INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700  800/521-0600
Effects of hydrocarbon sorption on biodegradation, emulsification capacity and microbial diversity of a mixed microbial population

Pointer, Judy Hill, M.S.
Rice University, 1994
RICE UNIVERSITY

EFFECTS OF HYDROCARBON SORPTION ON BIODEGRADATION, EMULSIFICATION CAPACITY AND MICROBIAL DIVERSITY OF A MIXED MICROBIAL POPULATION

by

JUDY HILL POINTER

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

APPROVED, THESIS COMMITTEE

C.H. Ward
C.H. Ward, Professor
Environmental Science and Engineering

Philip B. Bedient, Professor and Chair
Environmental Science and Engineering

Frederick B. Rudolph, Professor
Biochemistry and Cell Biology

Houston, Texas
April, 1994
ABSTRACT

Effects of Hydrocarbon Sorption on Biodegradation, Emulsification Capacity and Microbial Diversity of a Mixed Microbial Population

by Judy Hill Pointer

The presence of an adsorbent resin in broth/resin slurries exerted a selection pressure on a population of microorganisms isolated from gasoline contaminated soil. The resin acted as a non-degradable matrix to sorb and entrain the gasoline constituents and was used as a substitute for a homogeneous aquifer matrix of packed sand. When the resin and light non-aqueous phase liquids (LNAPLs) were present as the carbon source, two species, *Acinetobacter lwoffi* and *Pseudomonas fluorescens*, predominated over three other species in the original population. Concomitant with the diversity changes, emulsion production and degradation rates of low solubility compounds increased. At least two biosurfactants were produced, an extracellular surface tension reducer and an extracellular emulsifier. Only the emulsifier was associated with increased degradation and cell growth.
ACKNOWLEDGMENTS

I would like to thank my advisors for their invaluable assistance. Each has contributed greatly to my education and understanding of environmental science. I would like to especially thank my principal advisor, Dr. Herb Ward, for helping me to develop presentation and research skills.

In addition, many of the staff have guided me. Dr. Virginia Gordy provided patient guidance in laboratory techniques and was responsible for the GC/mass spectrometry results on the core samples reported in this thesis. She also lent a helping hand with the diversity study. Dr. Michele Thomas constantly guided my experimental efforts, offering valuable critiques as the work progressed. Robin Hazard helped with the development of the direct cell counting technique and aided in making media and preparing glassware. I also thank my fellow graduate students for their cooperation, patience and insights, as well as Exxon Co., USA for the gasoline blend used and Dow Chemical, Co. for some of the adsorbents that were tested. Additionally, I wish to thank Shell, Inc. for the donation of the DeNuoy Tensiometer that was used in the experiments.

Finally, I owe of debt of gratitude to my friend, Andy Procell, for his constant support and encouragement and to my daughter, Christine Pointer, for the many personal sacrifices she made in order to help her mom get the job done.
# TABLE OF CONTENTS

1 INTRODUCTION 1

2 LITERATURE REVIEW 7
2.1 Adaptation and Enrichment Cultures 7
2.2 Biodegradation and Bioavailability 10
2.3 Sorption/Desorption and Entrapment of Hydrocarbon 13
2.4 Biosurfactants 23

3 MATERIALS AND METHODS 36
3.1 Glassware 36
3.2 Media, Chemicals, Mixtures and Resins 36
3.2.1 Media 36
3.2.2 Chemicals and Liquid Organic Mixtures 37
3.3.2 Resins 38
3.3 Contaminated Soil 39
3.3.1 Sampling Site and Sampling Procedure 39
3.3.2 Soil Characteristics 41
3.4 Isolation of Microorganisms from Contaminated Soil 42
3.5 Experimental Systems and Techniques 44
3.5.1 Direct Cell Counting Technique 44
3.5.2 Preliminary Experimental System and Techniques 46
3.5.3 Growth of Soil Consortium on Various Carbon Sources 48
3.5.4 Growth of Soil Consortium at Various Carbon Concentrations 48
3.5.5 Growth and Surface Tension of Soil Consortium Without a Carbon Source 49
3.5.6 Adsorbents 49
3.5.6.1 Adsorbent Selection 49
3.5.6.2 Adsorbent Cleaning and Sterilization 53
3.5.6.3 Loading of Sorbate into the Adsorbent 54
3.6 Experimental Conditions for Experiments I, II, III, IV and V 58
3.6.1 Standard Procedures 60
3.6.1.1 Inoculation of Microcosms 61
3.6.1.2 Incubation of Microcosms and Agar Plates 62
3.6.1.3 Surface Tension Measurements 62
3.6.1.4 Emulsification Capacity Measurements 63
3.6.1.5 Gas Chromatography Extraction Procedure 64
3.6.1.6 Gas Chromatography 65
3.6.1.7 Viable Plate Counts and Purification of Microbes 66

4 RESULTS 67
4.1 New Methods Development 67
4.1.1 Direct Cell Counting Technique 67
4.1.2 Delivery System for a Sorbed Carbon Source 70
4.2 Preliminary and Control Experiments 77
4.2.1 Soil and Contaminant Characterization 78
4.2.2 Growth of Soil Consortium on Various Carbon Sources 80
4.2.3 Growth of Soil Consortium at Various Carbon Source Concentrations 81
4.2.4 Growth and Surface Tension of Soil Consortium Without a Carbon Source 82

4.3 Experiments I, II, III, IV, V 83
4.3.1 Experiment I: Growth Curve Trial Run Utilizing Three Different Treatment Regimes 84
4.3.2 Experiment II: Growth Curve Comparison of Aviation Gasoline and Hydrocarbon Mixture as Sole Carbon Source 85
4.3.3 Experiment IV and V: Cell Concentration, Surface Tension, Emulsification Capacity and Microbial Diversity 89
4.3.3.1  Effects of the Physical State of the Carbon Source on Cell Concentration  89
4.3.3.2  Effects of the Physical State of the Carbon Source on Surface Tension  90
4.3.3.3  Effects of the Physical State of the Carbon Source on Emulsification Capacity  92
4.3.3.4  Effects of the Physical State of the Carbon Source on Microbial Diversity  92
4.3.4   Experiment III: Disappearance of Hydrocarbon  99

5      DISCUSSION  104

6      CONCLUSIONS  111

APPENDIX 1  114

APPENDIX 2  115

REFERENCES  116
LIST OF TABLES

TABLE 2.4  
HLB ranges of biosurfactants and their application.  
28

TABLE 3.2.2  
Gas chromatographic analysis of aviation gasoline supplied by Exxon Co., USA.  
38

TABLE 3.3.1  
Traverse City Site Characteristics.  
40

TABLE 3.5.6.1  
Characteristics of resins that were tested as possible adsorbents.  
50

TABLE 3.5.6.3  
Gas chromatographic analysis of the stock batches of sorbed resin.  
57

TABLE 4.1.1  
Surface tension and 8 day growth curve of Traverse City contaminated soil microorganisms.  
70

TABLE 4.1.2a  
Growth of microbial consortium on 0.3% yeast extract in the presence of different resins.  
72

TABLE 4.1.2b  
Sorbed resin: sink or float by virtue of change in resin density after exposure to aviation gasoline.  
74

TABLE 4.2.1  
Gas chromatographic/mass spectrophometric analysis of contaminated core samples from the Traverse City spill site.  
80

TABLE 4.2.2  
Growth of soil organisms on various carbon sources.  
81

TABLE 4.2.4  
The mean surface tension and cell concentration of microcosms with no hydrocarbon source.  
83
TABLE 4.3.3  Results of Experiments IV and V: Cell concentration, surface tension and emulsification capacity of 140 hour microcosms.  91

TABLE 4.3.3.4a  Dominant colony types identified in the RU2SS2 core boring.  93

TABLE 4.3.3.4b  Diversity on R2A agar. Relative percents of the five dominant microbial species in the three different treatment regimes.  94

TABLE 4.3.3.4c  Species diversity on hydrocarbon fumes.  97

TABLE 4.3.4a  Time zero GC analysis of the amounts of hydrocarbon supplied to the microcosms when extracted immediately.  100

TABLE 4.3.4b  Time zero GC analysis of the amounts of hydrocarbon supplied to the abiotic microcosms when extraction was delayed 24 hours.  100

TABLE 4.3.4c  GC analysis of hydrocarbon recovery in three treatment regimes, at zero and 140 hours when extraction was delayed 24 hours.  101

TABLE 4.3.4d  GC analysis of hydrocarbon disappearance in microcosms after 140 hours of culture in each treatment regime  102

TABLE 5  Summary of results of experiments I through V  104
LIST OF FIGURES AND GRAPHS

FIGURE 2.3 Entrapment of liquid organic within aquifer pores, resulting in residual oil saturation. 15

FIGURE 2.4a Biosurfactant effects on phase barriers. 26

FIGURE 2.4b Structure of biosurfactant molecules. 29

FIGURE 3.3.1 A diagram of the Traverse City, Mi. spill site. 41

FIGURE 3.5.2 Preliminary experiment culture conditions. 47

FIGURE 3.5.6.1 Polymethacrylic, XAD-8 adsorbent. 53

FIGURE 3.6 Three different methods used to supply hydrocarbon to the microcosms. 59

FIGURE 3.6.1.4 Emulsification capacity scoring. 64

GRAPH 4.1.1a Comparison of direct cell counts to viable plate counts. 68

GRAPH 4.1.1b Whole cell surface tension of cultures with respect to time. 68

FIGURE 4.1.2 Comparison of resin pore diameters to the critical diameters of selected molecules. 76

GRAPH 4.2.3 Semilog plot of cell concentration in vapor cultures. 82
GRAPHS 4.3.2
(a & b)  
Cell concentration (a) and surface tension (b) of microbial consortium utilizing a hydrocarbon mixture of DMP, TMP and toluene as a carbon source.

GRAPHS 4.3.2
(c & d)  
Cell concentration (c) and surface tension (d) of microbial consortium utilizing aviation gasoline as a carbon source.
1 INTRODUCTION

The U.S. Environmental Protection Agency (EPA) Office of Underground Storage Tanks (OUST) identified about 128,000 leaking gasoline storage tanks nationwide in 1991, however it is estimated there are three million gasoline storage tanks located throughout the nation [Dowd, 1984]. If only 10% of these are leaking, there could be as many as 300,000 point source pollution problems to deal with in the future [Thomas et al., 1987]. Less than one sixth of the 128,000 identified tanks had been treated by 1991. Often the treatment consisted of simply removing the contaminated soil to another location for disposal [Hockman, 1992].

Gasoline from leaking underground storage tanks can seep into and contaminate aquifers, thus presenting a potential for human and economic harm. Gasoline in aquifer-derived irrigation waters can leave residues on crops that enter the food chain when harvests go to market. Hydrophobic petroleum products, like gasoline, may become more concentrated as they travel up the food chain. Even though intestinal absorption of hydrocarbons has been shown to be poor in humans, documentation from occupational exposures to hydrocarbons has indicated that some severe health effects may result from environmental exposure. Benzene exposure has caused leukemia in humans even though the exact mechanism is not known [Williams and Weisburger, 1991]. Large exposures to benzene vapors has been shown to cause cardiac arrhythmias and even sudden death [Andrews and Snyder, 1991]. Toxicity from alkylbenzenes has shown human central nervous system depression and n-alkane exposure has been associated with human
neurotoxicity [Andrews and Snyder, 1991]. Ingested and inhaled hydrocarbons have shown to cause a wide variety of tumors in rodents [Hewitt et al., 1991].

Because the potential for harm is so great, remediation of underground storage tank (UST) spill sites has been mandated by the federal government through several legislative acts: Resource Conservation and Recovery Act; Comprehensive Environmental Response, Compensation, and Liability Act; Clean Water Act; and the Safe Drinking Water Act. However, the remediation of identified sites has often been difficult and time consuming.

To help solve this problem the EPA, OUST has studied and promoted several state-of-the-art approaches to remediating UST spills. Their suggested approaches to remediation include such processes as physical containment (removal, barriers, slurry walls, liners, etc.); hydrodynamic controls (well systems, trenches); withdrawal and treatment (chemical, adsorption, ion exchange, stripping, incineration, filtration, density separation); and biological treatment (above ground bioreactors and in situ bioremediation). While some technologies are adequate for some situations, not all spill sites respond similarly. Usually it is necessary to use a combination of technologies to diminish contaminants to levels mandated by federal and state legislation.

One type of biological treatment suggested by OUST is in situ subsurface bioremediation. This is a process whereby indigenous microorganisms are stimulated to degrade contaminants in the subsurface.
To accomplish this, amendments such as oxygen, nitrogen, and phosphorus are injected into the subsurface to enhance rates of contaminant biodegradation [Thomas et al., 1990]. In situ delivery of nutrient amendments, was first attempted by Suntech, Inc. and patented by R. L. Raymond in 1974 [Raymond et al., 1975; Raymond et al., 1976; Raymond, 1978]. For a history of in situ subsurface bioremediation the reader is referred to a review by Thomas et al. [1989], entitled In Situ Biorestoration of Organic Contaminants in the Subsurface.

Of the different types of remediation technologies suggested by OUST, in situ bioremediation has some advantages. Unlike withdrawal and control methods, which immobilize or transfer contaminants to another part of the environment, biological processes can degrade hazardous compounds into innocuous material. By nature's design, microorganisms degrade naturally-occurring organic compounds. It is estimated that 90% of carbon on the earth is naturally cycled by microorganisms, principally bacteria and fungi [Stanier et al., 1986]. Degradation of hazardous organic compounds also occurs if adequate amounts of essential nutrients and electron acceptors are present. Effective biodegradation does not leave large amounts of potentially toxic residues that require long-term monitoring.

Another advantage of bioremediation over other remediation technologies is that it is more cost-effective because it requires relatively lower input of energy. Incineration can eliminate contaminants but is very energy demanding. A 1988 study suggested it costs an average of $63-$123/cubic yard of soil treated to bioremediate a gasoline spill [Ross et al.,
1988]. By comparison, disposal of contaminated soil in permitted facilities can average $300-$500/cubic yard, and incineration can run from $223-$1115/cubic yard. Incineration coupled with excavation can run as high as $2000/cubic yard [Fiorenza et al., 1991b; Ross et al., 1988].

There are, however, certain disadvantages to bioremediation. Some contaminants are recalcitrant to microbial degradation, even under good environmental conditions. In some cases the reason for recalcitrance is known, while in others the reason remains unclear [Bouwer, in press; Alexander, 1981]. It is known that sorption can render normally biodegradable contaminants recalcitrant. Contaminants can bind to carbon attached to aquifer solids and be trapped within the pore network of the subsurface soil matrix. Trapped or sorbed residual oil is unavailable for microbial attack and hence does not easily degrade biologically. Sorbed contaminants act as a continuous source of dissolved contamination.

Residual oil contamination has been referred to as 'rebound' contamination. The rebound effect is observed after aqueous phase contaminant concentrations are lowered by treatment and the extraction wells are turned off. The dissolved-phase contaminant concentration then appears to rebound back to pre-treatment levels as the trapped residual material continues to dissolve into the aqueous phase of the aquifer. It has been found that complete removal of LNAPL or sorbed contamination is very difficult, if not impossible. Therefore, residual oil often results in unacceptably high levels of contamination in ground water even after extraordinary measures have been taken to eliminate it [Lyon and Rhodes,
The rebound phenomenon can greatly increase the cost of *in situ* remediation, because of the extended clean-up time necessary. The added cost and time often makes this treatment technology less practical than other technologies recommended by OUST.

Because of this problem, methods to mobilize and degrade residual oil contamination during bioremediation have been studied. One method studied is the application of synthetic or biologically-produced surfactants (surface active agents) that could aid in the release of the trapped gasoline. Synthetic surfactants have been unpredictable for this purpose; they have been found to both enhance and retard oil degradation [Atlas, 1981]. Biosurfactants may prove to be more effective than synthetic surfactants because they are biodegradable and not toxic to microorganisms. Further knowledge of biosurfactants and their relationship to soil organisms, organic compounds and various soil matrices is needed to determine if biosurfactants are beneficial in mobilizing trapped residual oil contamination during bioremediation.

This thesis addresses the use of biosurfactants for petroleum hydrocarbon bioremediation. It was hypothesized that the physical state of a hydrocarbon would select for microorganisms that could utilize either the sorbed, soluble or vapor phases of the hydrocarbon. To test the hypothesis, a microbial consortium was isolated from gasoline-contaminated aquifer material and grown in the presence of sorbed, soluble or vapor phase hydrocarbon. To determine the effect of hydrocarbon state on the microbial consortium cell growth, biosurfactant production, hydrocarbon degradation
and microbial diversity were determined. The experimental system was designed to control variables, not to simulate an \textit{in situ} environment, and is only a model of altered carbon bioavailability.
2 LITERATURE REVIEW

2.1 Adaptation and Enrichment Cultures

Natural selection in microbial communities was first demonstrated in the 1800s by Sergius Winogradsky and Martinus Beijernick [Stanier et al., 1986]. The principle is based on the fact that only certain microbial species dominate in a mixed population of naturally-occurring microorganisms when they are exposed to specific conditions. In tandem with microbial growth, physiological characteristics useful for survival are expressed. In soil contaminated with gasoline, individual species that adapt to degrade the contaminants will have a competitive advantage over non-degrading species.

Adaptation is defined as an increase in the rate of degradation of a substrate after exposure to that substrate. Adaptation may occur as a result of 1) an increase in the number of degrading organisms, 2) depletion of a preferential substrate, 3) genetic changes, 4) enzyme induction, 5) removal of a toxicant, 6) adequate nutrients, and 7) the elimination of predators [Wiggins et al., 1987].

Thomas et al. [1989b] found that the microorganisms from contaminated soil at a creosote site were adapted to degrade components of the creosote, while organisms from the pristine soil were not. After a contaminant spill, the diversity of the microbial population in the affected area adapts and changes [Odu, 1972]. Aelion et al. [1987] found that
microorganisms in pristine soil adapted to degrade \textit{p}-nitrophenol and that the time required for adaptation was one to six weeks. However, \textit{p}-chlorophenol and ethylene dibromide were degraded more rapidly than the \textit{p}-nitrophenol and required no lag time before degradation began. When chlorinated benzenes were added to the soil, degradation was not detected.

One adaptation process may involve spontaneous random mutation or plasmid-mediated mutation. Alternately, an induction of specific microbial functions, involving suppression and/or enhancement of microbial enzymes capable of changing the indigenous population's metabolic capabilities may be at play. Additionally, simple selective forces may enhance the growth of certain microorganisms already present which already have the capacity to degrade the contaminants [Aelion et al., 1987]. Also, selective forces may enhance the population of microorganisms that survive by metabolizing the by-products of the hydrocarbon-degrading population. The process of cometabolism may be involved in the selection process. The net result of contaminant addition to soil matrixes is an initiation of adaptation processes that changes the make-up of the indigenous population and ensures the survival of the most competitive microorganisms.

Microbial functions that can enhance degradation are sometimes mutationally acquired by plasmid-borne vectors [Rusanskey et al., 1987; Pines and Gutnick, 1986; Wyndham, 1986]. Plasmids are packets of microbial DNA that can be transferred from one cell to the next by bacteriophage infections. The plasmid DNA is inserted into the bacterial cell cytoplasm but does not become integrated into the host cell DNA
[Stanier, 1986]. However, often this cytoplasmic DNA can impart new physiologic characteristics to the bacteria just as if it were a part of the cells inherited genes. Plasmids, associated with functional changes, have frequently been demonstrated in the bacterium *Acinetobacter calcoaceticus*. *Acinetobacter calcoaceticus* (RA57), containing plasmid pSR4 could disperse and grow on crude oil, but plasmid-free organisms could not. However, the plasmid-free organisms were able to grow on crude oil vapors, indicating the plasmid expressed functions necessary for liquid crude oil degradation that were not required for vaporized crude oil degradation [Rusanskey et al., 1987]. Wyndham [1986] found plasmid involvement in the adaptation of *Acinetobacter calcoaceticus* to degrade aniline. Pines and Gutnick [1986] demonstrated that a mutant strain of *A. calcoaceticus* TR3, was deficient of a polyanionic bioemulsifier produced in the wild type hydrocarbon degrading strain from which it was derived. The mutant was unable to grow on crude oil so they surmised the bioemulsifier produced by the wild type bacterium contributed to the oil degradation and was most likely a result of an acquired plasmid.

