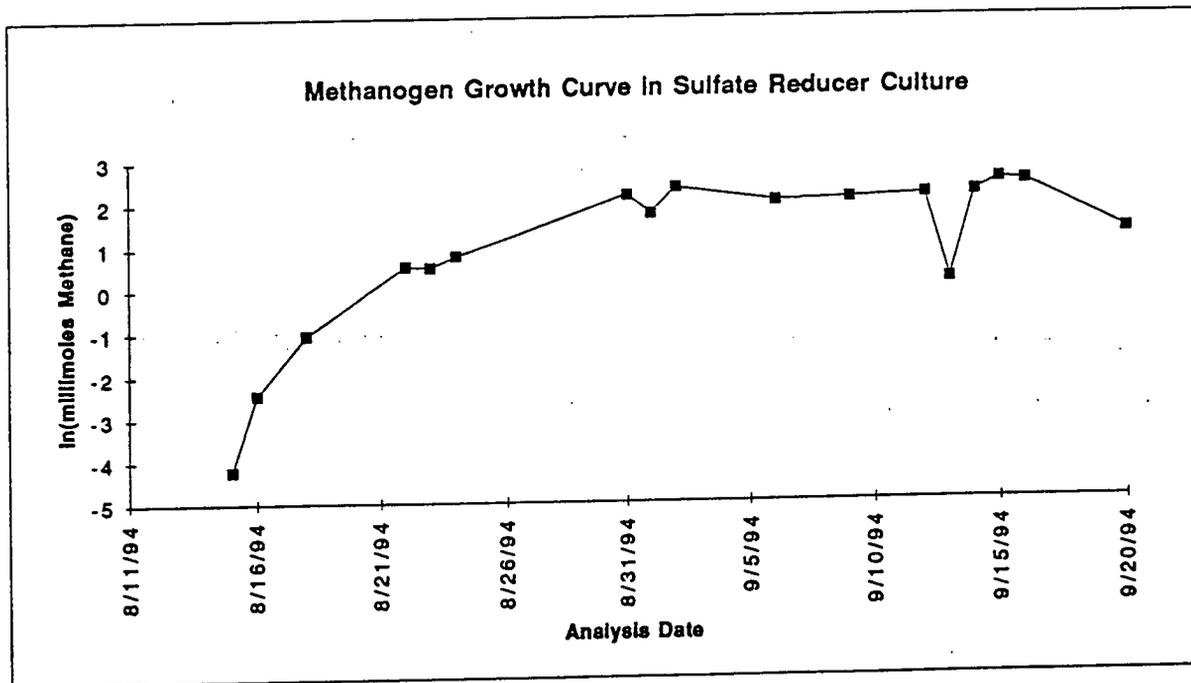


Figure 13. Natural log methanogen growth curve in sulfate reducer culture based on methane production.

### 3.2.4 Anaerobic/Methanogenic Culture:

Methanogens are anaerobes which use carbohydrates as energy sources and use  $\text{CO}_2$  in the place of  $\text{O}_2$  as an electron acceptor (Brock et al. 1984). Methanogens oxidize their energy source to  $\text{CO}_2$  and reduce  $\text{CO}_2$  to  $\text{CH}_4$ . If methanogens are operating in an environment,  $\text{CO}_2$  and  $\text{CH}_4$  gas should increase in the culture when  $\text{O}_2$  is not present.

Problems maintaining the culture gas-seal made data gathered for this culture difficult to interpret so a growth curve for this culture was not prepared. Figure 14 shows that  $\text{CO}_2$  and  $\text{CH}_4$  were present after 9/4/94 at concentrations higher than expected in ambient air. It is believed that  $\text{CH}_4$  production began to rise by the end of the experiment and that methanogens were operating. The presence of denitrifiers and sulfate reducers could not be verified in this culture. Culturing flasks will be redesigned for future studies to reduced the potential for leaks.

Figure 14 shows the moles of  $\text{N}_2$ ,  $\text{O}_2$ ,  $\text{CO}_2$ , and  $\text{CH}_4$  in the headspace of the methanogenic culture. The most obvious difference between this figure and those for the other anaerobes is the large amount of  $\text{N}_2$  and  $\text{O}_2$  in the headspace of this culture. Prior to 9/2/94, there was up to 70%  $\text{N}_2$  and 23%  $\text{O}_2$  in this culture's headspace. This is similar to the concentration of these gases in ambient air. It is believed that there was a gas leak in the lid valve for this culture which allowed air to enter the culture during analysis or incubation. Though attempts were made to control the leak during testing, the culture did not achieve anaerobic conditions until well into the culturing experiment (9/6/94).

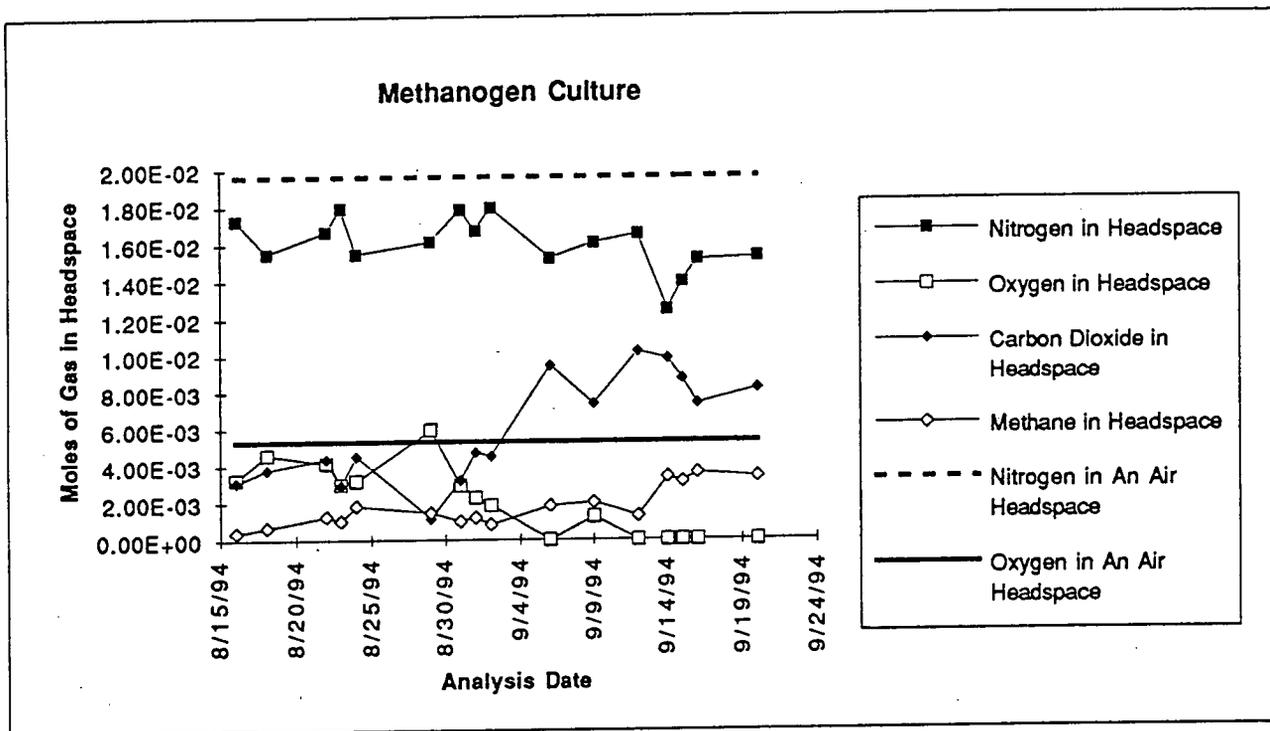


Figure 14. Headspace analysis of methanogen culture. Culture headspace initially purged with argon to remove all gases. Figure shows levels of nitrogen similar to ambient air.

## 4.0 Conclusions/Recommendations

### 4.1 Phase I - Abiotic/Unamended Soil Study

Phase I evaluated the transformations of a mixture of chlorinated solvents in sterile and unsterile OU 2 soil. The test objective of this Phase was to determine the types of chemical changes which occur when solvent mixtures contact the nonliving component of the Site soil or the existing microbial populations which have not been stimulated to grow.

Phase I included gathering data on the degree to which solvents are chemically altered due to contact with Site soil as judged by changes in concentration and structures of the solvents over time. A comparison of solvent transformations on sterile and nonsterile soil determined whether naturally occurring organisms in the soil could transform solvents without addition of nutrients and water and whether the abiotic portion of the soil could transform solvents as well.

The results of Phase I showed that the six solvents tested on sterile and nonsterile OU 2 soil steadily declined in concentration over a period of 71 days. Though the concentration of solvents decreased over time, no degradation products were measured in any sample of soil which was sterile or unamended with nutrients and water. Also, the concentration of all the solvents tended to decrease in a similar manner and rate.