Microorganisms with hydrocarbon degrading capabilities have been isolated using enrichment culture techniques. This procedure involves enrichment of hydrocarbon-degrading microorganisms by incubating carbon-free liquid microcosms or solid media culture plates in the presence of gasoline fumes. Growth in the liquid or solid medium suggests the metabolism of gasoline. However, this type of enrichment culture yields no information about microorganisms capable of degrading sorbed, entrained or liquid gasoline. Biodegradation of the low-solubility hydrocarbons in
gasoline, is dependent not only on microbial metabolic capacities but also on the microbial consortium's capability to enhance the bioavailability of low-solubility carbon substrates [Zajic and Seffens, 1984]. This may be especially true when the insoluble carbon substrate is sorbed or trapped within an aquifer matrix.

2.2 Biodegradation and Bioavailability

Hydrocarbons can be degraded by microorganisms either aerobically or anaerobically. Aerobic degradation of aliphatic gasoline constituents takes place by oxidation of the terminal carbon, dehydrogenation to the aldehyde, then further oxidation to the corresponding fatty acid. Subsequent beta oxidation yields fatty acids and acetic acids for use in the assimilative tricarboxylic acid cycle. Branched aliphatics are more difficult to degrade than n-alkanes, because of the steps needed for debranching prior to beta oxidation.

Aerobic degradation of aromatic compounds usually requires the removal and or rearrangement of substituents on the benzene ring. Oxygen is required as a co-substrate to produce the diol, catechol. Ring cleavage of catechol is catalyzed by oxygenases that produce muconic acid or semialdehyde. A decarboxylation in the meta pathway ultimately results in acetaldehyde and pyruvic acid. The ortho pathway yields succinic and acetic acids. These compounds, like those resulting from alkane degradation, can be used in assimilatory pathways. The oxygenase enzymes necessary to complete these metabolic activities must be encoded in the bacterial or
plasmid DNA and the enzymes must be expressed for hydrocarbon degradation to take place.

Biodegradation of gasoline contamination is not only dependent on the microbial population's metabolic competence but also on the availability of essential nutrients required for catabolism and biosynthesis. When high concentrations of carbon are present, oxygen becomes the limiting nutrient in aerobic systems [Odu, 1972]. Mineralization of high carbon and hydrogen content molecules (highly reduced) like gasoline, requires a greater oxygen to hydrocarbon molar ratio than mineralization of oxygen-containing carbon sources such as glucose. Ostendorf et al. [1991a] estimate that the complete mineralization of an octane molecule to CO$_2$ and H$_2$O requires $\approx 3.5$ oxygen to carbon mass ratio. By comparison, the oxygen to carbon mass ratio of glucose is $\approx 2.3$. The stoichiometric formulae presented below show the oxygen to carbon molar ratios of octane and glucose to be 1.6:1 and 1:1, respectively.

**Octane catabolism (mineralization)**

$$C_8H_{18} + 12.5 \text{O}_2 \rightarrow 8 \text{CO}_2 + 9 \text{H}_2\text{O}$$

**Glucose catabolism (mineralization)**

$$C_6H_{12}O_6 + 6 \text{O}_2 \rightarrow 6 \text{CO}_2 + 6 \text{H}_2\text{O}$$

Addition of oxygen to soil does not always ensure that oxygen is available to the organisms [Ostendorf, 1990]. Added oxygen may be unavailable in instances in which the pores of the matrix are blocked with
residual oil and oxygen diffusion is limited. Adding oxygen to such matrices results in poor remediation in the areas containing residual oil contamination.

In addition to oxygen limitation during bioremediation, nitrogen can be limiting and will affect the biosynthetic capacity and formation of new biomass if not supplied in adequate amounts. Nitrogen, in the form of a nitrate amendment, is much more soluble in water than oxygen but if unavailable, growth of contaminant-degrading microorganisms may be limited. The carbon to nitrogen molar ratio, for biosynthesis of new cells from octane is 8:1. Phosphorus is also required for cell metabolism and hence, contaminant degradation, but at lower levels (C:P molar ratios are ≈ 1.3:1) [Bouwer, in press].

**Biosynthesis from octane**

\[ C_8H_{18} + 7.5 \text{ O}_2 + \text{NH}_4 \rightarrow C_5H_7\text{NO}_2 \text{ (biomass)} + 3 \text{ CO}_2 + 7 \text{ H}_2\text{O} + \text{H}^+ \]

Contaminant biodegradation may be hindered or prevented when the hydrocarbon is unavailable for microbial attack. Contaminant bioavailability will be contaminant and site-specific and depend on whether the compound is insoluble, sorbed, toxic or below the threshold concentration for biodegradation. Uptake of hydrocarbons can be accomplished in three ways. Direct interaction and uptake of hydrocarbons, is most applicable when hydrocarbons of high aqueous solubility are being catabolized. The aqueous solubility of long chain alkanes is generally too low for this type of contact to be appreciable enough to sustain growth.
Secondly, microorganisms can produce solubilizing factors to render the hydrocarbon into small pseudosolubilized droplets which the microorganisms can transport into the cell. Thirdly, bacteria with hydrophobic cell walls can directly attach to large drops of aliphatic hydrocarbons. This is probably the primary means by which very insoluble liquid hydrocarbons are degraded [Gerson, 1993].

Numerous matrix and contaminant characteristics may either enhance or diminish bioavailability of substrate in subsurface environments. Matrix characteristics include soil and clay types, capillarity, heterogeneities, hydraulic conductivity, pore sizes, porosity, organic carbon-content and nutrient concentrations. Contaminant characteristics include concentration, age and types [Wilson et al., 1990]. The interaction of the contaminants with the matrix and the dynamics imposed by an ever changing biological component control the potential for microbial degradation. But one axiom will always hold true. Natural selection will always force the dominance of microorganisms with a competitive advantage.

2.3 Sorption/Desorption and Entrapment of Hydrocarbons

Hydrocarbons can be retained in soil matrices by two mechanisms; sorption and entrapment. Sorption of polar organic compounds may occur by ion exchange, ion pairing, acid-base interactions, or hydrogen bonding. The amount and distribution of organic carbon in the aquifer influences sorption of polar organic compounds.
In low carbon-content aquifers the predominant mechanism of hydrocarbon sorption is hydrophobic bonding [Wilson et al., 1990]. Non-polar organic contaminants, like gasoline, are hydrophobic. Hydrophobic compounds aggregate into drops that are excluded from the water phase of the aquifer. If the hydrophobic compounds are less dense than water, like gasoline, the drops tend to float on the water table, where they coalesce and concentrate into larger blobs. With time and repeated rainfall events, the rise and fall of the water table causes a smearing out of the blobs over the vadose zone/saturated zone interface. The weathered blobs of gasoline become entrapped within the aquifer pores because of the capillary pressures that develop within the pore matrices as the water table rises and falls (see Figure 2.3) [Wilson et al., 1990]. London-van der Waals dispersion forces between the sorbate (hydrocarbon) and sorbent (aquifer matrix) supplement this mechanism.

Gasoline trapped in pore spaces of the capillary fringe can volatilize into the gaseous phase of the vadose zone, however, they have minimal opportunity to solubilize into the water phase of the saturated zone because coalescence of the oil blobs decreases the organic/aqueous surface area available for diffusion. Therefore, the trapped gasoline constituents are excluded from the aqueous phase of the aquifer and hence unavailable for microbial degradation. Ostendorf et al. [1991] located a 0.2 m thick lens of heavy contamination at the capillary fringe of a 22 year-old aviation fuel spill site in Traverse City, Michigan. Attempts to remove the trapped aviation fuel, by increasing the ground water flow with pumping wells,
could not increase the hydraulic conductivity enough to overcome the capillary pressures within the pore network of the aquifer.

Figure 2.3. Entrapment of liquid organic hydrocarbon within aquifer pores, resulting in a residual oil saturation. After Wilson, et al. [1990].

A = maximum oil saturation. B = residual oil saturation. In the pore to the left, the pore aspect ratio (body/throat ratio) is high, resulting in a snap off and entrapment of a single oil blob. In the pores on the right, the smaller diameter (rt) pore has greater capillary pressure. Capillary pressure forces water into the smaller pore first. The water rushes through and by-passes the organic liquid in the larger pore. A stable interface is formed on the down stream side of the oil blob. The blob is trapped.
If the aquifer matrix is hydrophilic, as most are, the adhesive forces between the water-wet surfaces and the aqueous liquid will be greater than the forces between the water-wet surfaces and the hydrophobic organic liquid. A meniscus is formed between the two immiscible fluids when they are in contact with the water-wet pore surface. The contact angle of the meniscus will be acute, and a thin layer of water will line the pore, surrounding the organic liquid. A capillary pressure is created. Capillary pressure is related to the interfacial tension or surface tension as below:

\[
P_c = \text{capillary pressure} \\
\text{IFT} = \text{interfacial surface tension} \\
\varnothing = \text{contact angle} \\
rt = \text{pore radius}
\]

\[
P_c = \frac{2(\text{IFT})(\cos \varnothing)}{(rt)}
\]

The contact angle and pore radius are inversely proportional to the capillary pressure. The interfacial tension is directly proportional to the capillary pressure. Decreases in capillary pressure mean less organic liquid will become entrapped and less force is needed to mobilize the oil that is trapped. If the pore body is made more hydrophobic by adsorption of surfactant, the contact angle will increase, and again the oil will be more easily mobilized. When the contact angle is 90°, \( \cos \varnothing = 0 \) and there is no capillary pressure.

Physical parameters of the aquifer matrix are more important than properties of the organic liquid in the capillary trapping mechanism. Unrelated compounds can result in similar amounts of trapped oil in the same matrix. In short columns of packed Sevilleta soil, Wilson et al. [1990]
demonstrated that Soltrol-130 (a mixture of C10 to C13 paraffins), perchloroethylene (PCE), and kerosene, resulted in similar amounts of residual organic liquid saturation. Water saturated columns of the packed soil were first saturated with each compound, one at a time, under low injection pressures. After stabilization the soil columns were flooded with water at low velocities until a stable organic residual existed. The remaining amount of constituents were measured, compared, and found to range from 27.1 ± 1.7 to 26.3 ± 2.2 percent in repeated trials. However, when residual Soltrol saturation was measured in the same manner in a soil with an almost identical grain size curve, Traverse City soil, the residual organic liquid percent was found to be almost 10% lower. Wilson and colleagues hypothesized that small-scale layering of a small amount of fine clay in the Sevilleta soil, not present in the Traverse City soil, may have accounted for the difference. They concluded that clay layering could have produced fine heterogeneities in the packed columns or the fine clay particles may have swollen and altered the liquid organic trapping within the pores of the Sevilleta soil.

The entrapment mechanism has less significance in sediments and slurries, but still functions inside microcapillary veins of individual particles. In sediments, like those found on lake bottoms, hydrophobic compounds can be trapped inside micropores of various solid organic material, which in part (along with direct sorption) accounts for the immobilization of toxic components that may harm the aquatic environment if released [Subba-Rao et al., 1982; Lyon and Rhodes, 1991].
The degree of sorption/desorption and degradation may be affected by pH and temperature. Lemley et al. [1988] demonstrated the effect of pH and temperature on biodegradation of Aldicarb and its intermediates. It was found that pH in the range of 4.9 to 8.3 had little effect on substrate sorption of Aldicarb in a sandy loam soil, but a significant effect on Aldicarb degradation. As pH rose, the degradation of Aldicarb switched from oxidation to hydrolysis, resulting in the production of different intermediates. An increase in temperature decreased sorption and increased degradation of all the Aldicarb species. These results correlated well with results from batch and field studies of Aldicarb degradation, unlike results of solution studies without soil present; therefore, they concluded that the presence of a sorptive soil was necessary for accurate prediction of pH dependent Aldicarb degradation.

The degree of sorption/desorption may be affected by chemical properties of the sorbent and the sorbate, particularly polarity. Ogram et al. [1985] found that sorption of 2,4-dichlorophenoxy acetic acid (2,4-D) increased with increasing organic carbon content in Webster, Eustis and Cecil soils or clays. However, sorption of the negatively charged 2,4-D was inhibited on negatively charged Montmorillonite clay with no organic-carbon content.

Sorption of a contaminant to sediment has been reported to inhibit its biodegradation. Steen et al. [1980] showed the inhibition of phthalate ester degradation when it was sorbed on sediment. He offered a modification of
the Monod microbial rate degradation kinetics to include a rate-limiting sorption term as follows:

\[-d(S_T) = k_2 \frac{(S_T)(B)}{1 + K_p(r)}\]

- $S_T$ = concentration of the substrate
- $B$ = concentration of the bacteria
- $K_p$ = measured partition coefficient for the test compound with respect to sediment
- $r$ = sediment: water mass ratio
- $k_2$ = second-order rate constant

Steen and his colleagues' results indicated that sorption of phthalate ester was much more rapid than degradation and that sorption affected the kinetics solely by decreasing the amount of substrate available.

In addition, Subba-Rao et al. [1982] demonstrated that various Montmorillonite clay suspensions in lake water prevented the complete mineralization of benzylamine when the compound was present in low concentrations. At high benzylamine concentrations, mineralization from solution was faster than desorption of the compound from the clay. However, after the solution concentration of the compound had decreased, the mineralization rate slowed. They suggested ion exchange sites on the clay were competing with the bacteria for the benzylamine. As long as the concentration of the compound remained low in solution, availability of the active sites on the clay would continue to pull the benzylamine out of solution, hence making it unavailable for microbial degradation.

Biodegradation rates are dependent on both the soil or sediment (sorbent) characteristics and the type of microorganisms that are degrading the substrate (sorbate). Siragusa et al. [1986] measured the sorption rate of
p-nitrophenol from solution onto estuarine bottom sediment and found it to be rapid. He and colleagues found the desorption of the compound from the sediment to be slow. However, the rate of measured anaerobic biodegradation was less than the rate of contaminant desorption. In contrast, aerobic degradation of p-nitrophenol was faster than desorption of the compound from the sediment, suggesting the likelihood that contaminant desorption rates and hence the character of the sediment may be more important for aerobic degradation than it is for anaerobic mineralization.

In addition to contaminant sorption, bacteria may be sorbed to soil surfaces as well. Lavie et al. [1986] found that respiration was reduced in cultures of *Histoplasma capsulatum* when the mycelium was bound to Montmorillonite, Kaolinite and Attapulgite clays. The cell wall of *H. capsulatum* is composed of polymers of glucose (glucans) which can form hydrogen bonds with the clays. They surmised the reduction in respiration was not due to a decrease in nutrient availability due to sorption, but to a decrease in mycelia surface area available for substrate uptake. This indicated that soil-surface binding of organisms may decrease subsurface degradation rates.

Contrary to the above, some investigations have shown that some bacteria degrade substrates when attached to a solid surface. Ogram et al. [1985] tested three mathematical models against empirical results for the biodegradation of 2,4-D in soils. The model that best fit the data proposed that both solution phase and sorbed phase bacteria degraded only solution phase 2,4-D.
Hydrophobic bacteria can adhere to contaminated aquifer matrices. Rosenberg [1981b] demonstrated that bacteria can adhere to hydrophobic polystyrene and used this principle to develop a simple screening technique for hydrophobic bacteria. He suggested that organisms that adhered to the polystyrene were more likely to be hydrocarbon-degraders because of their hydrophobic cell walls. Lyklema et al. [1989] determined that bacterial sorption to sulfonated polystyrene surfaces were of the weak Van der Waals type. Minimal shear forces broke the attachments and revealed the adherence was reversible and characteristic of non-specific secondary minimum coagulation.

A high rate of contaminant desorption can increase the toxic effect of contaminants in an aquifer. Weissenfels et al. [1992] found that biodegradation of polycyclic aromatic hydrocarbons (PAHs) sorbed to a low carbon-content soil released toxic compounds into the fluid phase. Biodegradation of PAHs in a second soil with higher carbon-content was less; however fewer toxic compounds were detected in the fluid phase. In the higher carbon-content soil they identified two phases of PAH sorption. The fast-sorption phase was completed in hours. The slow-sorption phase that followed, lasted several days. The material sorbed to the high-carbon content soil in the first phase could be removed by biodegradation, but the slowly sorbed material (about 28% of the total) could not be degraded and resulted in a residual non-degradable oil saturation. Weissenfels and his colleagues then replaced the soils in the microcosms with XAD-2 resin. Hydrophobic XAD-2 resin has a strong affinity for PAHs. When the resin was present, no biodegradation took place. Apparently, the resin and
microorganisms were competing for the oil. This suggested that sorbents (either carbon in soils or resins in laboratory cultures) may hinder contaminant biodegradation. And, even though biodegradation rates are higher in less sorptive soils, biotoxicity of the contaminants may be greater. If contaminant desorption causes a more hazardous situation than irreversible sorption, even though it aids degradation, it may be best to simply leave sorbed contaminants alone.

In contrast to the Weissenfels et al. [1992] experiments, Wszolek and Alexander [1979] demonstrated an instance where the contaminant degradation rate out-competed the rate of contaminant sorption. In their experiments the microbial consortium used only alkylamines that were in solution and did not degrade compounds when in an alkylamine-clay complex. Higher rates of mineralization were found with larger cell inoculums and lower biodegradation rates were determined for compounds with higher molecular weights within a homologous series of sorbed n-alkylamines. The abiotic desorption rate for the alkylamine-clay complexes was determined and compared to the rate of biotic degradation of the alkylamines; they found that more compound was degraded than could be accounted for by abiotic desorption alone. They surmised that the microorganisms were somehow causing the contaminant desorption rate to increase in the biotic cultures.

If microorganisms can enhance the desorption of organic pollutants, as hinted at in Wszolek and Alexander's work, then the sorption of toxic
substrates to the subsurface matrix can not be relied upon to ensure biotoxic compounds stay sorbed and thus harmless to the environment. Evidence suggests adaptation of indigenous microflora *in situ* may result in enhanced microbial emulsification capabilities. Francy et al. [1991] found that indigenous bacteria from the contaminated soil of the Traverse City aviation fuel spill site had better emulsifying capacities than bacteria from the pristine soils at the site. If adaptation is accompanied by the excretion of extracellular products, like emulsifiers, that enhance desorption of recalcitrant hydrocarbons, the complete, long-term immobilization of toxic substrates would not be possible, except in sterile environments.

2.4 Biosurfactants

The concept of using biosurfactants to aid oil spill cleanup is an outgrowth of microbial enhanced oil recovery (MEOR). Injecting surface-active agents that mobilize the oil increases the yield of low oil bearing formations. It is believed that biosurfactants have an advantage over synthetic surfactants for oil spill clean up because they are not toxic and can be injected into the subsurface without fear of ground water contamination or environmental harm. There are numerous commercial and industrial applications for biosurfactants including cosmetics, food, pulp and paper, textiles, and ore-processing [Cooper, 1986b]. Biosurfactants with unique characteristics may be produced by strain-selecting techniques and overproducing bacterial mutants. If the cost of bacterial fermentation can be justified by increases in the quantity, effectiveness and efficiency of
biosurfactants, then biosurfactant technology may replace the use of synthetic surfactants in many cases.