There was a statistically significant difference between loss of solvents from the sterilized samples versus the unsterilized unamended samples. The sterilized vials tended to lose more solvent than the unsterilized vials. This may be due to the effects of the sterilization process on the integrity of the sample vials. Apparently, the autoclaving process which heats the vials to 121° C and 21 psi increases the tendency of the vials to leak solvents which are added to the vials after autoclaving.

This evidence leads to the conclusion that no solvent transformations occurred when the six solvents of interest were placed in contact with sterile and unamended OU 2 soil over the 71-day test. Loss of the solvents from the sample vials due to migration through the septa is a probable explanation for solvent disappearance in the samples.

This phase provided valuable information to be used in the design of future biological experiments in which solvent transformations are studied. From this information it is known that: 1) significant solvent transformations should not occur due to solvent contact with the nonliving portion of the soil, 2) significant solvent transformations should not occur due to the action of existing soil microorganisms which are not stimulated to grow with the addition of water or nutrients, 3) measures to reduce the loss of solvents from sample containers during experimentation should be considered in future studies, and 4) other sterilization techniques may have to be considered, since autoclaving sample vials may decrease the ability of the vials to retain solvents.

#### 4.2 Phase II - The Biotic Study

Phase II evaluates the presence of four populations of microorganisms in OU 2 soil which are known to transform chlorinated solvents. Also, this phase examines the ability of these four populations to transform a mixture of six chlorinated solvents including PCE, TCE, methylene chloride, carbon tetrachloride, 1,1,1-TCA, and 1,1,2-TCA. Phase II was approximately 50% complete at the time of this report. This report provided data gathered only for the first test objective of this phase (enrichment of the four populations from OU 2 soil). Information regarding the populations' ability to transform the solvents will be provided in a final report at the conclusion of Phase II.

The test objective addressed by FY-94 activities for Phase II was to determine if four microbial populations which have shown potential for biotransformation of chlorinated solvents in literature could be enriched in the Site soil. The goal of this portion of Phase II was to enrich

these key solvent transforming microbial populations from a sample of surface soil from OU 2 IHSS 110. These key populations included methanotrophs, denitrifiers, sulfate reducers, and methanogens.

Four enrichment cultures were prepared. Each one of the cultures was designed to support one of the key populations and encourage its growth in the laboratory. Headspace gas analysis followed the growth in the cultures over 36 days and identified the types of populations which were growing.

The results of this portion of Phase II show that denitrifiers, sulfate reducers, and methanogens were enriched from OU 2 soil. These organisms were relatively easy to grow in the laboratory, requiring only anaerobic conditions and nutrient additions.

Enrichment culturing of the aerobic methanotrophic microorganisms was more difficult than for the anaerobic bacteria. These organisms require methane as an energy source making culture maintenance more difficult. Phase II provided evidence which suggests that methanotrophs were enriched from OU 2 soil. However, further culturing is required to verify the presence of methanotrophs.

Phase II data showed that more than one anaerobic population could be enriched in the same culture flask under denitrifying and sulfate reducing conditions. Headspace gas analysis data verified the presence of denitrifiers and methanogens in the culture specific for denitrifying organism enrichment. Also, the presence of denitrifiers, sulfate reducers, and methanogens was verified in the culture specific for sulfate reducing organism enrichment.

In summary, all three anaerobic populations of interest were successfully enriched from OU 2 soil. Further culturing studies will verify the growth of the aerobic methanotrophic population in OU 2 soil.

#### 4.3 Recommendations

The findings of the portion of Phase II completed in FY-94 are encouraging. Three of the four microbial populations of interest have been successfully enriched from OU 2 soil with relative ease. Adaption of all four populations to chlorinated solvents has begun.

It is the authors' recommendation that Phase II of this study continue since the growth of the microorganisms from OU 2 was successful. Completion of Phase II will provide data which shows whether OU 2 organisms can transform chlorinated solvents. Phase III will determine to what extent the organisms can treat a mixture of chlorinated solvents in soil such as found in OU 2.

If Phase II and Phase III of this study are successful, in that they show microorganisms in OU 2 can be stimulated to degrade the mixture of chlorinated solvents found in the soil, in situ or ex situ treatment of solvent contaminated material from OU 2 or other OUs may be a viable option for Site environmental restoration.

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