The term biosurfactant refers to all substances produced by microorganisms that display surface-active properties. These surface-active agents have effects at solid-liquid, liquid-liquid, and liquid-air interfaces. Interfaces that simulate residual contamination in aquifers can be artificially produced in laboratory cultures in microcosms containing culture medium, liquid-organic substrate and a solid matrix. The aqueous and organic liquids are the liquid phases and the soil matrix or artificial matrix, the culture vessel walls, and the bacteria are the solid phases. A vapor phase also exists in sealed microcosms. It consists of the head space in the vessel, but can also include suspended air bubbles in rapidly mixed solutions. The effects produced by the biosurfactants take place at interfaces of the phase barriers in the microcosms. The interfaces affected are the solid/aqueous liquid, solid/organic liquid, aqueous liquid/organic liquid, aqueous liquid/vapor, and organic liquid/vapor interfaces (Figure 2.4a). At low concentrations biosurfactants will preferentially sorb to these interfaces and significantly reduce the amount of work required to maintain the barriers. The minimum amount of work required to maintain a phase barrier is termed the surface free energy and the amount of energy required per unit area is termed surface or interfacial tension [Zajic and Panchal, 1976].

\[ W_{\text{min}} = (g) \times A \]  
\[ W_{\text{min}} = \text{Surface Free Energy} \]  
\[ g = \text{Surface Tension} \]  
\[ A = \text{Area} \]
Biosurfactants affect the dynamics of chemical transfers across phase barriers. When surfactants accumulate at vapor/liquid interfaces, they can reduce the surface tension of the liquid. If hydrocarbons are in the head space of a microcosms, the accumulated surfactants may accelerate the hydrocarbons' rate of transfer into the aqueous phase. If solid/liquid interfaces are involved, the wetting behavior of the solid can be lowered or enhanced depending on its hydrophobicity. Cooper et al. [1980] demonstrated that microbial produced substances have this capability. He and colleagues demonstrated that *Thiobacillus thiooxidans* produces phospholipids that enhance the wettability of sulfur particles, thus enhancing the suspension of the sulfur particles in aqueous solutions. Other biosurfactants have been found that improve the mechanical dewatering of fuel grade peat by making the organic solids in the peat less water-wet, therefore making it easier to remove the water [Cooper et al., 1986a].

At liquid/liquid barriers, biosurfactants affect interfacial tension by suspending the oil phase in the water (o/w) or suspending the water phase in the oil (w/o). Biosurfactant molecules that act at liquid/liquid barriers to produce suspensions are called bioemulsifiers. Bioemulsions consist of microscopic droplets ranging in size from 0.1 to 100 μm diameter. The o/w suspensions are sometimes referred to as micellar solutions. Biosurfactants have two functions when emulsifying; they stabilize the droplets, by decreasing coalescence of like liquids, and aid in the formation of new droplets. Microemulsions of droplets ranging in size from 100 to 600 Å are also possible. Microemulsions, unlike emulsions, are optically clear.
Figure 2.4a Biosurfactant effects on phase barriers.

- Surfactant accumulates at vapor/water interface → reduction of surface tension
- Surfactant accumulates at oil/water interface → reduction of interfacial tension
- Surfactant accumulates at hydrophilic solid/water interface → changes surface to oil wet
- Surfactant accumulates at hydrophobic solid/water interface → changes surface to water wet
- Water in oil (w/o) makes an emulsion
- Oil in water (o/w) makes a micellar solution
When emulsions and microemulsions increase the amount of an immiscible organic liquid in water beyond its normal aqueous solubility, it is referred to as solubilization or pseudosolubilization. Increased solubilization increases the bioavailability of an organic liquid to bacteria and may increase the rate of degradation. Organisms growing on hydrocarbons often produce biosurfactants, though this is not a prerequisite for biosurfactant production [Rosenberg et al., 1979; Reddy et al., 1982; Efroyimson and Alexander, 1991].

All surface active molecules, whether their primary action is to reduce surface tension, alter surface wettability or create emulsions, contain a common characteristic - they are amphiphatic and have at least one hydrophobic and one hydrophilic moiety on each molecule. This structure allows the molecule to be attracted to both hydrophobic and hydrophilic components of a system and, in the process, bring the immiscible phases of compounds into contact.

The ratio of hydrophilic to hydrophobic moieties of a surface active molecule is called the hydrophilic-lipophilic balance (HLB). To determine the HLB ratio of a surface active agent, the molecular weight of the hydrophilic part of the molecule is multiplied by twenty, then divided by the molecular weight of the whole molecule. Knowing the HLB of a surface active molecule can be useful in predicting its action on phase interfaces in a system, as to whether it acts mainly as an emulsifier, a solubilizer or a wetting agent. W. C. Griffin [1954] reported a semi-empirical method for
predicting the application of surface active molecules based on their HLB values. In Griffin’s scheme, the value ten (10) represents an even hydrophilic-lipophilic balance of the molecule (See Table 2.4). Zajic and Panchal [1976] emphasized that usually a blend of surface active agents of varying HLB ranges was suitable for most commercial applications of surface active agents; enhancement of the mixing of immiscible solutions was usually best achieved by a variety of actions at the phase interfaces in a three-phase system.

<table>
<thead>
<tr>
<th>HLB range</th>
<th>application</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-6</td>
<td>w/o emulsifier</td>
</tr>
<tr>
<td>7-9</td>
<td>wetting agent</td>
</tr>
<tr>
<td>10-18</td>
<td>o/w emulsifier</td>
</tr>
<tr>
<td>13-15</td>
<td>detergent</td>
</tr>
<tr>
<td>15-18</td>
<td>solubilizer</td>
</tr>
</tbody>
</table>


The hydrophilic heads of biosurfactant molecules are usually more complex than the hydrophobic tails. In general, the polar head, if anionic, is a carboxyl, phosphate or sulfate group; a cationic polar head may contain amine groups. The non-polar tail of biosurfactant molecules is usually a saturated or unsaturated fatty acid. Most biosurfactants are glycolipids. Glycolipids include the trehalose mycolates and esters and the rhamnolipids.
and sophorolipids. Phospholipids and neutral fatty acids, which also have biosurfactant properties, are produced by some hydrocarbon-degrading bacteria and yeast [Zajic and Seffens, 1984]. Lipopeptide surfactants have been found in *Bacillus* sp., the most studied of which is surfactin [Zajic and Seffens, 1984]. Surfactin is a cyclic lipopeptide that lyses red blood cells and lowers surface tension. Figure 2.4b shows some representative biosurfactant molecules.

Figure 2.4b Structure of biosurfactant molecules. Adapted from "Production of Biosurfactants" in *Biosurfactants* [Desai and Desai, 1993].

Sophorose Lipid
\[
\begin{align*}
\text{CH}_2\text{CH} & \quad \text{CH} \\
\text{CH} & \quad \text{O} \\
\text{CH}_2\text{CH} & \quad \text{CH} \\
\text{OH} & \quad \text{O} \\
\text{CH}_2\text{CH} & \quad \text{CH}
\end{align*}
\]

Rhamnose Lipid
\[
\begin{align*}
\text{CH}_3 \\
\text{CH}_2\text{CH} & \quad \text{O} \\
\text{CH} & \quad \text{O} \\
\text{CH}_3 & \quad \text{O} \\
\text{CH} & \quad \text{O}
\end{align*}
\]

Surfactin
\[
\begin{align*}
\text{L Leu} & \quad \text{D Leu} \\
\text{L Asp} & \quad \text{L Val}
\end{align*}
\]

Sophorolipid from *Torulopsis* sp., Rhamnolipid produced by *Pseudomonas aeruginosa*, and Surfactin from *Bacillus subtilis*.

Microbiologically produced polymeric surface active agents have also been identified but their structures are less well defined. These substances tend to be bioemulsifiers that do not lower surface tension. The most studied compound of this type has been named emulsan. It is produced by a strain
of *Acinetobacter calcoaceticus* and is an extracellular heteropolysaccharide that is a very powerful emulsion stabilizer [Rosenberg et al., 1979]. Other polymeric biosurfactants have been identified, such as biodispersan, from another *A. calcoaceticus* strain [Rosenberg, 1988], liposan from *C. lipolytica* [Kappeli and Fiechter, 1977], and mannoprotein emulsifiers from *S. cerevisiae* [Cameron et al., 1985]. All of these contain sugar backbones and protein. Some also have a lipid component.

Generally the structure of a biosurfactant is bacterium- and substrate-specific. Changes in the structure of the chemical moieties found on either the tail or head end of the molecules can be made by altering the growth substrate or the culture conditions of microcosms in which the biosurfactants are produced [Cooper, 1986b]. Biosurfactant synthesis can be either *de novo* or substrate-dependent. *De novo* synthesis involves the biosynthesis of the moieties of the biosurfactant from molecules produced by the microorganism. Substrate-dependent synthesis involves the biosynthesis of the moieties of the biosurfactant molecule directly from all or parts of the carbon substrate on which it is growing [Reddy et al., 1983; Falatko and Novak, 1992]. Both *de novo* and substrate-dependent synthesis of the moieties is followed by linkage of the moieties by the microorganism to produce the total biosurfactant molecule [Desai and Desai, 1993].

Substrate specificity, or the lack of it, by biosurfactants for particular types or physical states of hydrocarbons has been demonstrated. Reddy et al. [1982] grew three yeast and one bacterial species on liquid n-hexadecane and found that removal of the emulsifying and solubilizing factors of the
culture by EDTA decreased the growth of these organisms on the liquid hydrocarbon. However, removing the factors had no affect on microbial growth when soluble hexadecane was substituted for the liquid n-hexadecane substrate. In 1983 Ready and co-workers reported isolation of a heat-stable polymeric substance from *Pseudomonas* PG-1, containing protein, carbohydrates and lipids from the organisms growing on the liquid pristane. They found two solubilizing fractions produced by the *Pseudomonas* that they thought were responsible for the degradation of liquid pristane. When the fractions were tested abiotically for solubilizing ability, one fraction showed a specificity for solubilizing the liquid pristane over other liquid n-alkanes; however, the second fraction, an emulsifier, produced a non-specific solubilization of the liquid n-alkanes in addition to the liquid pristane. They proposed that the first solubilizing factor played the predominant role in the uptake of the pristane after it had come in contact with the bacteria, while the non-specific emulsifying factor aided the degradation of pristane and the other n-alkanes by enhancing their transport to the organism.

Biosurfactant molecules can be extracellular or cell-associated. Cell mediated biosurfactants may affect the growth of specific microbial strains in the presence of sparingly soluble substrates. Rosenberg and Rosenberg [1981a] reported the isolation of a mutant strain of *Acinetobacter calcoaceticus* RAG-1 that was unable to adhere to liquid hexadecane. Gentle agitation of both the adhering wild type and nonadherent mutant type culture resulted in decreased growth in only the nonadherent mutant cultures; however, addition of an emulsan to the mutant cultures resulted in
comparable growth on the hydrocarbon. They concluded that adherence of the RAG-1 bacteria to the hydrocarbon played a crucial role in its degradation in the absence of the added emulsan. Later, E. Rosenberg and colleagues identified the emulsifying agent in the wild type RAG-1 cultures as a galactosamine-aminouronic polysaccharide [Rosenberg et al., 1983].

Efroymson and Alexander [1991] demonstrated that an organic solvent added to hexadecane- and naphthalene-grown cultures of an Arthrobacter sp. increased mineralization of both hydrocarbons. The bacteria did not excrete any solubilizing products, but analysis revealed that some were adhered to the solvent-water interface. Addition of a surfactant (Triton X-100) inhibited the mineralization of the solvent-dissolved hexadecane but increased the rate of degradation of the solvent-dissolved naphthalene. The surfactant, therefore, seemed to aid the degradation of the more aqueous soluble compound, naphthalene, but inhibit the degradation of the less aqueous soluble compound, hexadecane. They proposed the attached bacteria were degrading dissolved hydrocarbons in the aqueous phase and that adherence was more important in degradation of less soluble compounds than it was for soluble compounds.

An organism can produce more than one type of biosurfactant and does not necessarily need an insoluble hydrocarbon as a growth substrate to do so. Cooper and Goldenberg [1987] studied two biosurfactant-producing Bacillus species. The first species produced a product with strong emulsifying capacities but did not reduce surface tension when growing on water-soluble substrates (sucrose, tryptic soy broth and yeast extract). The
second species produced two biosurfactants on the same substrates. The glucosamine product isolated from this species was a bioemulsifier, which was associated with the capsule of the bacteria. The second product isolated, a monoglyceride, lowered the surface tension. Because these two products were difficult to separate, they suggested that earlier reports of biosurfactants that expressed both physiological characteristics may have actually been mixtures of products.

The concentration of surface active agent necessary to ensure its effectiveness was related to its efficiency [Zajic and Seffens, 1984]. These authors defined efficiency as the concentration required to produce a significant reduction in the surface tension of water. Effectiveness of a surfactant was defined as the minimum value to which a biosurfactant could lower the surface tension. This minimum value is related to the critical micelle concentration (CMC) of a biosurfactant. CMC is controlled by steric factors of the molecule that result in attractive and repulsive forces between its functional groups and the functional groups of the aqueous and organic liquids. It is commonly believed that the more a biosurfactant can reduce surface tension (or interfacial tension), the better it will promote solubilization and thus degradation of hydrophobic contaminants.

Present research indicates that there is much more involved in biosurfactant-mediated degradation of hydrocarbons than the surface tension reducing ability of the biosurfactant. Aronstein, Alexander and co-workers demonstrated in a series of experiments [Aronstein et al., 1991; Aronstein and Alexander, 1992] that surfactants below their CMC concentrations aided
in the degradation of phenanthrene and biphenyl. Concentrations as low as 10 and 100 \( \mu \text{g/g} \) of nonionic biosurfactants enhanced both desorption and degradation in aquifer material, and higher concentrations inhibited degradation, possibly by virtue of toxicity. However, the extent of desorption did not always predict the degree of biodegradation. Two possibilities were offered for this phenomenon. First, it was possible that the surfactants increased the rate of the desorption, thus increasing the rate at which the hydrocarbon became available to the microorganisms. Second, they surmised that the biosurfactants may have altered the strength of the sorption complex, thereby making hydrocarbon more available to the organisms without increasing the appearance of the substrate in the solution. They also found that lower organic content of the soil (0.4\% compared to 32.9\%) significantly enhanced biosurfactant performance.

The types, uses and interactions of synthetic surfactants have been thoroughly studied and many good reviews exist. The reader is referred to the texts, *Surfactants and Interfacial Phenomena*, edited by M. J. Rosen, 1989 and *Emulsions and Solubilization*, edited by K. Shinoda and S. Friberg, 1986. Microbial-produced compounds have unique and complex properties. It is apparent that much needs to be clarified about the exact mechanisms of biosurfactant enhancement of biodegradation before biosurfactants can be effectively used to enhance contaminant degradation *in situ*. Biosurfactants have been intensively investigated in recent years. Two texts, *Biosurfactants and Biotechnology* (1987) and *Biosurfactants* (1993) have excellent explanations of the recent advances in this field.
3 MATERIALS AND METHODS

3.1 Glassware

All glassware was acid-washed in concentrated sulfuric acid to which a strong oxidant was added (Nochromix cleaning solution, Godax Laboratories, Inc., Pawling, NY), then rinsed in distilled water and air dried. Openings were then covered with aluminum foil and the glassware was baked in a 123°C oven for 3 hours. This treatment sterilized the glassware and eliminated any residual trace organics present. Metal utensils were treated in a like manner except that a methanol rinsing was substituted for acid washing. Plasticware, Teflon-lined Mininert (Pierce, Rockford, IL) gas-tight valves and other Teflon apparatus was washed in laboratory-grade detergent, then rinsed in 18 MΩ/cm deionized water, Milli-Q (Millipore Corp., Bedford, MA) and in methanol. These air-dried materials were sterilized at 20 psi for 25 minutes, and then baked in a 70°C oven for a minimum of 24 hours before use.

3.2 Media, Chemicals, Mixtures and Resins

3.2.1 Media

Aqueous media were prepared using 18 MΩ/cm deionized water. The pH was adjusted between 7.0 and 7.2 with NaOH or HCl and then the media were filter sterilized through 0.2 μm disposable filters (Nalge, Co.,
Rochester, NY). Solid media were steam sterilized at 20 psi for 25 minutes at 100°C, cooled, then poured, while warm, into plates or slants as needed. Media used were as follows:

1. **Soil-Washing Medium** [Bone and Balkwill, 1986]
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Na4P2O7•10 H2O</strong></td>
<td>0.05 M</td>
</tr>
<tr>
<td>polyvinylpyrrolidone</td>
<td>0.1%</td>
</tr>
</tbody>
</table>

2. **Mineral Salts Medium** [Ridgeway et al., 1990] - HCMM2;
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KH2PO4</strong></td>
<td>1.36 grams/liter</td>
</tr>
<tr>
<td><strong>Na2HPO4</strong></td>
<td>1.42 grams/liter</td>
</tr>
<tr>
<td><strong>KNO3</strong></td>
<td>0.5 grams/liter</td>
</tr>
<tr>
<td><strong>(NH4)2SO4</strong></td>
<td>2.38 grams/liter</td>
</tr>
<tr>
<td><strong>MgSO4•7H2O</strong></td>
<td>0.05 grams/liter</td>
</tr>
<tr>
<td><strong>CaCl2</strong></td>
<td>0.01 grams/liter</td>
</tr>
<tr>
<td><strong>H3BO4</strong></td>
<td>0.00286 grams/liter</td>
</tr>
<tr>
<td><strong>MnSO4•H2O</strong></td>
<td>0.00154 grams/liter</td>
</tr>
<tr>
<td><strong>Fe(NH4)2(SO4)2•H2O</strong></td>
<td>0.00353 grams/liter</td>
</tr>
<tr>
<td><strong>CuSO4•H2O</strong></td>
<td>0.00039 grams/liter</td>
</tr>
<tr>
<td><strong>ZnCl2</strong></td>
<td>0.00021 grams/liter</td>
</tr>
<tr>
<td><strong>CoCl2•6H2O</strong></td>
<td>0.00041 grams/liter</td>
</tr>
<tr>
<td><strong>Na2MoO4•2H2O</strong></td>
<td>0.00025 grams/liter</td>
</tr>
</tbody>
</table>

3. **Phosphate-Buffered Saline (PBS)**
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Na2HPO4</strong></td>
<td>1.24 grams/liter</td>
</tr>
<tr>
<td><strong>NaH2PO4•H2O</strong></td>
<td>0.18 grams/liter</td>
</tr>
<tr>
<td><strong>NaCl</strong></td>
<td>8.5 grams/liter</td>
</tr>
</tbody>
</table>

4. **1/2 Strength Nutrient Agar** (Difco Laboratories, Detroit, MI) in 18 MΩ/cm deionized water

5. **R2A Agar** (Difco Laboratories) in 18 MΩ/cm deionized water

6. **Noble Agar** (Difco Laboratories) in HCMM2 medium
3.2.2 Chemicals and Liquid Organic Mixtures

All chemicals used for media preparation were of Reagent grade or higher purity and purchased from standard chemical houses (J.T. Baker Chemical Co., Phillipsburg, NJ; Fisher Scientific, Pittsburgh PA; Mallinckrodt Inc., Paris, Kentucky; Aldrich Chemical Co., Milwaukee, WS). Liquid hydrocarbons were of two types. The first was an aviation gasoline blend supplied courtesy of Exxon Chemical Co. USA, Baytown, TX. Its content was stated as being 10% aromatic and 90% aliphatic hydrocarbons upon receipt. Gas chromatographic analysis of this blend was performed in our laboratories. Composition is presented in Table 3.2.2. Other liquid hydrocarbons used were 2,3-dimethylpentane, 97% purity; and 2,2,4-trimethylpentane, 99.7% purity; and toluene, 99.8% purity (Aldrich Chemical Co.). These three chemicals were used individually in some preliminary experiments, but later were combined in a hydrocarbon mixture of 45:45:10 (DMP, TMP, toluene).

Solvents used for extraction and cleaning of gas chromatographic equipment were dichloromethane, 99.9% purity (Aldrich Chemical Co.) and methanol, High Purity for Purge & Trap Analysis (Aldrich Chemical Co.). p-Cumene and p-cymene were purchased from Supelco, Inc., Bellefonte, PA. These two chemicals were used as extraction and internal standards. Organic analytical standards for identification were purchased in kit form from Supelco (PolyScience Corp., Niles, IL). Liquid hydrocarbons were sterilized, when necessary, by passing through 0.45 μm Teflon filters.
Table 3.2.2 Gas chromatographic analysis of aviation gasoline supplied by Exxon Co. USA.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Compound</th>
<th>Exxon blend</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/kg</td>
<td>% mass of blend</td>
</tr>
<tr>
<td>1</td>
<td>2,3-dimethylbutane</td>
<td>4.63</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>benzene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2,3-dimethylpentane</td>
<td>1.75</td>
<td>1.7</td>
</tr>
<tr>
<td>4</td>
<td>2,2,4-trimethylpentane</td>
<td>29.91</td>
<td>29.6</td>
</tr>
<tr>
<td>5</td>
<td>2,5-dimethylhexane</td>
<td>3.85</td>
<td>3.8</td>
</tr>
<tr>
<td>6</td>
<td>2,3,4-trimethylpentane</td>
<td>12.38</td>
<td>12.2</td>
</tr>
<tr>
<td>7</td>
<td>toluene and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,3-dimethylhexane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2,2,5-trimethylhexane</td>
<td>3.66</td>
<td>3.6</td>
</tr>
<tr>
<td>9</td>
<td>ethylbenzene</td>
<td>&lt;PQL*</td>
<td>29.7</td>
</tr>
<tr>
<td>10</td>
<td>m-xylene</td>
<td>&lt;PQL</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>o-xylene</td>
<td>&lt;PQL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unknowns</td>
<td>14.90</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td>total hydrocarbon</td>
<td>101.16</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* PQL: Practical quantitation limit

3.2.3 Resins

Gas chromatographic resins tested for suitability as a gasoline sorbent, included Amberlite XAD-4, Amberlite XAD-8, Amberlite IRC-50, Amberlite IRA-938 (Rohm and Haas Co., Philadelphia, PA) and XUS
400285.00L, XUS 40323.00 (Dow Chemical Co., Beaumont, TX). More details on these resins can be found in section 3.5.6.1 of this document.

3.3 Contaminated Soil

3.3.1 Sampling Site and Sampling Procedure

The contaminated material used for the experiments came from a Traverse City, MI US Coast Guard Station. The area was the site of an aviation fuel spill that took place in 1969. This spill site has been studied extensively and some basic characteristics of the site have been compiled in Table 3.3.1.

The subsurface material was obtained by aseptic core boring utilizing a procedure developed by Dunlap et al. [1977] and modified by Wilson et al. [1983]. The drilling contractor was The Traverse Group, Inc. The boring procedure used employed a specially fabricated hollow-stem auger and ensured that samples would not be contaminated with surface biota. The sample used for soil isolations in these experiments came from 17 to 19 feet beneath the surface and was located at the water table near a previous core boring designated AV-D-6. The area was within the contaminated lens. Fig 3.3.1 is a diagram indicating the site of the core borings and showing the location of some of the previous core borings. The boring for this sample is designated RU2 on the diagram. It is located approximately 200 feet from the source of the spill.
Table 3.3.1 Traverse City Site Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>water temp. 11° - 12° C, pH near neutral</td>
</tr>
<tr>
<td>original amount spilled = 25,000 gallons</td>
</tr>
<tr>
<td>depth to water table = 16 feet, high and low water table = 15 to 17 feet</td>
</tr>
<tr>
<td>unconsolidated, sandy, homogenous matrix</td>
</tr>
<tr>
<td>first pilot scale bioremediation test, 1988</td>
</tr>
<tr>
<td>hydraulic conductivity = 1.0 x 10^{-2} cm/s</td>
</tr>
<tr>
<td>matrix maximum organic liquid saturation = 86.1%</td>
</tr>
<tr>
<td>porosity = 35%, bulk density = 1.723 g/cm³</td>
</tr>
<tr>
<td>residual oil saturation = 17.6% ≈ 0.02 kg hydrocarbon/kg wet soil</td>
</tr>
<tr>
<td>particle density, 2.65 ± 0.01 g/cm³</td>
</tr>
<tr>
<td>carbon content of subsurface matrix, 0.01%</td>
</tr>
</tbody>
</table>

[Ostendorf et al., 1991b; Ostendorf, 1990; Rifai, 1989; Kampbell et al., 1990; Francy et al., 1991; Ward et al., 1989].
3.3.2 Soil Characteristics

The borings were placed into one-quart sterile mason jars and shipped to our labs on ice, where they were stored at 4°C for approximately 2 months. Discreet areas of oily sand, intermittent with relatively clean sand,
were observed in the soil samples before they were used. Using aseptic technique, these areas were broken up and the sample was mixed to homogeneity before allocating the soil into one-pint mason jars. Samples for analysis and soil isolations were taken from the one-pint jars.

Contaminant concentrations were determined by gas chromatography/mass spectrometry (GC/MS). A Hewlett Packard Co. (Wilmington, DE) gas chromatograph/mass spectrophotometer equipped with an Ultra 2 (Hewlett Packard Co.) non-polar column (5% diphenyl and 95% dimethylpolysiloxan), 0.22 mm x 25 m was used. Constituent determination and concentration estimation was made by comparing unknowns in the samples to standards. The moisture content of the soil was determined gravimetrically and the number of microorganisms per gram of soil was determined by plate counts of colony forming units (cfu) on one half-strength Nutrient Agar (Difco Laboratories) plates.

3.4 Isolation and Quantification of Microorganisms from Soil

A method for removing organisms from soil samples, previously developed in our laboratories [Fiorenza, 1991a], was modified to include steps to remove all silt particles. This was done so that carbon in the silt could not serve as an alternative carbon source for the microorganisms in the experiments.

A known amount of contaminated soil (10 g) was removed from the Teflon-sealed mason jar designated RU2SS2b. The soil was washed three
times with 10 ml of 0.1% PVP medium. Each washing was vortexed for 1 minute at a setting of ten. All washings were collected into one tube and differentially centrifuged. The supernatant fluid was collected from an initial spin of 482 g for 10 minutes. This was followed by a 12,000 g spin for 15 minutes. This pelleted the organisms and removed most of the sand and silt from the sample. The pellet was resuspended in 100 ml of HCMM2 medium amended with 0.3% yeast extract. The cells plus debris were incubated at 22°C with gentle stirring on a magnetic stir plate.

After 3 days, the culture flask was turbid and a small rim of brown silt rimmed the flask at the medium/air interface. Approximately 30 ml of the turbid broth was removed to a centrifuge tube and the cells plus some carry-over silt were centrifuged at 12,000 g for 15 minutes. The supernatant fluid was discarded and the remaining (pink over brown) layered pellet was resuspended in 35 ml of HCMM2 medium by vigorous vortexing for 1 minute. The suspension was centrifuged again and a small portion of the pellet was stained with crystal violet. Microscopy revealed that non-microbial particulate debris and microorganisms remained in the brown- and pink-layered pellet. Only the upper pink layer of the pellet was resuspended in medium with gentle aspiration from a Pasteur pipette. The suspension was transferred to a fresh centrifuge tube and the more firmly packed brownish layer of the pellet was discarded. The cells from the pink layer were centrifuged as before and rechecked for debris by crystal violet staining. Again some debris was visible. The procedure was repeated once more, at which time, no debris was observed by microscopy. The washed pellet was resuspended in 10 ml of HCMM2. Appropriate dilutions were
made in crystal violet staining solution and the cell concentration was determined by a direct cell counting technique discussed in section 4.5.1 of this document.

3.5 Experimental Systems and Techniques

3.5.1 Direct Cell Counting Technique

A direct cell counting technique was developed so that inoculum size could be standardized and cell concentrations could be measured quickly. Flasks and microcosms were inoculated with cells that had been centrifuged at 12,000 g for 15 minutes, then resuspended in a known volume of medium inside the centrifuge tube. One tenth ml of the suspension was used to determine cell concentration and then a portion of the remaining cell suspension was appropriately diluted to deliver a known number of cells to each culture vessel. When determining cell concentration in microcosms during an experiment, 0.1 ml of the liquid phase was removed after vortexing the vial for 4 seconds.

To count the cells, the 0.1 ml of cell suspension was diluted in a staining solution. The solution was made weekly, by adding 12 ml of crystal violet (Difco Laboratories) directly to 100 ml of either PBS or mineral salts medium, pH 7.0 to 7.2. The staining solution was filtered through a 0.2 µm filter to remove gross stain particulate and then stored at 4°C prior to use. To standardize inoculum size at the start of each experiment, 0.1 ml sample of cell suspension from the cell suspension tube
was serially diluted in the staining solution. To determine cell concentration during the experiment, 0.1 ml of liquid phase from the microcosms was diluted from 1:2 to 1:150 in the same staining solution. The appropriate dilution was selected by estimating the cell concentration visually, then using that estimation to mathematically determine the dilution that would approximate 50 to 100 cells per microscopic field.

Immediately after dilution, clean lint-free hemacytometer counting chambers were carefully filled with the stained, cell suspension that had been dispersed by vortexing. The chambers were put in a sealed plastic box with some water in the bottom to retard evaporation and the box was placed in a 4°C refrigerator for a minimum of 1 hour before counting. Cells could be stored in this manner for 2.5 hours without appreciable alteration in cell counts. This cool down period was necessary to make the cells easier to count by 1) decreasing bacterial motility, and 2) causing the bacteria to settle on the surface of the counting chamber, thus decreasing the depth of field in which the bacteria were observed. After refrigeration, each chamber was counted within 15 minutes before the bacteria regained motility as the chambers warmed to room temperature.

The chambers were counted at 400X (40 X objective and 10 X ocular) on bright field on a Nikon Optophot microscope (Nippon Kagaku, USA, Inc., Garden City, NY). Four fields of 1/250 cubic mm volume were counted per each dilution. The counts from all four fields were averaged and
the mean was multiplied by 250,000 (hemacytometer volume dilution) and by the staining dilution to determine the number of cells per cubic centimeter (cells/ml).

Cell concentration determinations from direct cell counting were correlated with the results of counting colony forming units (cfu) by the viable cell spread plate method in an experiment where both techniques were used simultaneously. A 7-day growth curve comparing both counting techniques was determined as part of a larger experiment during which cell surface tension readings and cell concentrations were determined in triplicate. The culture flasks were batch sampled. Vapor from a hydrocarbon mixture of DMP, TMP and toluene was used as the carbon source. The cell inoculum was $1 \times 10^7$ cell/ml of HCMM2 medium. The cell concentrations were measured at eight time periods, both by direct and plate counts. Results of the correlation can be found in section 4.1.1 of this document.

3.5.2 Preliminary Experimental System and Techniques

In preliminary experiments, except where stated otherwise, organisms were grown in the presence of a soluble carbon source dissolved in HCMM2 medium or in HCMM2 medium contained in 250 ml biometer flasks with vapor-phase hydrocarbon as the carbon source. In the case of vapor-phase hydrocarbon, liquid hydrocarbon was allowed to volatize into the headspace of the biometer flask then dissolve into the medium, until solubility was reached (Figure 3.5.2). All flask cultures were sampled for various
parameters, batch-wise, when necessary through Teflon-lined needles inserted into the flask through Teflon-coated stoppers. The flasks were incubated at room temperature (22° to 25° C) on an horizontal reciprocal shaker (Eberbach Inc., Ann Arbor, MI) set at 90 strokes/minute on a 1/2 inch stroke path. In later experiments, specially-designed vapor microcosms were used and destructively sampled when necessary. The microcosm culture system will be discussed in detail in section 3.6. The specially-designed microcosms were incubated on the same shaker placed inside a humidified culture chamber (Sherer-Gillett Co., Marshall, MI) with temperature set at 22° C and humidity at 60%.

Figure 3.5.2 Preliminary experiment culture conditions
3.5.3 Growth of Soil Consortium on Various Carbon Sources

Growth of the soil consortium on various carbon sources was assessed in 250-ml biometer flasks to which 100 ml of HCMM2 had been added. In some cases the mineral medium was supplemented with yeast extract in concentrations of 0.03 or 0.3%. To assess the consortium's ability to metabolize hydrocarbon, yeast extract was eliminated from the medium and liquid hydrocarbon was placed in the side arm of the biometer flask. The flasks were sealed and incubated at room temperature (22° to 25°C) on magnetic stir plates or on a reciprocal shaker for up to 16 days. Hydrocarbons tested as carbon sources were aviation gasoline blend or 2,3-dimethylpentane and 2,2,4-trimethylpentane, singly and in combination. Also tested was a mixture of the latter two compounds to which toluene had been added. Growth was considered positive when visible turbidity of the culture broth was detected and was qualitatively ranked from 0 to 4. Results can be found in section 4.2.2 of this document.

3.5.4 Growth of Soil Consortium at Various Carbon Concentrations

Growth of the soil consortium on various amounts of a liquid hydrocarbon mixture supplied to the 10-ml microcosms in the form of vapor was determined over a 235 hour period. Each vapor microcosm had 200 mg of clean XAD-8 (Rohm and Haas Co.) resin floating in the outer well in order to include a check for the effect on growth of adding this sorbent to the culture system. Ten ml of $1 \times 10^7$ cells/ml was inoculated into the outer chamber. Various volumes of liquid hydrocarbon mixture (0.04, 0.08, 0.4,
or 0.8 ml) were placed in the inner well. The number of microorganisms was determined by direct cell counts at five sampling times (Section 4.2.3).

3.5.5 Growth and Surface Tension of the Soil Consortium Without a Carbon Source

In a similar experiment, the growth of the soil consortium with no carbon source was checked. This experiment lasted for 188 hours and measurements were taken at three sampling times. As before, the number of cells/ml was determined by direct cell counts. In addition, surface tension was monitored in the samples. The microcosms had 100 mg, instead of 200 mg, of clean XAD-8 (Rohm and Haas Co.) resin in the outer well and there was no liquid hydrocarbon added to the inner wells. The initial cell inoculum was the same. Results can be found in section 4.2.4 of this document.

3.5.6 Adsorbents

3.5.6.1 Adsorbent Selection

To assess the effects of the various physical states of the carbon source on the microbial consortium, it was necessary to select an appropriate sorptive matrix for the hydrocarbon mixture. Several different gas chromatographic resins were evaluated for this purpose. Some of the resins' characteristics, as reported by the manufacturers, can be found in Table 3.5.6.1.
Table 3.5.6.1 Characteristics of resins that were tested as possible adsorbents.

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Company</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amberlite XAD-4</td>
<td>Rohm and Haas Co.</td>
<td>Polystyrene/DVB</td>
</tr>
<tr>
<td>Amberlite XAD-8</td>
<td>Rohm and Haas Co.</td>
<td>Polymethacrylic (acrylic ester)</td>
</tr>
<tr>
<td>Amberlite IRC-50</td>
<td>Rohm and Haas Co.</td>
<td>DVB/methacrylic acid polymer</td>
</tr>
<tr>
<td>Amberlite IRA-938</td>
<td>Rohm and Haas Co.</td>
<td>Amine/DVB/Styrene copolymer</td>
</tr>
<tr>
<td>XUS 40285.00L</td>
<td>Dow Chemical Co.</td>
<td>DMA/Styrene/DVB/Cl-Met copolymer</td>
</tr>
<tr>
<td>XUS 40323.00</td>
<td>Dow Chemical Co.</td>
<td>DVB, EVB copolymer</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>adsorbent</th>
<th>type</th>
<th>°C</th>
<th>stability</th>
<th>specific gravity g/cc</th>
<th>water slb</th>
<th>surf area m²/g</th>
<th>% H2O</th>
<th>pore radius range, Å</th>
<th>avg. pore dia, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>XAD-4</td>
<td>non-ionic non-polar</td>
<td>250</td>
<td></td>
<td>(1.02)</td>
<td></td>
<td>725</td>
<td></td>
<td>30 - 1,100</td>
<td>40 - 60</td>
</tr>
<tr>
<td>XAD-8</td>
<td>non-ionic intermediate polarity</td>
<td>250</td>
<td></td>
<td>(1.09)</td>
<td></td>
<td>160</td>
<td>52</td>
<td>30 - 1,100</td>
<td>240</td>
</tr>
<tr>
<td>XUS 40323.00</td>
<td>non-ionic non-polar</td>
<td>250</td>
<td>inert</td>
<td>(1.07)</td>
<td>none</td>
<td>650 min c</td>
<td>40 - 48</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>XUS 40285.00L</td>
<td>low-polarity anionic mostly hydrophobic</td>
<td>230</td>
<td>stable</td>
<td>(1.07)</td>
<td>none</td>
<td>800 min c</td>
<td>45 - 55</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>IRA-938</td>
<td>anion</td>
<td>60</td>
<td></td>
<td>(1.07)</td>
<td>inslb</td>
<td>7.3</td>
<td>72</td>
<td>25,000 - 250,000</td>
<td></td>
</tr>
<tr>
<td>IRC-50</td>
<td>cation</td>
<td>100</td>
<td>stable</td>
<td>(1.1 - 1.4)</td>
<td>inslb</td>
<td>1.8</td>
<td>45</td>
<td>200 - 2000</td>
<td></td>
</tr>
</tbody>
</table>

Note:
- a burn at °C: the maximum temperature to which the resin can be heated before it burns;
- b water slb: aqueous solubility;
- c min: minimum surface area.
The resins being considered for selection were twice rinsed with 100 ml of 18 MΩ/cm sterile water. After each rinse the water was aspirated off. Then each resin was washed twice with 35 ml of methanol. After each rinse the methanol was aspirated off. The cleaned resins were placed in sterile petri plates and baked in a 70°C oven for 24 hours till they appeared completely dry. The clean, dry resins were used in cultures and tests as described below.

Selection of adsorbent was determined by testing several different resins for their biodegradability. Lack of growth, scored by visual turbidity on a scale of 0 to 4, when compared to control cultures containing a yeast extract carbon source, meant the particular resin could not support microbial growth and was not biodegradable. The resins were also tested for toxicity to microbial growth by culturing soil microbes in the presence of the resin plus 0.3% yeast extract as a carbon source. In this case, lack of or diminished growth when compared to controls meant that the particular resin was toxic to microbes. Results of these tests can be found in section 4.1.2 of this document.

In addition, each resin was tested for its effect on surface tension under abiotic conditions. In this case the resin was simply placed in sterile mineral medium and over a period of 14 days was periodically checked for any significant change in surface tension.

Resins were qualitatively checked for their ability to sorb hydrocarbon (aviation gasoline) by a simple 'sink or float' test. In this test, the resins were
first cleaned and dried as above, then 5 ml of aviation gasoline was added to
each resin in a tube. Tubes were vortexed frequently for the next 24 hours
to ensure ample sorbate/adsorbent contact time. The excess gasoline was
removed by vacuum, then each tube was placed under a gentle stream of
filtered, zero nitrogen until it appeared dry and the resin beads no longer
stuck together (a matter of minutes). Finally 10 ml of mineral medium,
supplemented with 0.3% yeast extract, was added to each tube. After
vortexing the tubes for 1 minute, the resins either sank or floated. Floating
resins were assumed to have had enough gasoline sorbed within them to
make the loaded resin less dense than water. Resins that sank were
assumed to be incapable of sorbing enough hydrocarbon. It must be noted
that water-saturated clean resins always sank as their true wet densities were
all greater than water, however after clean resins were baked dry, they
floated. The tubes of hydrocarbon sorbed resins were checked over the next
14 days to see if they remained floating, and remained sterile (by virtue of
turbidity). Results of this test can be found in section 4.1.2. Of the many
different types of adsorbents tested, only one, Amberlite XAD-8 (Rohm and
Haas Co.) was used routinely in later experiments. See Figure 3.5.6.1 for the
chemical structure of this macroreticular polymer.
3.5.6.2 Adsorbent Cleaning and Sterilization

A cleaning method that was a modification of the manufacturer's recommended procedure was developed [Rohm and Haas Gas Chromatography Resins Manual]. First the resin was loaded into a glass column and backflushed with 5 bed volumes of 18 MΩ/cm water. Second, the resin was down washed with a minimum of 5 bed volumes of methanol. Third, the resin was dried in the column by passing a stream of zero nitrogen over it. Several columns full of cleaned resin were combined into one prepared glass culture dish and placed in a 70°C oven and baked for several days to drive off all moisture.
Next the resin was autoclaved in the same glass culture dish for 25 minutes at 20 psi at 100°C. It was checked for sterility by placing approximately 0.5 g into each of five, 10 ml culture vials containing HCMM2 and 0.3% yeast extract. Four of the five vials were contaminated with turbid growth within one week. The autoclaving process was repeated two more times before all five test vials remained uncontaminated for two weeks. A gram portion of the cleaned resin was checked by gas chromatography for impurities (peaks in the range of hydrocarbons that were to be used in the experiments) and none were detected.

3.5.6.3 Loading of the Sorbate into the Adsorbent

The clean, sterile resin was divided into batches and some of it was loaded with hydrocarbon mixture, some of it with aviation gasoline and some was left unloaded as a clean resin control.

First, a stock batch of liquid hydrocarbon mixture was made by weighing appropriate amounts of 2,3-dimethylpentane, 2,2,4-trimethylpentane and toluene into an acid-cleaned, sterile bottle. The result was a 45:45:10% mixture (wt/wt) of these compounds. This mixture and the aviation gasoline blend were stored at -20°C, and were the only sources of hydrocarbon used for the final five experiments.

Both the aviation gasoline and hydrocarbon mixture were loaded into the clean sterile resin in the same manner. An appropriately cleaned and sterilized Ace glass column (Ace Glass Inc., Vineland, NJ) was assembled
inside a sterile cabinet with Teflon end caps and valves. Each end had a Teflon filter attached in place before the column was weighed. The column top cap was removed inside the sterile cabinet and approximately 15 grams of the cleaned resin was poured in. After the cap was replaced, the column was weighed again and the amount of clean resin was determined gravimetrically.

After returning the column to the sterile cabinet, 50 ml of liquid hydrocarbon was forced in through the bottom valve and filter of the column by syringe. The resin inside the columns expanded about 23% of its length within the first 15 minutes and bubbles that formed in the column were tapped out. After 2 hours of standing, the bottom valve was opened and the excess organic liquid was drained off through the filter, then a zero nitrogen gas line was attached to the bottom filter/valve assembly. A vacuum pump was attached to the top filter/valve assembly. Pump and air pressure were applied simultaneously to the column.

A visual line of drying resin could be observed in the column. When this line reached one half the column distance (a matter of minutes), the column was inverted and the pressure and vacuum lines were reversed. The drying line was then traced up the remaining length of the column. When dry, the resin would fall freely back and forth inside the column. The column was again weighed and the sorbate/adsorbent ratio was calculated.

Four batches of resin sorbed with the hydrocarbon mixture were made. All four batches were combined into one stock jar. The average
amount of hydrocarbon mixture sorbed by the resin was 33.45 % (w/w) (range; 29 to 39%). The amount of aviation gasoline sorbed was 29.55 % (only one batch was made). The densities of both the liquid aviation gasoline and the liquid hydrocarbon mixture was 0.726 g/cc. From these facts it was determined that 150 mg of the hydrocarbon-sorbed resin would contain 50 mg of the hydrocarbon mixture. Likewise, 169 mg of the aviation gasoline sorbed resin would contain 50 mg of aviation gasoline. Both stock batches of sorbed resin were tightly sealed with Teflon lids, placed within a bell jar and stored at 22° to 25°C to minimize condensation.

Gas chromatographic analysis for quantity and quality were performed on both stocks of sorbed resins. Gas chromatographic readings were made on a Hewlett-Packard Gas Chromatography model HP 5890A equipped with an HP 3392A Integrator and a flame ionizing detector (Hewlet Packard Co.). The gas chromatograph was equipped with a 100% dimethylpolysiloxone (non-polar) column, 5m x 0.53 mm x 2.65 μm film thickness. The injection stream was split 4 to 1. Initial temperature was 30°C with a 0.5 minute equilibration time. Temperature was ramped at a rate of 10°C/minute. The final temperature was 157°C. The detector temperature was 225°C. The composition and concentrations of the stocks are reported in Table 3.5.6.3.
Table 3.5.6.3 Gas chromatographic analysis of the stock batches of sorbed resin.

<table>
<thead>
<tr>
<th>RT c</th>
<th>Hydrocarbon</th>
<th>Sorbed aviation gasoline d</th>
<th>%</th>
<th>Sorbed hydrocarbon mixture e</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.28 2,3 dimethylbutane</td>
<td>0.6</td>
<td>10.505</td>
<td>22.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.83 benzene</td>
<td>19.7</td>
<td>19.744</td>
<td>42.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5.18 2,3 dimethylpentane</td>
<td>2.256</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.60 2,2,4 trimethylpentane</td>
<td>7.908</td>
<td>15.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6.49 2,5 dimethylhexane</td>
<td>a 20.523</td>
<td>a 41.0</td>
<td>b 15.964</td>
<td>b 34.5</td>
</tr>
<tr>
<td>6</td>
<td>6.90 2,3,4 trimethylpentane</td>
<td>3.182</td>
<td>6.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>7.01 toluene and 2,3</td>
<td>unknowns</td>
<td>5.989</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>7.61 2,2,5 trimethylhexane</td>
<td>9.74</td>
<td>o-xylene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>9.07 ethylbenzene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.24 m-xylene</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i i</td>
<td>9.74 o-xylene</td>
<td>100</td>
<td>46.207</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

_ extraction efficiency 100 92.4_

a toluene and 2,3-dimethylhexane co-elute, b toluene only, c retention time, d 50 mg of aviation gasoline sorbed to 119 mg of resin, e 50 mg of hydrocarbon mixture sorbed to 100 mg of XAD - 8
3.6 Experimental Conditions for Experiments I, II, III, IV and V

It was necessary to design an experimental system in which all factors could be controlled while one parameter (the physical state of the carbon source) was manipulated. One experimental design was used for all experiments, after the preliminary experiments were completed. Some of these experiments were growth curves (Experiments I and II), others were destructively sampled at only two time periods (Experiments III, IV and V). The experimental system devised (Figure 3.6) for experiments I through V utilized:

i.) a consortium of organisms isolated from Traverse City contaminated material (core boring RU2SS2),

ii.) a defined mixture of hydrocarbons, and

iii.) the presence or absence of a sorptive matrix (XAD - 8).

Microcosms were constructed of 25-ml glass scintillation vials in which a piece of glass tubing had been fused to the interior to form a small inner well 20 mm high. (Figure 3.6) Liquid hydrocarbon was added to the inner vial in those experiments in which vapor-phase hydrocarbon was as the carbon source. The microcosms were sealed using a Teflon gas-tight screw cap. The carbon source was either sorbed within the resin which floated upon the aqueous layer (S) or allowed to vaporize from the inner well, then solubilize into the aqueous phase (V). An intermediate condition, that included air-filled clean resin in contact with the aqueous phase and vaporized hydrocarbon, was also included (R + V).
Figure 3.6 Three different methods used to supply hydrocarbon to the microcosms.

<table>
<thead>
<tr>
<th></th>
<th>S sorbed hydrocarbon</th>
<th>R+ V clean resin + vapor hydrocarbon</th>
<th>V vapor hydrocarbon no resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>hydrocarbon (mg)</td>
<td>50</td>
<td>43.8</td>
<td>44.5</td>
</tr>
<tr>
<td>% 2,3 DMP</td>
<td>22.7</td>
<td>45.8</td>
<td>45.2</td>
</tr>
<tr>
<td>% 2,2,4 TMP</td>
<td>42.7</td>
<td>10.4</td>
<td>10.3</td>
</tr>
<tr>
<td>% toluene</td>
<td>34.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrocarbon (ml)</td>
<td>0.07 (50 mg)</td>
<td>0.07 (50 mg)</td>
<td></td>
</tr>
<tr>
<td>ppm per microcosm</td>
<td>5000</td>
<td>5000</td>
<td>5000</td>
</tr>
<tr>
<td>resin (mg)</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>cells/ml</td>
<td>$1 \times 10^7$</td>
<td>$1 \times 10^7$</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>medium, 10 ml</td>
<td>HCMM2</td>
<td>HCMM2</td>
<td>HCMM2</td>
</tr>
<tr>
<td>microcosm capacity</td>
<td>21.5 cc</td>
<td>21.5 cc</td>
<td>21.5 cc</td>
</tr>
</tbody>
</table>

a 50 mg (0.07 ml) of aviation gasoline was substituted for hydrocarbon mixture in some microcosms in Experiment II. b % amount determined by gas chromatography analysis after extraction of the entire microcosm contents (liquid-phase, resin and vapor).

c 119 mg of resin was substituted in some microcosms for 100 mg in Experiment II.
Standardization of each component in each treatment regime was, in part, unsuccessful due to the fact that the resin drying process changed the relative percents of the gasoline components in the hydrocarbon mixture in the S regime. The most volatile component, 2,3 DMP, was lower in S than in the V or R+V treatments, while the less volatile component, toluene, was enriched. Therefore, the S treatment regime had the same total amount of hydrocarbon per microcosm, but the relative percents of the three constituents were not the same as the V and R+V treatments.

The cell inoculum was standardized by inoculating the microcosms with an automatic repeating pipettor. The total amount of resin and sorbed hydrocarbon present were standardized by weighing either the loaded or clean resin into the outer chamber of each microcosm. The amount of liquid (to vapor) hydrocarbon was standardized by placing the organic liquid within the inner well of each microcosm with a Teflon automatic repeating pipettor. The microcosms were then color coded and destructively sampled at different times in order to measure parameters.

3.6.1 Standard Procedures

Several standard procedures were used in all experiments. All operations requiring sterility were performed inside either a Class II, horizontal laminar flow cabinet (NuAire Inc., Plymouth, TX) or a Class III, vertical laminar flow containment cabinet (Baker, Inc., Portland, Maine). Both cabinets were equipped with high efficiency particulate filters to ensure a sterile working environment.
Microcosms were sampled for cell concentration, surface tension, emulsification capacity, hydrocarbon concentration and diversity of the microbial consortium. Triplicate microcosms were sampled for hydrocarbon concentration in all experiments and quintuplet microcosms were sampled for the other parameters assayed in Experiments III, IV and V. All measurements were repeated 3 or 4 times for each microcosm sampled. Microbial diversity was determined using five pooled microcosms from each regime at the beginning and end of Experiment V. The contents of the pooled microcosms were plated in triplicate and two to three plates of each dilution series were used for each analysis.

3.6.1.1 Inoculation of Microcosms

Microcosms were inoculated with sorbent, hydrocarbon and cell inoculum derived *de novo* for each experiment from the same jar (RU2SS2b) of contaminated Traverse City soil, as quickly as possible during the set up of each experiment. First, all microcosms were color coded and hydrocarbon-sorbed resin or clean resin was weighed into the outer chamber of each. The microcosms were quickly sealed with Teflon gas-tight caps. Next, the cell concentration of the microbial mixture was determined and a stock inoculum of $1 \times 10^7$ cells/ml of HCMM2 was made and placed in a 1000 ml Erlenmeyer flask. A magnetic stir bar was added to the flask and it was gently stirred and cooled to 4°C before and during inoculation in order to inhibit growth while all microcosm manipulations were being completed. Abiotic controls received HCMM2 medium without the microbial mixture from another flask that was treated in a like manner. Thirdly, 10 ml of either
the cell inoculum or the control medium was delivered to the outer well of five microcosms at a time with an automatic repeating pipettor. Finally, hydrocarbon mixture was delivered to the inner well of the five microcosms with a Teflon-lined automatic repeating pipettor, when needed and then the five microcosms were quickly resealed. The same stock hydrocarbon mixture was used for all experiments (in addition, an aviation gasoline mixture was used in Experiment II). The same stock hydrocarbon mixture was also used in emulsification tests and fume/agar plate cultures. With the exception of the first step - weighing in the resin and labeling the microcosms, the set up for each experiment was completed within 1 hour.

3.6.1.2 Incubation of Microcosms and Agar Plates

Microcosms were incubated on a horizontal reciprocal shaker, set at 90 strokes/minute with a 0.5 inch stroke path, which was placed inside a humidified growth chamber set at 60% humidity and 22 °C. Agar plates and slants were incubated at 22° to 25°C. Noble agar (Difco Laboratories) plates, prepared with HCM2 medium were incubated in the presence of hydrocarbon fumes inside a sealed fume cabinet at room temperature.

3.6.1.3 Surface Tension Measurements

Each microcosm was vortexed for 4 seconds to evenly resuspend and redistribute the cells before samples were removed. One tenth ml of the cell suspension was removed from each microcosm for direct cell counts. Surface tension readings were determined using a DuNouy ring interfacial
tensiometer model 70545 (CSC Scientific, Fairfax, VA) using 9 ml of the remaining cell suspension.

Nine ml of the liquid from the microcosm chamber containing intact cells and culture fluid was used for cell-mediated surface tension readings. For extracellular surface tension measurements, the liquid contents from the chamber of each microcosm was centrifuged at 12,000 g for 15 minutes, and 9 ml of the cell-free supernatant fluid was tested. Samples were placed in beakers and allowed to sit undisturbed for 30 minutes before measurements began in order to allow sufficient time for surface tension to form.

Each microcosm was measured 3 times with a 30 minute interval between each reading. The result, in dyne/cm, was corrected mathematically using a table of correction factors found in the CSC DuNouy Tensiometer Instruction Manual. Each sample surface tension reading was normalized to the cell concentration determined for that specific microcosm.

3.6.1.4 Emulsification Capacity Measurements

Emulsification capacity was analyzed using a modification of a method developed by Francy et al. [1991], that was in turn modified from Broderick and Cooney [1979]. After vortexing each microcosm for 4 seconds, the cap was removed and 0.1 ml of culture broth was sacrificed for a direct cell count to determine cell concentration. Nine ml of the remaining cells and culture fluid was then removed to a methanol washed and baked centrifuge tube. Using the cell concentration determination, the cells were
diluted to a concentration of $5 \times 10^8$ cells/ml with HCMM2 medium. After vortexing again, 4 ml of the culture was immediately placed in a 13 mm x 125 mm glass screw cap tube. The remaining culture fluid was centrifuged at 12,000 g for 15 minutes to pellet the cells. After pelleting, 4 ml of the cell-free supernatant fluid was placed in a second screw cap tube. One ml of the hydrocarbon mixture was layered over the top of the cell-free or cell containing fluids in each tube. Each tube was vortexed at a setting of 10 for 10 seconds. All tubes were left undisturbed for 2 hours before they were scored for emulsification capacity on a scale of 0 to 4 (Figure 3.6.1.4). This modified procedure normalized the cell concentration in each sample so accurate comparisons of emulsification capacity could be made.

**Figure 3.6.1.4 Emulsification capacity scoring**

![Figure 3.6.1.4 Emulsification capacity scoring](image)

---

3.6.1.5 Gas Chromatography Extraction Procedure

Gas-tight sealed microcosms were frozen at -20°C after 0 or 140 hours of incubation then held for 24 hours before extraction. All injections and extractions were accomplished through Teflon gas-tight valves with gas-
tight syringes equipped with Teflon-lined needles. The entire contents of each frozen microcosm was extracted with 2 ml of cold dichloromethane after the microcosm had been spiked with 11.7 μl (10 mg) of p-cymene, to determine extraction efficiency. After injection, the microcosms were laid on their sides in a tray and placed in a 4°C reciprocating shaker at 220 rpm while thawing. After thawing (≈ 1 to 1.5 hours), they were inverted and refrozen for at least 1 hour. This allowed the liquid lower organic-layer to be easily separated from the solid frozen aqueous-layer. Approximately 1 ml of the organic-layer from each microcosm was placed in a Teflon-sealed vial and stored at -20°C until gas chromatographic analysis could be performed.

3.6.1.6 Gas Chromatography

On the day of analysis, the gas chromatograph was calibrated with known standards of the hydrocarbon mixture. A 1 to 1000 dilution of the extractant from each microcosm was made in cold dichloromethane. The dilution was spiked with an internal standard of 10 μl (10 μg) of a stock 1 to 1000 dilution of p-cumin in methanol. One μl injections of each dilution were run on the gas chromatograph at a range of 3 and an attenuation of 3, in most cases. The chromatogram readouts were corrected individually using a mathematically determined correction factor derived from the known amount of p-cymene spiked in prior to extraction. Extraction efficiency, as determined by recovery of p-cymene, was within 10% of the expected value.
3.6.1.7 Viable Plate Counts and Purification of Microbes

Viable counts were determined for both the Traverse City soil, and on culture fluid after 140 hours of culturing under the various treatments of Experiment V. The spread plate technique was used and two types of media were used for the assay, R2A agar and Noble agar (Difco Laboratories). In all cases, three dilution series of each treatment group were plated. The number of colonies were counted on plates that contained between 30 and 300 colonies for statistical significance. The colony numbers were counted after the second day on the R2A agar plates and after 3 to 5 days on Noble agar plates.

Within 7 to 10 days, colonies on R2A plates had grown to a size and appearance that enabled differentiation of the colony type. At this time, differential counts of the various colony types were made on dilution plates that had well-spread colonies. The colony types were labeled alphabetically and a description was recorded. Then an attempt to purify each colony type was made. Each isolate was streaked three times on R2A agar and eventually five dominant species were identified and confirmed by Microbial Specialist, Inc., Houston, Texas.
4 RESULTS

4.1 New Methods Development

Two initial problems to the experimental system design were encountered. A quick and accurate method was needed to standardize the number of cells delivered to each microcosm and a method to deliver quantitative amounts of sorbed hydrocarbon to each microcosm was needed.

4.1.1 Direct Cell Counting Technique

A direct cell count technique, that relied on staining and microscopic counting of the bacteria was first used in a batch experiment. A growth curve of soil isolates using both the direct cell count method and viable count technique was performed (see Methods 3.5.1). The carbon source for this trial was vapors of aviation gasoline. The results of the two methods were compared, and the pros and cons of each technique were determined.

The results of the two methods are depicted in Graph 4.1.1a. Cell concentration, determined by either sampling method, indicated the cultures remained in log phase past the 75-hour sample point. The surface tension of the medium decreased during log phase, ≈ 15 dynes per cm (Graph 4.1.1b). After 140 hours, both direct and viable cell counts indicated the culture remained in stationary phase for the next two days during which time the surface tension remained relatively constant.
Graph 4.1.1a Comparison of direct and viable cell counts.

n = 3; Δ LOGPCC: log of cell concentration determined by viable plate counts; O LOGHCC: log of cell concentration determined by direct cell count

Graph 4.1.1b Whole cell surface tension of cultures with respect to time.
The direct cell count technique was adequate for quickly determining cell concentration in this experiment. Direct cell count results were not identical to the viable plate method results but showed a correlation (Table 4.1.1). The direct cell count technique's average standard deviation was slightly less than the viable count technique's, 14% compared to 20%. Both methods were more accurate after the cells entered stationary phase growth. Direct cell counts were on an order of 0.3 to 0.7 logs higher than the viable counts.

The direct count technique had some advantages over the viable count method. Direct count cell concentrations were determined within one or two hours of sampling. The viable count method required a 2 to 3 day waiting period before individual colony forming units were large enough to be counted. This eliminated the viable count method for real-time standardization of cell inoculum. Large amounts of agar medium were required for the viable count method but not for direct cell counts. A sophisticated light microscope and practice in viewing and recognizing bacterial cells were required for direct counting and this might make the technique more subjective than the viable count method. This subjectivity could be diminished by using the same operator throughout the experiments. When this was done, relative comparisons of cell counts throughout the experiment were assumed to be meaningful.
Table 4.1.1 Surface tension and 8-day growth curve of contaminated Traverse City soil microorganisms.

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Direct counts</th>
<th>Viable counts</th>
<th>Surface tension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>time in hours</td>
<td>cell concentration cells x 10^6</td>
<td>logd</td>
<td>cell concentration cells x 10^6</td>
</tr>
<tr>
<td>0</td>
<td>10.0 ± nd</td>
<td>7.0</td>
<td>5.2 ± nd</td>
</tr>
<tr>
<td>69</td>
<td>18.8 ± 5.3</td>
<td>7.3</td>
<td>8.0 ± 3.0</td>
</tr>
<tr>
<td>75</td>
<td>31.4 ± 4.8</td>
<td>7.5</td>
<td>14.1 ± 2.2</td>
</tr>
<tr>
<td>92.5</td>
<td>41.0 ± 4.5</td>
<td>7.6</td>
<td>9.2 ± 1.5</td>
</tr>
<tr>
<td>96.5</td>
<td>43.0 ± 8.4</td>
<td>7.6</td>
<td>9.9 ± 2.2</td>
</tr>
<tr>
<td>140.5</td>
<td>68.6 ± 1.1</td>
<td>7.8</td>
<td>12.8 ± 1.1</td>
</tr>
<tr>
<td>146.5</td>
<td>69.3 ± 6.2</td>
<td>7.8</td>
<td>13.9 ± 2.7</td>
</tr>
<tr>
<td>164.5</td>
<td>69.7 ± 7.7</td>
<td>7.8</td>
<td>13.5 ± 2.6</td>
</tr>
</tbody>
</table>

logd: log of the direct count concentration, logv: log of the viable count concentration

4.1.2 Delivery System for a Sorbed Carbon Source

The premise of the study was that the physical state of a hydrocarbon would select for microorganisms that could utilize either the sorbed, soluble or vapor phases of a hydrocarbon. This premise required the development of an appropriate sorptive matrix for delivery of organic hydrocarbon to the microbial population. Important matrix characteristics were as follows:

i. Sorption: It must sorb, trap or entrain gasoline.

ii. Non-biodegradable: The matrix must not be used as a carbon source by the microbial consortium.
iii. Non-toxic: The matrix must not hinder the growth of the microorganisms.

iv. Sterile: The matrix must be able to be sterilized.

v. Pure: The matrix must not be contaminated with other organic carbon sources.

vi. No surface tension effect: The matrix must not affect surface tension of the cultures.

The logical choice of natural subsurface material as a sorptive matrix was rejected, primarily because of the purity requirement. The Traverse City subsurface material was reported to have 0.01% (10,000 ppm) carbon-content. While carbon acts as an attractant to the hydrophobic gasoline contaminants \textit{in situ}, it may also act as an alternate carbon source for microorganisms. Carbon-free sand (silica dioxide) was also eliminated as a possible matrix for the microcosms because it was unable to sorb or trap hydrocarbon in sand-slurry microcosm cultures. Therefore, several gas chromatographic resins of varying qualities were assessed by a series of preliminary experiments for the above criteria.

The six resins tested are all used primarily to concentrate organic carbons from waste water prior to gas chromatographic analysis. This ability is due to their polymeric natures, rigid macroreticular structures and large internal surface areas. They differ in chemical nature and hydrophobicity. The various polarities of the adsorbents affect their propensity for various sorbates. To determine which resin would perform best, it was necessary to test each one.
In one experiment, six resins were tested for biodegradability or toxicity. All samples in the test were inoculated with microorganisms from the Traverse City contaminated soil and monitored for 16 days for turbidity. The test revealed that none of the resins were toxic to the microbial population when 0.3% yeast extract supplemented HCMM2 medium was used as the carbon source (Table 4.1.2a). No growth was detected when the yeast extract was not included in resin containing cultures indicating that none of the resins were biodegradable (results not shown). At the end of this test the samples were checked for surface tension. There was no reduction in surface tension when compared to the biotic controls that contained no resin, indicating that none of the resins had reduced the surface tension. Thus, criteria ii, iii and vi were met by all six resins.

Table 4.1.2a Growth\(^a\) of microbial consortium on 0.3% yeast extract in the presence of different resins.

<table>
<thead>
<tr>
<th>Resin</th>
<th>Carbon Source</th>
<th>day 0</th>
<th>day 1</th>
<th>day 2</th>
<th>day 4</th>
<th>day 5</th>
<th>day 6</th>
<th>day 8</th>
<th>day 10</th>
<th>day 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>XAD-4</td>
<td>yeast extr</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>XAD-8</td>
<td>yeast extr</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>XUS 40323</td>
<td>yeast extr</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>XUS 40285</td>
<td>yeast extr</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>IRA 938</td>
<td>yeast extr</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>IRC 50</td>
<td>yeast extr</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>none</td>
<td>none</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>none</td>
<td>yeast extr</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>none</td>
<td>none</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>none</td>
<td>yeast extr</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Growth determined via turbidity and ranked on a scale of 0 (none) to 4 (most).
Resins were subjected to a qualitative test to determine their ability to sorb hydrocarbon. If a sufficient amount of light density gasoline (sp. gv. 0.726 g/cc) was sorbed within a resin, the resin would float upon the surface of the medium. Aviation gasoline-sorbed resins were vortexed with 0.3% yeast extract HCMM2 medium and checked over the next 14 days in the 'sink or float' test (Table 4.1.2b). The anionic and cationic resins (IRA-938 and IRC-50, Rohm and Haas Co.) immediately sank upon addition of the medium containing yeast extract. The low-polarity, anionic resin XUS 40285 (Dow Chemical Co.) floated for 8 days before sinking. The remaining three non-ionic resins, XAD-4, XAD-8 (Rohm and Haas Co.) and XUS 40323 (Dow Chemical Co.), continued to float for the entire term of the test, indicating they could retain sufficient amounts of gasoline. Dry resins not treated with aviation gasoline as above, but vortexed with the HCMM2-yeast extract medium, sank immediately after vortexing.

Turbid growth was only detected in the XAD-8 resin samples during this test. Apparently, simple methanol rinsing was insufficient for sterilization of the resin. At a later date, an attempt to dry heat sterilize the resin at 123°C for 3.5, hours was made and failed. The resin decomposed (as determined by gas chromatography) even though the manufacturer stated it was stable to 250°C. Autoclaving proved to be successful, but only after three sterilization periods of 25 minutes each at 120 psi and 100°C. After sterilization, the resin was checked by gas chromatography for impurities. No peaks, other than the peaks of the solvent used for extraction, were identified.
Table 4.1.2b Sorbed resin: sink or float by virtue of change in resin density after exposure to aviation gasoline

<table>
<thead>
<tr>
<th>Resin</th>
<th>day 0</th>
<th>day 1</th>
<th>day 3</th>
<th>day 5</th>
<th>day 6</th>
<th>day 7</th>
<th>day 8</th>
<th>day 11</th>
<th>day 13</th>
<th>day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>XAD-4</td>
<td>growth&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>F or S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XAD-8</td>
<td>growth&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>F or S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>XUS 40323</td>
<td>growth&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>F or S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>XUS 40285</td>
<td>growth&lt;sup&gt;a&lt;/sup&gt;</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>F or S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IRA 938</td>
<td>growth&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>F or S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IRC 50</td>
<td>growth&lt;sup&gt;a&lt;/sup&gt;</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>F or S&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Growth determined via turbidity on a scale of 0 (none) to 4 (most);  
<sup>b</sup> S = sink; F = float; S* = gradually sinking resin

At this stage, XAD-8 resin was selected over the other two non-ionic resins for two reasons. First, XAD-8 was reported to have the highest pore radius of the three. Pore radius can influence the migration of large molecules in and out of the macroreticular structures of resins. It was noted that while the critical diameters of a biosurfactant molecule (surfactin), a typical aliphatic molecule and an aromatic molecule were much smaller than the average pore diameter of XAD-8, this was not true for the other two resins and biosurfactant molecules might be excluded from migration into their smaller pores (Figure 4.1.2). Also a typical bacterium of 1 μm (10,000 Å) diameter would be excluded, physically, from migrating to the interior of the pore network of the XAD-8 resin and also the other resins.
Since bacteria, but not typical biosurfactant molecules, would be excluded from the XAD-8 resin, it was hoped that the microcosm design would select for microorganisms producing extracellular biosurfactants that aided the removal of hydrocarbon sorbed to the interior of the resin matrix. Second, it was hoped the intermediate polarity of the resin would better mimic hydrophilic aquifer surfaces and favor hydrocarbon retention by capillary trapping mechanisms.
Figure 4.1.2 Comparison of resin pore diameters to the critical diameters of selected molecules. (1 mm = 2 Å).

Quantitative loading of XAD-8 resin proceeded as described in Materials and Methods, 3.5.6.3. Gas chromatographic analysis of the loaded
resin indicated that the more volatile component (DMP) was diminished and the toluene was enriched compared to the original liquid hydrocarbon stock solution. The original liquid stock of hydrocarbon mixture was 45:45:10 (DMP, TMP, toluene) respectively. The recovery from the sorbed resin after the entire microcosm extraction of both the aqueous phase and the sorbed phase was 23:43:35. An attempt was made to extract only the aqueous phase of microcosms containing sorbed resin at time zero, however the amounts recovered of the individual constituents was so low as to be below PQLs (results not shown). It was undetermined if the Exxon blend aviation gasoline was affected similarly because of the large proportion of unknown constituents and the co-elution of some constituents. The variation in proportions was probably due to the drying step of the resin loading process. However, without drying, the loaded resin stuck together and obviously contained a great deal of free-phase liquid organic. Because it was thought that free-phase liquid organic might interfere or alter the growth or surfactant expression of the microorganisms, this difference in proportions was quantified and then tolerated. Refer to Table 3.5.6.3 for the constituent relative percents of the XAD-8 sorbed aviation gasoline and hydrocarbon mixtures.

4.2 Preliminary and Control Experiments

Preliminary experiments were conducted to: i., characterize the soil biology and soil contaminants; ii., determine the soil bacteria's ability to grow on various carbon sources; iii., determine the concentration of
hydrocarbon needed in vapor microcosms and; iv., assess the possibility of surface tension effects with no carbon source present.

4.2.1 Soil and Contaminant Characterization

Soil moisture content was determined gravimetrically to be 16.7% (w/w). Viable counts using one-half strength Nutrient agar (Difco Laboratories) using the spread plate technique were performed on the RU2SS2 core sample. The number of heterotrophic organisms/gram sediment were estimated. Three replicates yielded $7.1 \pm 0.1 \times 10^7$ cfu/gram of dry soil.

Previous fuel carbon analysis near the area and depth of the RU2SS2 core boring (core boring AV-D-6) indicated fuel carbon concentrations were between 1000 to 3000 ppm [Ostendorf et al, 1991b]. Gas chromatographic analysis, performed in our laboratory, revealed present total hydrocarbon contamination was around 39 ppm (Table 4.2.1), indicating a considerable decrease in contamination from the previous report. The exact reason for the decrease in fuel carbon contamination from previous reports is not known. However, the present boring was assumed to contain microorganisms adapted to degrading gasoline and able to tolerate hydrocarbon concentrations as high as 3000 ppm.

Gas chromatography revealed that the toluene component of the aviation gasoline contaminant co-eluted with 2,3-dimethylhexane. To overcome this, the possibility of using a defined carbon source, similar to
aviation gasoline, was investigated. Two compounds present in gasoline 2,3-dimethylpentane and 2,2,4-trimethylpentane, resulted in peaks distinct from toluene on the GC. They represented a range of solubility similar to that found in aviation gasoline. If the three components were mixed in a 45:45:10 ratio (w/w), they would represent the 10% aromatic, 90% aliphatic composition of aviation gasoline. This ratio was determined to have the same density as aviation gasoline (0.726 g/cc) and all three compounds were available commercially. Therefore, testing of these three components, as an alternate carbon source for the microbial population, was undertaken.
Table 4.2.1  Gas chromatographic/mass spectrophotometric analysis of contaminated core samples from the Traverse City spill site.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aq. slb. b</th>
<th>VP c °C at 1 atm</th>
<th>RU1SS2 mg/kg, n = 3</th>
<th>RU2SS2 mg/kg, n = 3</th>
<th>% mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2,3-dimethylbutane</td>
<td>22.5 ppm</td>
<td>54</td>
<td>&lt;PQL*</td>
<td>&lt;PQL</td>
<td></td>
</tr>
<tr>
<td>2 benzene</td>
<td>80.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3 2,3-dimethylpentane</td>
<td>5.3</td>
<td>69.4</td>
<td>1.72 ± 0.47</td>
<td>&lt;PQL</td>
<td>2.4</td>
</tr>
<tr>
<td>4 2,2,4-trimethylpentane</td>
<td>2.4</td>
<td>99.2</td>
<td>7.38 ± 1.46</td>
<td>8.80 ± 0.09</td>
<td>22.3</td>
</tr>
<tr>
<td>5 2,5-dimethylhexane</td>
<td>1.5</td>
<td>108.1</td>
<td>2.28 ± 0.42</td>
<td>1.45 ± 0.02</td>
<td>2.3</td>
</tr>
<tr>
<td>6 2,3,4-trimethylpentane</td>
<td>2.3</td>
<td>113.5</td>
<td>4.51 ± 0.92</td>
<td>5.75 ± 0.10</td>
<td>14.2</td>
</tr>
<tr>
<td>7 toluene and 2,3-dimethylhexane a</td>
<td>554</td>
<td>110.6</td>
<td>3.00 ± 0.66</td>
<td>4.45 ± 0.12</td>
<td>10.2</td>
</tr>
<tr>
<td>8 2,2,5-trimethylhexane</td>
<td>1.2</td>
<td>115.6</td>
<td>4.07 ± 0.54</td>
<td>5.15 ± 0.08</td>
<td>12.7</td>
</tr>
<tr>
<td>9 ethylbenzene</td>
<td>136.2</td>
<td>&lt;PQL</td>
<td>&lt;PQL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 m-xylene</td>
<td>139.1</td>
<td>&lt;PQL</td>
<td>&lt;PQL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 o-xylene</td>
<td>144.4</td>
<td>&lt;PQL</td>
<td>1.61 ± 0.07</td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>unknowns</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>total hydrocarbon</td>
<td>33.80</td>
<td>38.73</td>
<td></td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

a co-elutes with toluene; b Aq. slb.: aqueous solubility; c VP: vapor pressure; * PQL: Practical quantitative limit; ND: not detected.

4.2.2  Growth of Soil Consortium on Various Carbon Sources

Two tests were performed in biometer flasks to determine the ability of the microbial consortium to grow on various carbon sources when supplied in vapor form (Tables 42.2a and b). Similar growth occurred when aviation gasoline, 0.3% yeast extract supplemented mineral medium or toluene in combination with DMP and TMP were used as the carbon sources. DMP or TMP, singly or in combination, could not support growth.
Table 4.2.2 Growth of microbial consortium on various carbon sources.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aviation gasoline blend (Exxon)</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>TMP:DMP, 1:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TMP:DMP:TOI, 1:1:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>0.03% yeast extract</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
<td></td>
</tr>
</tbody>
</table>

b.  

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>10</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TMP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TMP:DMP, 1:1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.3% yeast extract</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^a\) Growth determined via turbidity, 0 = none, 4 = most.

4.2.3 Growth of Soil Consortium at Various Carbon Source Concentrations

A 10-day growth curve was determined for the microbial consortium in microcosms amended with various amounts of hydrocarbon mixture supplied to the inner well in vapor cultures (Graph 4.2.3). There was no difference in the cell concentration when different amounts of hydrocarbon were added to the inner well, suggesting that growth was not limited by lack of carbon source at the smallest amount of 0.04 ml (29 mg), nor was growth increased at the greatest amount of 0.8 ml (581 mg). After 163 hours, the cell concentration at all carbon concentrations decreased and the cultures appeared to be in stationary phase growth from about 110 hours onward. In this experiment, increasing the amount of hydrocarbon in the inner well
neither extended the duration of the stationary phase or advanced the onset or rate of the log phase growth. This suggests that the concentration of soluble carbon available was the same, within the range tested, regardless of the amount of hydrocarbon initially supplied.

Graph 4.2.3  Semilog plot of cell concentration in vapor cultures.

4.2.4  Growth and Surface Tension of Soil Consortium Without a Carbon Source

In the absence of a carbon source, the microbial consortium failed to grow over a 188 hour time period (Table 4.2.4). The absence of growth confirmed that extraneous forms of carbon had been successfully eliminated from the system. However, cell concentration in the cultures did not significantly decrease.
Surface tensions remained constant throughout the test, suggesting that dead or dormant cells would not affect surface tension. In a separate test (results not shown), it was determined that initial cell concentrations of the consortium, from $1 \times 10^7$ to $1 \times 10^8$ cells/ml of mineral salts medium, did not significantly affect surface tension measurements at time zero.

Table 4.2.4 The mean surface tension and cell concentration of microcosms with no carbon source

<table>
<thead>
<tr>
<th>time in hours</th>
<th>abiotic microcosms + clean resin</th>
<th>biotic microcosms + clean resin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>surface tension</td>
<td>cell concentration</td>
</tr>
<tr>
<td></td>
<td>dynes/cm</td>
<td>dynes/cm</td>
</tr>
<tr>
<td></td>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td>0</td>
<td>73.1 ± 0.1</td>
<td>nd ab</td>
</tr>
<tr>
<td>140</td>
<td>nd a</td>
<td>nd ab</td>
</tr>
<tr>
<td>188</td>
<td>nd a</td>
<td>nd ab</td>
</tr>
</tbody>
</table>

$^a$ nd: not done; $^b$ no cells were inoculated into these samples and no turbidity was detected throughout the test period, subsequently, no cell counts were attempted.

4.3 Experiments I, II, III, IV, V

In the last 5 experiments, growth of a microbial population isolated from the contaminated sediment was assessed. In each of these experiments, the consortium was grown on three different physical states of the carbon source. In the V (vapor) treatment, the hydrocarbon was supplied to the microbes solely by distillation of hydrocarbon into the head space of the microcosm and subsequent solubilization into the aqueous medium. In the S (sorbed) treatment, the carbon was supplied to the microbes in a sorbed state (i.e.; sorbed into the macroreticular structure of the XAD-8 resin). Unlike
the V state, hydrocarbon was in direct contact with the aqueous medium of the culture. An intermediate treatment, designated R + V (resin plus vapor), consisted of clean (non-sorbed) resin floating on the aqueous medium and vaporized hydrocarbon, as in the V state (refer to Figure 3.6).

Experiment I was a trial run to work out mechanics of the experimental design. Experiment II assessed the similarities between surface tension reductions and cell concentrations when the soil consortium was grown on aviation gasoline or the hydrocarbon mixture. Experiment III assessed the disappearance of the components of the hydrocarbon mixture in the three culture systems. Experiment IV determined the cell concentration, and biosurfactant production (surface tension reducers and emulsifiers) when the soil consortium was grown on the hydrocarbon mixture. Experiment V was a repeat of Experiment IV with an additional assessment of the change in diversity of the types of organisms present.

4.3.1 Experiment I: Growth Curve Trial Run Utilizing Three Different Treatment Regimes

The first of the five experiments was a 136 hour growth curve involving 72 microcosms in which surface tension and cell concentration was measured. Many mistakes were made and mechanical difficulties were detected and corrected before the next experiment. The results were not statistically significant and are not reported. However, some general trends were evident and later proved to be valid. There was an increase in cell
concentration in the S regime when compared to the V regime and a general decrease in surface tension in all treatments over 136 hours.

4.3.2 Experiment II: Growth Curve Comparison of Aviation Gasoline and Hydrocarbon Mixture as Sole Carbon Sources

Experiment II was designed to measure growth of the microbial consortium on two different carbon sources (the hydrocarbon mixture and the aviation gasoline blend) for a 284 hour (13 day) period. This experiment was done in duplicate microcosms and therefore statistical analysis was not performed. The data points are simply the average of both microcosms in each treatment group and only general trends were suggested. The results are depicted in graphs 4.3.2 a, b, c, d. From this experiment it was determined that the microorganisms responded similarly both in growth pattern and surface tension reduction to the hydrocarbon mixture and the aviation gasoline. It was also determined that by 140 hours all systems were in stationary phase growth and the maximum surface tension reduction had been reached.

Regardless of the physical state of the carbon source, a trend toward greater cell concentration was observed in the S microcosms and toward a lesser cell concentration in the V microcosms. Growth in the R + V microcosms was somewhere in-between. The number of samples were too few to perform meaningful statistics on cell concentrations results. It must be noted that the S treatment contained a significantly higher proportion of toluene (in microcosms utilizing the hydrocarbon mixture for
growth) than either the V or R + V treatments, ≈ 35% as compared to ≈10%, respectively. The variation in the constituent percents could not be determined for the aviation gasoline treatments, but it is assumed that a like difference probably existed (i.e.; enrichment of the less volatile constituents of the blend in the S treatment when compared to the R + V and V treatments).

Surface tension reductions were similar among the different treatments and both carbon sources. However, at 44 hours the surface tension in the vapor cultures was above 60 dynes/cm, while it was in the 50’s in all resin containing cultures. In future experiments the number of sampling times was decreased in order to increase the number of samples analyzed per time. The sample times chosen for future analysis were zero and 140 hours.
Graphs 4.3.2  Cell concentration (a) and surface tension (b), of microbial consortium utilizing a hydrocarbon mixture of DMP, TMP and toluene as a carbon source

\[ \Delta \text{VHC: V regime, O RVHC: R + V regime, ■ SBDHC: S regime} \]
Graphs 4.3.2  Cell concentration (c) and surface tension (d) of microbial consortium utilizing aviation gasoline as a carbon source

\[c.\]

\[d.\]

\(\Delta\) VAVGS: V regime, O RVAVGS: R + V regime, ■ SBDAVGS: S regime
4.3.3 Experiment IV and V: Cell Concentration, Surface Tension, Emulsification Capacity and Microbial Diversity

Table 4.3.3 reports cell concentration, surface tension and emulsification capacity data for the microcosms of both Experiment IV and Experiment V. Microcosms of the three treatment groups were sampled at 140 hours. The experimental setup and sampling were identical in both experiments. Experiment V was carried out approximately a month after Experiment IV was completed and represents a replication of Experiment IV. Both experiments contained 45 microcosms each, devoted to measuring cell concentration, surface tension and emulsification capacity, in the three treatment groups. Both experiments also included an additional 15 microcosms which were used as abiotic controls. The abiotic microcosms contained clean resin and hydrocarbon mixture in the inner well, as in the R + V group, but were not inoculated. During Experiment V, additional microcosms were included to test for microbial diversity in the various treatment groups. The results of the microbial diversity study are discussed in section 4.3.3.4.

4.3.3.1 Effects of the Physical State of the Carbon Source on Cell Concentration

In both experiments the cell concentration of the three treatment groups was greatest when the hydrocarbon was initially supplied in a sorbed state. Cell concentration was least when the hydrocarbon was supplied in a vapor state. Generation time, of the microbial consortium, ranged from
20 hours for the S treatment to 24.1 hours for the V treatment with the R+V treatment at 21.5 hours.

4.3.3.2 Effects of the Physical State of the Carbon Source on Surface Tension

Similar surface tension reductions took place in all treatment groups when compared to the abiotic controls. Surface tension dropped approximately 15 dynes/cm on average in all treatments, indicating that surface tension was not affected by variations in the physical states of the carbon source. The surface tension reductions were primarily an extracellular phenomenon because there was no significant difference between the cell-mediated and extracellular surface tension readings in any group. Normalization to cell concentration showed that surface tension was not correlated to the cell concentration of the total consortium. However, this does not exclude the possibility that distinct microbial populations might have had some correlation.
Table 4.3.3 Experiment IV and V Results: Cell concentration, surface tension and emulsification capacity of 140 hour microcosms.

<table>
<thead>
<tr>
<th></th>
<th>Biotic: sorbed</th>
<th>Biotic: clean resin + vapor</th>
<th>Biotic: Vapor</th>
<th>Abiotic control:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>R + V</td>
<td>V</td>
<td>R + V</td>
</tr>
<tr>
<td>number of cells $\times 10^7$ per microcosm</td>
<td>IV 162.0 ± 27.9</td>
<td>96.1 ± 20.6</td>
<td>57.0 ± 6.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>V 141.0 ± 16.3</td>
<td>88.1 ± 7.7</td>
<td>56.1 ± 3.1</td>
<td>0</td>
</tr>
<tr>
<td>a $n = 15$</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
</tr>
<tr>
<td>surfactant measurements</td>
<td>cell mediated</td>
<td>extracellular</td>
<td>cell mediated</td>
<td>extracellular</td>
</tr>
<tr>
<td>surface tension</td>
<td>IV 57.4 ± 3.0</td>
<td>56.8 ± 2.8</td>
<td>57.0 ± 2.7</td>
<td>57.0 ± 1.6</td>
</tr>
<tr>
<td>per microcosm</td>
<td>V 58.5 ± 1.0</td>
<td>58.2 ± 1.8</td>
<td>59.3 ± 2.4</td>
<td>58.5 ± 1.8</td>
</tr>
<tr>
<td>dyne cm$^{-1}$</td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
</tr>
<tr>
<td>normalized surface tension $\times 10^{-7}$</td>
<td>IV .367 ± .058</td>
<td>.374 ± .099</td>
<td>.588 ± .159</td>
<td>.672 ± .242</td>
</tr>
<tr>
<td>dyne * ml * cell$^{-1}$ * cm$^{-1}$</td>
<td>V .400 ± .067</td>
<td>.437 ± .046</td>
<td>.658 ± .056</td>
<td>.685 ± .107</td>
</tr>
<tr>
<td>c emulsification capacity of 2.0 $\times 10^9$ cells</td>
<td>IV 2.1 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>1.3 ± 0.3</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>V 3.6 ± 0.7</td>
<td>2.6 ± 0.7</td>
<td>2.9 ± 0.2</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
</tr>
</tbody>
</table>

a $n$ = the number of samples analyzed

b NA = not applicable. Cell mediated surfactant measurements were performed on whole culture broth. Extracellular surfactant measurements were performed on the broth supernatant after the cells had been removed.

c Emulsification of abiotic controls is not normalized per cells.
4.3.3.3 Effects of the Physical State of the Carbon Source on Emulsification Capacity

Emulsification capacity, like surface tension reduction, was primarily an extracellular phenomenon because cell-free culture broth showed only a slight drop in capacity over whole cell broth measurements. Emulsification capacity was greatest in the S group, least in the V group and the R+V group was somewhere in between. This indicated that emulsification capacity, unlike surface tension reduction, correlated with the presence of hydrocarbon in a sorbed state.

4.3.3.4 Effects of the Physical State of the Carbon Source on Microbial Diversity

A microbial consortium was first isolated from the Traverse City sediment (RU2SS2 core boring) and used to 1) identify the colony types present in the sediment and 2) propagate a culture of the consortium to use as an inoculum for the experiment. To identify the colony types, an aliquot of the original cell suspension was serially diluted and plated on R2A agar plates three days before the start of the experiment. After 5 days of growth on the plates, five distinct colony types were recognized. The morphologies of the colonies were distinguished from each other and assigned an alphabetical number, (a) through (e). All of the microorganisms that could be identified are obligate aerobes, with the exception of *Alcaligenes denitrificans*, which is a facultative anaerobe (Table 4.3.3.4a).
To propagate a culture of the consortium to use as an inoculum for the experiment, the remainder of the original cell suspension was placed in a flask of HCMM2 medium supplemented with 0.03% yeast extract. After 3 days of incubation, cells from this culture were prepared for inoculation into the microcosms, as described in Materials and Methods section 3.4. Three 50-ml aliquots of the prepared inoculum material was serially diluted and plated on R2A agar, and Noble agar incubated in fumes. After 5 days of growth on R2A agar, colonies of identical morphology to colonies (a) through (e), as identified 2 days before in the original material, were counted (Table 4.3.3.4a, columns two and three). Additional colony types were not observed.

Table 4.3.3.4a Dominant colony types identified in the RU2SS2 core boring and counted in the time zero cell inoculum of Experiment V.

<table>
<thead>
<tr>
<th>Colony designation</th>
<th>Dilution plate counted</th>
<th>No. cfu</th>
<th>Genus, species</th>
<th>Gram stain motility</th>
<th>Form</th>
<th>Colony morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>10^5</td>
<td>12</td>
<td><em>Acinetobacter lwofli</em></td>
<td>neg nonmotile</td>
<td>coco bacillus</td>
<td>large white convex gooey</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>10^5</td>
<td>11</td>
<td><em>Pseudomonas paucimobilis</em></td>
<td>neg motile</td>
<td>rod</td>
<td>small medium orange gooey</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>10^5</td>
<td>1</td>
<td><em>Pseudomonas fluorescens</em></td>
<td>neg motile</td>
<td>rod</td>
<td>small white to tan, egg shaped</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d</td>
<td>10^5</td>
<td>5</td>
<td><em>Alcaligenes denitrificans</em></td>
<td>neg nonmotile</td>
<td>rod</td>
<td>small clear roundgooey</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>10^5</td>
<td>nd</td>
<td>unidentified ^a Gram negative bacilli</td>
<td>neg nonmotile</td>
<td>rod</td>
<td>large runny, gooey egg, sunny side up</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals of 5 colony types</td>
<td>10^5</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td>nd: none detected; ^a unidentified organism e (See Appendix 1).</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>134</td>
<td>163</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The percent of each colony type present in the inoculum was determined by counting the number of a particular colony type at the $10^4$ and $10^5$ plates, and dividing by the total number of colonies at those dilutions. Because dilution plates with concentrated cell suspensions were too crowded for colony selection (i.e. $10^3$ and $10^2$ dilution plates), colony types that may have been present in these plates, but not at higher dilutions, would not be identified, therefore, organisms that may have been present at lower percentages than the 5 dominant organisms probably escaped detection. In Table 4.3.3.4b, relative percents of the five dominant microbial species in the three treatment groups, before and after 140 hours of incubation, are compared.

Table 4.3.3.4b. Diversity on R2A agar. Relative percents of the five dominant microbial species in the three different treatment regimes

<table>
<thead>
<tr>
<th>Genus species</th>
<th>0 hours</th>
<th></th>
<th>140 hours</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>inoculum %</td>
<td>S %</td>
<td>R + V %</td>
<td>V %</td>
</tr>
<tr>
<td>Acinetobacter lwoffi</td>
<td>30</td>
<td>47</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas paucimobilis</td>
<td>40</td>
<td>0.3</td>
<td>21</td>
<td>38</td>
</tr>
<tr>
<td>Alcaligenes denitrificans</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>14</td>
<td>51</td>
<td>44</td>
<td>22</td>
</tr>
<tr>
<td>(e) Gram negative bacilli</td>
<td>0.7</td>
<td>1.3</td>
<td>3</td>
<td>27</td>
</tr>
<tr>
<td>total colonies identified</td>
<td>163</td>
<td>369</td>
<td>201</td>
<td>182</td>
</tr>
</tbody>
</table>
The diversity of the microbial species varied between the various treatment groups. In the sorbed hydrocarbon treatment (S), Acinetobacter lwoffi accounted for 47% of the identified colony types. Colonies of this organisms were not detected in the vapor treatment, yet it accounted for 30% of the colonies identified in the original microbial inoculum. Alcaligenes denitrificans remained around the same relative percent in the original soil and the vapor culture but was not detected in cultures when resin was present. The absence of Alcaligenes denitrificans, a facultative anaerobe, in conjunction with the presence of obligate aerobes in the 140 hour cultures, indicated that the microcosms were not oxygen depleted. Pseudomonas paucimobilis and organism (e) colonies were both detected in larger percentages in the vapor treatments (V) when compared to the treatments that contained resin. Pseudomonas fluorescens grew best in the sorbed hydrocarbon treatment groups. Colonies of this bacterium were detected in gradually increasing percentages from the vapor state (22%) to the sorbed state (51%).

Supplying the carbon to the consortium in a sorbed state restricted the growth of more species than it enhanced. Hydrocarbon in the solubilized state in the V treatments maintained the growth of four species. In the R + V cultures three species dominated, whereas in the S cultures only two dominated. When the carbon available for degradation was sorbed onto the XAD-8 resin, the microbial population that utilized it for growth became less diverse than when the hydrocarbon was supplied solely in the vapor form.
There was concern that microbial growth on R2A agar plates (Table 4.3.3.4a) might not detect some of the organisms that were present in the cell inoculum derived from the Traverse City core boring. In Experiment V, this possibility was investigated by plating dilutions of the 0-hour cell inoculum and of the five pooled microcosms of each treatment, after 140 hours of growth, on Noble agar. These plates were incubated in fumes of the hydrocarbon mixture for one month. By counting the number of 0-hour cell inoculum colonies found on the Noble agar plates and dividing by the number of 0-hour cell inoculum colonies found on the R2A agar plates at the $10^3$ dilution, it was estimated that about 12% of the microorganisms were capable of growing in Noble agar/hydrocarbon fume conditions. Therefore, Noble agar plates supplied with hydrocarbon fumes as the carbon source appeared to be a less likely method to detect members of the microbial consortium than the R2A agar plate method.

To determine if the types of microorganisms that grew on Noble agar with hydrocarbon fumes were different than the types that grew on R2A agar, an attempt was made to identify the colonies that grew under these conditions. The cell inoculum at 0 hours and after 140 hours, from the three treatment groups, were observed over a 1-month period. Growth in hydrocarbon fumes on Noble agar plates was slow compared to R2A agar plates, in all groups. The colonies did not display a recognizable morphology and it was necessary to restreak the colonies on R2A agar before identification could be made. After a few days of growth on R2A agar, the restreaked colonies reverted to their typical morphology. It was not possible to get accurate relative percents of the various colony types from
the different treatments while the colonies were growing on Noble agar, because of the difficulty in identifying them while on this medium. However, after restreaking on R2A medium, it could be determined which colony types were present and then an association between the colony type and the species it represented could be made. Therefore, only a qualitative species diversity was obtained. The presence of each species was recorded and is reported in Table 4.3.3.4c.

Table 4.3.3.4c Species diversity on hydrocarbon fumes.

<table>
<thead>
<tr>
<th>Genus species</th>
<th>Cell suspension</th>
<th>Cell suspension</th>
<th>S</th>
<th>R+V</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter lwaffi</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas paucimobilis</em></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes denitrificans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>(e) Gram negative bacilli</td>
<td>+*</td>
<td>+*</td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

* Organism (e) was only detected as a satellite of *Pseudomonas fluorescens*. Conditions prior to plating were either (cell suspension derived from contaminated sediment) at 0 hours or 140 hours of V (vapor phase carbon source), R+V (clean resin and vapor carbon source) or S (sorbed carbon source). Note: 8-day-old data for the three treatment groups was not done.

Only two of the species, *Pseudomonas fluorescens* and the Gram negative bacilli, grew well in these conditions and persisted in most of the treatment groups of the 0 and 140 hour series for one month. Two other colony types, *Acinetobacter lwaffi* and *Pseudomonas paucimobilis* were detected in 8-day-old plates of the original cell suspension, but after one
month of incubation the former died off and only one colony of the later
type could be detected on the plates (Table 4.3.3.4c).

*Pseudomonas fluorescens* was the only species that consistently grew
on fumes of the hydrocarbon mixture in Noble agar plates. The Gram
negative bacilli, organism (e), was only found as a satellite organism
surrounding the periphery of a few colonies of *Pseudomonas fluorescens*.
However, many colonies of *Pseudomonas fluorescens* were found that did
not have this bacilli surrounding it. In contrast, growth of this species in
R2A agar plates was not associated with *Pseudomonas fluorescens*.
Organism (e) may have been growing on byproducts of *Pseudomonas
fluorescens* which suggests a communalism between these two organisms
under hydrocarbon fume culture conditions.

Colonies of *P. fluorescens* were distinctively different under
hydrocarbon fume culture conditions when compared to R2A agar colonies
of the same species. After one month of incubation in the presence of fumes
the colonies were very large and dark brown to black in color. In R2A agar
culture the colonies were medium to large and tan to whitish in color.
Apparently, growth of *P. fluorescens* in fumes of gasoline components on
Noble agar plates results in dark pigment production.

When the consortium was cultured under fume conditions on Noble
agar plates, and its growth was compared to growth on R2A agar plates; 1)
fewer organisms grew, 2) fewer species were detected, and 3) properties of
the colonies, such as pigmentation and growth pattern were affected.
Therefore, it was determined that R2A agar plate culture of the microbial consortium would be a better method to use when determining the diversity of the consortium on various hydrocarbon states.

4.3.4 Experiment III: Disappearance of Hydrocarbons

In Experiment III an attempt was made to determine the amount of hydrocarbon degraded by the various treatment regimes. The disappearance of the various constituents were measured by gas chromatography in gas-tight sealed biotic and abiotic microcosms at zero and 140 hours. Extractions of the entire contents of the microcosms within 1 hour of set up yielded greater than 90% recovery of the total hydrocarbon supplied to the microcosms (Table 4.3.4a). During the experiment, there was a 24 hour wait period before extraction, because of time limitations imposed by the set up and sampling processes. During this period the microcosms were stored in a freezer at -20°C. Using this procedure, abiotic 0 time amounts of the mixture were decreased from the total amount of hydrocarbon mixture (50 mg) that had been supplied to each microcosm during set up (Table 4.3.4b). It is believed that rapid vaporization of the hydrocarbon mixture into the head space of the microcosms during freezing caused the decrease in the amounts that could be recovered at 0 time during the experiment.
Table 4.3.4a  Time 0 gas chromatographic analysis of the amounts of hydrocarbon supplied to abiotic microcosms when extracted immediately. *

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>R + V</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>relative % of total extracted</td>
<td>mg</td>
</tr>
<tr>
<td>DMP</td>
<td>10.505</td>
<td>22.7</td>
<td>19.814</td>
</tr>
<tr>
<td>TMP</td>
<td>19.744</td>
<td>42.7</td>
<td>20.686</td>
</tr>
<tr>
<td>toluene</td>
<td>15.964</td>
<td>34.5</td>
<td>4.709</td>
</tr>
<tr>
<td>total HC recovered</td>
<td>46.207</td>
<td>100.0</td>
<td>45.209</td>
</tr>
<tr>
<td>total HC supplied</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Extraction efficiency</td>
<td>92 %</td>
<td>91 %</td>
<td>94 %</td>
</tr>
</tbody>
</table>

* Zero hour cultures were iced down and extracted within one hour of inoculation. n = no. of samples

Table 4.3.4b  Time 0 gas chromatographic analysis of the amounts of hydrocarbon supplied to abiotic microcosms when extraction was delayed 24 hours. **

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>R + V</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>relative % of total extracted</td>
<td>mg</td>
</tr>
<tr>
<td>DMP</td>
<td>5.04±0.22</td>
<td>22.6</td>
<td>14.41±0.58</td>
</tr>
<tr>
<td>TMP</td>
<td>9.69±0.31</td>
<td>43.4</td>
<td>14.60±0.60</td>
</tr>
<tr>
<td>toluene</td>
<td>7.59±0.12</td>
<td>34.0</td>
<td>3.26±0.16</td>
</tr>
<tr>
<td>total HC</td>
<td>22.31±0.62</td>
<td>100.0</td>
<td>32.27±1.34</td>
</tr>
<tr>
<td>total HC supplied</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Extraction efficiency</td>
<td>45 %</td>
<td>65 %</td>
<td>55 %</td>
</tr>
</tbody>
</table>

** Zero hour cultures were stored at -20°C for 24 hours prior to extraction. n = no. of samples
At 140 hours the entire contents of biotic and abiotic microcosms were extracted after the microcosms were stored at -20°C for 24 hours. Table 4.3.4c presents these results.

Table 4.3.4c Gas chromatographic analysis of hydrocarbon in three treatment regimes at 140 hours when extraction was delayed 24 hours **

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>R + V</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg</td>
<td>mg</td>
</tr>
<tr>
<td>abiotic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>biotic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMP</td>
<td>4.83±0.30</td>
<td>4.32±0.11</td>
<td>12.01±0.26</td>
</tr>
<tr>
<td>TMP</td>
<td>9.42±0.44</td>
<td>8.73±0.39</td>
<td>12.37±0.51</td>
</tr>
<tr>
<td>toluene</td>
<td>7.47±0.15</td>
<td>3.81±0.25</td>
<td>2.96±0.31</td>
</tr>
<tr>
<td>total HC</td>
<td>21.72±0.89</td>
<td>16.86±0.26</td>
<td>27.34±1.08</td>
</tr>
</tbody>
</table>

** Zero hour cultures were stored at -20°C for 24 hours prior to extraction. n = no. of samples

In abiotic microcosms the concentrations of the less soluble components of the hydrocarbon mixture, TMP and DMP, are reduced in all treatments when compared to abiotic 0 hour extractions; however they were dramatically reduced in the vapor treatment series. It was believed that the extraction process was deficient in removing hydrocarbons present in the headspace of the microcosms. Apparently, most of the two less soluble hydrocarbons, DMP and TMP, primarily existed in the headspace in the V treatment group. In contrast the toluene, which is approximately 100 times more soluble than the aliphatics, was not significantly reduced in any of the 140 hour abiotic groups when compared to the 0 hour abiotic groups. It appears that the resin in the S and R+V treatments may have acted as a sink for the less soluble hydrocarbons, thus facilitating extraction. This suggests
that the bioavailability of the less soluble aliphatc fraction, TMP and DMP, of the hydrocarbon mixture to the aqueous phase was increased when the resin was present. This may, in part, account for the increased growth and increased hydrocarbon disappearance in the resin containing cultures.

The concentration of hydrocarbon in each biotic sample was subtracted from the corresponding abiotic sample to determine the disappearance of the hydrocarbons in the different treatments (Table 4.3.4d). Data were analyzed for significance, using EXCEL software and a two-sample Student's t-Test assuming unequal variances. There was a significant decrease in both the TMP and DMP hydrocarbon concentrations (p < .05) in the S and R + V treatments, however, there was no decrease in the concentration of these compounds in the V treatment (p > .05). No difference (p > .05) in the amounts of TMP or DMP degraded was detected between the S and R + V treatments. For total hydrocarbon degradation and toluene degradation, significance was at p < .05 when the difference between all three groups or any two groups were compared.

Table 4.3.4d Gas chromatographic analysis of hydrocarbon disappearance in microcosms after 140 hours of culture in each treatment regime.

<table>
<thead>
<tr>
<th>(n = 3)</th>
<th>S</th>
<th>R + V</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>relative % of total</td>
<td>mg</td>
</tr>
<tr>
<td>DMP</td>
<td>0.52±0.22</td>
<td>10.7</td>
<td>0.17±0.07</td>
</tr>
<tr>
<td>TMP</td>
<td>0.69±0.29</td>
<td>14.2</td>
<td>0.21±0.07</td>
</tr>
<tr>
<td>toluene</td>
<td>3.66±0.39</td>
<td>75.2</td>
<td>1.90±0.10</td>
</tr>
<tr>
<td>total HC</td>
<td>4.87±0.73</td>
<td>100.1</td>
<td>2.28±0.19</td>
</tr>
</tbody>
</table>

n = no. of samples
Assuming disappearance correlated with degradation, one must conclude that the microorganisms were growing primarily on the more soluble aromatic portion of the hydrocarbon mixture, the toluene. All treatments preferentially removed toluene. Removal of toluene was greatest in the S treatment and least in the V treatment, but toluene was also initially present in a greater concentration in the S treatment when compared to the V treatment (≈ 16 mg compared to ≈ 5 mg). It must be noted that the initial amount of toluene was approximately the same in the R + V treatment and the V treatment and the amount of toluene disappearance between these two treatments was not significant. Therefore, it is unlikely that the presence of the resin in the cultures aided toluene degradation. However, degradation of the low solubility aliphatic compounds (DMP and TMP) was only precluded in the microcosms that did not contain resin. For the aliphatic compounds, it may be possible that the presence of the resin enhanced their degradation.
5 DISCUSSION

Table 5 is a simplified summary of the experimental results. It denotes some positive relationships between bacterial species, hydrocarbon degradation and surfactant production. As can be seen, the dominant species found in the contaminated soil changed with the culture conditions. By analyzing these relationships some general conclusions can be made and some speculations as to the necessity for specific physiological traits needed for the degradation of sorbed gasoline components can be made.

<table>
<thead>
<tr>
<th>Species present</th>
<th>Dominant Species</th>
<th>ST</th>
<th>EC</th>
<th>Tol</th>
<th>DMP</th>
<th>TMP</th>
<th>CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>S A. lwofii</td>
<td>A. lwofii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>P. paucimobilis</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>P. fluorescens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram negative bacilli (e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R+V A. lwofii</td>
<td>A. lwofii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>P. paucimobilis</td>
<td>P. paucimobilis</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>P. fluorescens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram negative bacilli (e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V P. paucimobilis</td>
<td>P. paucimobilis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>P. fluorescens</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram negative bacilli (e)</td>
<td>Gram negative bacilli (e)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. denitrificans</td>
<td>A. denitrificans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| SI A. lwofii    | ST: surface tension reduction; EC: emulsification capacity; Tol: toluene degradation; DMP: DMP degradation; TMP: TMP degradation; CC: cell concentration ranked from the greatest (3) to the least.
A major concern when comparing results from the three treatments, is that the sorbed treatment (S) had a relative higher percentage of toluene than the other two. Therefore, differences that may have only been associated with relative amounts of toluene may have skewed the data. However, the toluene concentrations in the R + V and V treatments were the same. Therefore, the R + V treatment is a better sorbed hydrocarbon standard to compare the V (soluble hydrocarbon) treatment results against.

As expected, both cell concentration and the degradation of total hydrocarbon were positively related. In treatments where the cell concentration was greatest, the degradation of hydrocarbon, especially of toluene (as measured by disappearance), was also greatest. It appears that cell concentration was a good indicator of the relative amounts of total hydrocarbon degraded. However, when considered individually, the total amounts of TMP or DMP degraded were insignificant to the total amount of toluene degraded in any treatment. Therefore, cell concentration is probably more closely associated with toluene degradation than it is with the total hydrocarbon mix degradation.

The degradation rate of the sorbed carbon source in the S and R + V treatments was similar to previous investigative findings. Oberbremer and Müller-Hurtig [1989] found the degradation rate by a mixed microbial community in a hydrocarbon/soil mixture to be very high, 25.7 g/kg dry soil per day. The addition of sophorose lipid biosurfactant (HLB of 6.87) increased this rate to 46.5 g/kg of dry soil per day [Oberbremer et al., 1990].
Others have reported oil degradation rates of 0.02 to 1.38 g/kg dry soil per day. Equating the mg amount of sorbing resin present with a comparable amount of sorbing soil, the amount degraded in the S regime was 8.37 g/kg of dry resin per day. The R + V regime degradation rate was 4.94 g/kg of dry resin per day.

A maximum of 9.8% and 4.6% of the theoretical amount of available hydrocarbon was degraded within the 140-hour time period using an initial mass percent of 50% hydrocarbon/resin in the S and R + V treatments, respectively. Oberbremer and coworkers [1990] reported 95% degradation after only 57 hours of culture using an initial hydrocarbon/soil concentration of 13.5%. The strength of the resin-sorption complex in the present experiment was probably greater than the strength of the soil-sorption complex in their experiments. This may have accounted for the slower rate of degradation observed.

Oberbremer and colleagues, [1990] found that degradation was a two-stage event in their experiments. The more soluble naphthalene was degraded first, while the less soluble paraffins were only degraded in a second stage that followed the production of interfacial tension reducing agents by the microbial consortium. Degradation in these experiments was also accompanied by extracellular production of surfactants, both an emulsifier and a surface tension reducer. Stages were not detected nor measured due to end-point sampling in the present experiment, but the more soluble toluene was preferentially eliminated over the less soluble aliphatics. The emulsifier was only present when the least soluble constituents, the
aliphatics, were degraded. Stages may have been detected if more sampling times had been included and the time period had been extended past that needed for total degradation of the toluene.

It is important that the biosurfactants produced in these experiments were extracellular. Numerous investigators [Rosenberg, et al., 1983; Rosenberg and Rosenberg, 1981a; Reddy, et al., 1983; Efroymson, et al., 1991] have reported the presence of cell-mediated emulsifiers that alter the hydrophobicity of the bacterial cell wall and facilitate direct contact of the bacteria with the liquid organic. The present experiment was designed to minimize direct contact of bacteria with the organic phase by sorbing the organic liquid into the interior of the XAD-8 macroreticular pore network in the S treatment. It is probable that in the R + V treatment, solubilized and vaporized hydrocarbon also migrated into the interior of the clean resin that was supplied to microcosms. The large internal surface area of the XAD-8 resin (160 m²/g) does not exclude sorption of hydrocarbon to the exterior of individual resin beads, but it greatly favors interior trapping of the organic liquid. Since the pore diameters of the resin were small, they physically excluded migration of bacterial cells into the interior of the resin beads. Low-solubility organic liquids, like TMP and DMP, inside the resin would have to be mobilized out of the individual beads before bacterial contact could be made. This condition may have favored the production of extracellular surfactants that were capable of migrating into the interior of the resin and mobilizing the hydrocarbons by altering wetting behavior or by solubilizing hydrocarbons in o/w emulsions.
At least two types of surface active agents were produced by the microbial population. The agent that was responsible for the surface tension reduction was present in all treatments, regardless of the physical state of the hydrocarbon. If molecules that reduce surface tension accumulate at the vapor/aqueous barrier, it would be logical to assume their presence would aid degradation of hydrocarbons that must cross this barrier before being solubilized. One might have expected a decrease in this biosurfactant when hydrocarbons were trapped within the resin and already in contact with the aqueous phase. But this was not the case. Apparently the production of the surface tension reducing biosurfactant was independent of, or unaltered by, the physical state of the hydrocarbon source. However, this does not exclude the possibility that it aided hydrocarbon degradation in some other way.

In the present experiment, emulsification capacity was dependent on the physical state of the hydrocarbon and the presence of particular microorganisms. A change from more to less diverse was detected in the microbial consortium when resin was available to enable hydrocarbon sorption as opposed to only solubilized hydrocarbon. The diversity change was evident, though not as great, even in the R + V treatment. The diversity change detected in the R + V treatment could not have been skewed by the relatively greater amount of toluene as it might have been in the S treatment. Concomitant with the diversity change an emulsifier was produced and A. lwaffi, a species not detected in the V treatment, became one of two or three dominant species in the S and R + V treatments, respectively. Emulsification capacity, normalized to total cell concentration, increased
with increasing dominance of *A. lwoffi* in the resin containing microcosms, implicating this species as the producer of the emulsifier. Apparently, the genetic attributes of *A. lwoffi* did not give it a competitive edge when the hydrocarbon was not sorbed or otherwise present in direct contact with the aqueous phase. The other dominant species, *P. fluorescens*, was competitive regardless of the state of the hydrocarbon, suggesting it may have been responsible for the surface tension reducing biosurfactant.

Previous investigators [Oberbremer, et al., 1990; Wszolek and Alexander, 1979; Efroymsen and Alexander, 1991] have reported that biosurfactants enhanced the degradation of less soluble constituents of hydrocarbon mixtures over the more soluble members of the mixtures. In culture environments where the only means of hydrocarbon bioavailability is limited to vapor/aqueous transfer, minimally soluble compounds may not be present in high enough concentrations in the culture medium to initiate enough surfactant production to aid in solubilization of the hydrocarbon. The present experiments support those conclusions. Degradation of the most insoluble components of the hydrocarbon mixture, TMP and DMP, was detected only when XAD-8 resin was present (S and R + V treatments) and when *A. lwoffi* and the emulsifier were present. And, in preliminary experiments attempts to grow the soil isolates on solubilized single carbon sources of TMP or DMP failed.

With the exception of surface tension reduction, in every case, the values of measured parameters (hydrocarbon disappearance, emulsion production and cell concentration) of the R + V treatment was in-between
the measured values of these parameters in the S and V treatments. This suggests that the hydrocarbon sorption was primarily responsible for the changes in the parameters. The loading process for the resin used in the S treatment ensured that hydrocarbon was entrained within the interior of the macroreticular structure of the resin or sorbed to the exterior of the resin bead, and therefore not initially available to the aqueous phase as a solubilized hydrocarbon source. In the R+V treatment, entrained hydrocarbon was not initially available but this did not exclude the possibility of hydrocarbon sorption directly onto the resin from the vapor and aqueous phases. In either case, the area of direct surface contact between the hydrocarbon and the aqueous phase was probably increased in the S and R + V treatments over that in the vapor microcosms. Therefore, population adaptation and emulsification capacity increases in the resin containing treatments may have been a result of, rather than a cause of, the increased bioavailability and subsequent degradation of the hydrocarbons.
6 CONCLUSIONS

Sorbed gasoline constituents are responsible for a residual oil contamination that is difficult to bioremediate. In these experiments, degradation of sorbed and entrained hydrocarbon, within or on, a non-ionic polymeric resin (XAD-8) was associated with an emulsifier, possibly produced by *A. lwaffi*. It is not known if the emulsifier caused the increase in hydrocarbon degradation and cell concentration that were observed. The increase in emulsification capacity may have only been a result of an increase in bioavailability of the hydrocarbon to the aqueous layer and a subsequent increase in its degradation.

It was determined that a change in the diversity of the microbial population took place when the hydrocarbon was entrained or sorbed to the resin. The change in the population resulted in a less diverse population where only two species, *A. lwaffi* and *P. fluorescens* dominated over three other species in the original microbial consortium. The emulsification capacity of the microbial population that degraded sorbed or entrained hydrocarbon was greater than the capacity of the population that degraded solubilized hydrocarbon. It was also determined that the ability of the consortium to produce a surface-tension reducing biosurfactant was not associated with sorbed hydrocarbon.

The positive relationship between the increase in emulsification capacity and the increase in degradation of the less soluble components of a hydrocarbon mixture, DMP and TMP, along with an increase in toluene
degradation, suggests that sorbed or entrained hydrocarbon in the S and R + V treatment groups may have affected the degradation process by exerting selection pressure on the indigenous population to produce the emulsifier. In all treatment groups the total hydrocarbon concentration was the same, but in one sorbed treatment group (S), the toluene constituent constituted a relatively larger proportion of the total hydrocarbon source (35% as opposed to 10%). However, in the other sorbed treatment group (R + V), the toluene constituent was the same as a comparable treatment group that contained only solubilized hydrocarbon (10%). Therefore, increased emulsification capacity and hydrocarbon degradation demonstrated a positive relationship with a sorbed hydrocarbon source as opposed to solubilized hydrocarbon source, at least for the R + V treatment group.

The toluene constituent was the primary component degraded in the hydrocarbon mixture by all treatment groups and it is not determinable from the present experiments how much this factor influenced the degradation results. Therefore, further experimentation is needed to clarify this point and to determine if the emulsifier produced, when the hydrocarbon was sorbed or entrained as opposed to solubilized, actually aided in the degradation process or was simply a result of increased bioavailability due to increased surface area contact between the organic and aqueous phases of the culture system.

Many more questions remain to be answered. Were only the surface-tension reducing and emulsifying biosurfactants produced? The emulsifying agent may have been a mixture of more than one surface active agent as Cooper and Goldenberg [1987] found in their experiments with *Bacillus* sp.
Did the surfactants only aid the degradation of hydrocarbon by the species producing it? It is possible that *P. fluorescens* opportunistically degraded hydrocarbon that was mobilized and/or solubilized by emulsion produced by *A. lwofii*. To what degree are microorganisms capable of producing biosurfactants of specificity? When considering the variety of interfaces in a subsurface environment and the possible aggregation of biosurfactants at various interfaces, it seems logical that a variety of amphiphatic molecules would be needed to do the job. Perhaps Zajic and Seffens [1976] were right when they suggested a mixture of surfactants is usually best for most applications. Through selection pressure it is possible that the indigenous microorganisms at the Traverse City site adapted to express just the right mix of surfactants. Developing artificial culture conditions that closely mimic the true environment may be the only way to get accurate information as to the capacity of microorganisms for bioremediation of residual oil saturation.
APPENDIX 1

Organism (e) identified as follows:

Glucose oxidizer, MacConkey-positive, oxidase-positive, unidentifiable gram negative bacilli, not one of the following:

Achromobacter xylosoxidans  Pseudomonas mallei
Agrobacter radiobacter  Pseudomonas mendocina
Brucella sp.  Pseudomonas mesophilica
EF-4b  Pseudomonas paucimobilis
EO-2  Pseudomonas pickettii
Flavobacterium breve  Pseudomonas pseudomallei
Flavobacterium meningosepticum  Pseudomonas putida
Flavobacterium multivorum  Pseudomonas putrefaciens, biotype 1
Flavobacterium spiritivorum  Pseudomonas stutzeri
Flavobacterium sp (Ilb)  Pseudomonas thomassii
Pseudomonas aeruginosa  Pseudomonas vesicularis
Pseudomonas cepacia  Va-1
Pseudomonas diminuta  Vb-3
Pseudomonas fluorescens  Vd (Achromobacter sp)

by Microbiology Specialists; Inc. 8911 Interchange Drive, Houston, Texas 77054-2507
APPENDIX 2

Acronyms Found In Text:

EPA: U.S. Environmental Protection Agency
OUST: Office of Underground Storage Tanks
LNAPL: Light non-aqueous-phase liquid
UST: underground storage tank
MEOR: microbial enhanced oil recovery
HLB: hydrophilic-lipophilic balance
CMC: critical micelle concentration of a biosurfactant
cfu: colony forming units
DMP: 2,3-dimethylpentane
TMP: 2,2,4-trimethylpentane
2,4-D: 2,4-dichlorophenoxy acetic acid
GC: gas chromatography
GC/MS: gas chromatography/mass spectrometry
REFERENCES


Raymond, R. L. (1978) "Environmental Bioreclamation" in Proc. of *1978 Mid-Continent Conference and Exhibition on Control of Chemicals and Oil Spills.*


