Kirschner, Larry E., Bioremediation Potential of the Microflora in a Chlorinated Alkene Contaminated Industrial Leachate. Doctor of Philosophy (Biology), May, 1993. 115 pp., 23 tables, 16 illustrations, references, 89 titles.

Three major microbial subpopulations from an industrial leachate stream were characterized with respect to their bioremediation potential, and particular aspects of a cometabolically active subpopulation were determined. Viable microbial populations in the leachate and surrounding soil were quantified prior to the demonstration of potential trichloroethylene (TCE) degrading subpopulations. Procedures for isolation of leachate subpopulations of cometabolic interest consisted of enrichment with toluene, methane, or propane. Isolates using these carbon sources were obtained from the leachate. Toluene-oxidizing isolates included *Pseudomonas* species, *Alcaligenes* species, and *Acinetobacter.* For methane enrichments, a type-I methanotroph appeared to be the predominant bacterial strain. Propane-oxidizing cultures were assayed for their TCE degrading abilities, and were used to explore the heretofore unaddressed question of preferential expression of terminal or subterminal oxidative pathways during trichloroethylene (TCE) degradation. The specific oxygen uptake rate (SOUR) for selected propane-oxidizing isolates was determined by challenging whole cell preparations with intermediates of propane and propionate metabolism. SOUR and TCE degradation data for the isolates (2 *Mycobacterium* strains, a *Rhodococcus* species, and a *Nocardia* species) and a reference culture, *Mycobacterium vaccae* JOB5 suggested that both terminal and subterminal oxidative pathways were utilized for TCE degradation.

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BIOREMEDIATION POTENTIAL OF THE MICROFLORA IN A CHLORINATED ALKENE CONTAMINATED INDUSTRIAL LEACHATE

DISSERTATION

Presented to the graduate Council of the

University of North Texas in Partial

Fulfilment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

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Denton, Texas

May, 1993

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CHAPTER I

INTRODUCTION

The presence of xenobiotic chemicals such as tetrachloroethylene (PCE), trichloroethylene (TCE), dichloroethylene (DCE), and vinyl chloride (VC) in the environment has lead to the recognition of the need for effective treatment methods for contaminated soil and groundwater. A survey of water supplies in the U. S. showed that two-carbon chlorinated chemicals made up ten of the fourteen most common volatile organic chemicals (VOC) in drinking water. As an example of the prevalence of VOC's in the environment, in the mid-1980's, sixty percent of the drinking water supply closures in Massachusetts were the result of the presence of these chemicals in water (Moore et al., 1989). Among the contaminating chemicals, chlorinated alkenes rank as one of the most common (Winter et al., 1989). Since these chlorinated aliphatic hydrocarbons (with the exception of VC) are considered recalcitrant to direct aerobic microbial attack, alternative methods must be found if biological processes are to be used for degradation and mineralization. In terms of aerobic bioremediation processes at the present time, cometabolic methods provide the only effective biological strategy for aerobically treating chlorinated alkenes.

This work was initiated to address the problem of TCE contamination in an industrial leachate stream. The plan of action was to first document the presence of healthy microbial populations in contaminated soil adjacent to the French drain leachate system as well as the leachate itself. Then specific subpopulations (of potential interest for their cometabolic properties) were isolated from the leachate, and characterized. From these subpopulations, one

group, the propane-oxidizing bacteria were further characterized for their TCE degrading capabilities and the expression of specific oxidative pathways during TCE degradation. Of particular interest was the question of whether or not subterminal or terminal oxidation was required for cometabolic degradation of TCE. The requirement for expression of a specific pathway, such as subterminal oxidation, if not readily expressed by enriched cultures, could make the bacteria unsuitable for use in bioremediation processes.

Degradative reactions for TCE. Trichloroethylene (TCE) is one of the most common groundwater contaminants in the environment. It persists for several reasons. In addition to primary factors such as poor containment and disposal practices, halogenated alkenes are produced as a natural consequence of abiotic and biotic transformation of halogenated alkanes. TCE and its tetra- and dichloro-homologs have proven to be recalcitrant to conventional treatment procedures that utilize direct aerobic microbial attack. Until the early 1980's, both tetrachloroethylene (PEC) and TCE were considered resistant to microbial degradation (Kästner, 1991).

Although persistent in certain environments, chlorinated alkenes are transformed and mineralized by several mechanisms. These include abiotic weathering and anaerobic microbial degradation. Mammalian liver enzymes (cytochrome P-450) also break down these compounds, but in doing so, toxic intermediates are formed. Regardless of the degradative system involved, the chlorinated alkenes are degraded by only three major chemical mechanisms: substitutions; dehydrohalogenations; and oxidation-reduction reactions (Vogel et al., 1987).

Substitution reactions- This reaction type is characterized by the abiotic exchange of substituent groups on the parent molecule and is of more importance in the breakdown of halogenated alkanes than halogenated alkenes. In the environment, these reactions are

generally slow with half-lives of the products ranging from days to centuries. The rates of abiotic substitutions in aqueous environments are increased by high ionic strengths and pH values greater than pH 11. Below pH 11, pH dependent substitution reactions are generally not observed. In aqueous media, hydroxylations are thought to be the most prevalent type of substitution:

Substitution reactions can also occur as conjugations with sulfide, as shown below:

The rate at which substitution occurs partially depends on the degree of halogenation of the compound undergoing the reaction. Generally, as chlorination increases, the rate of substitution decreases. Monochloroalkanes have half-lives of about one month; polychlorinated alkanes are less likely to undergo substitution. Some halogenated compounds, including breakdown products, have extremely long half-lives (Table 1). It should be noted, however, that the half-life numbers given are probably only theoretical in many cases. Freedman and Gossett (1991) showed that dichloromethane, when supplied in concentrations that did not inhibit methanogenesis, can be used as a sole carbon source by methanogenic bacteria.

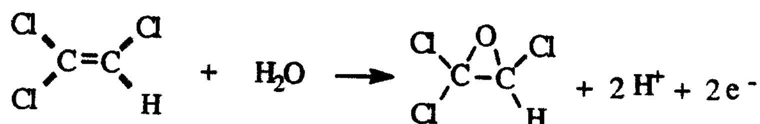
Dehydrohalogenation reactions- Reactions of this type are important to bioremediation processes because they are a source of chlorinated alkenes in the environment. These reactions proceed by the removal of a halogen atom from one carbon, followed by the removal of a hydrogen atom from an adjacent carbon, as shown below:

Table 1
Estimated Half-lives of Chlorinated Alkenes
at 20 °C

Chlorinated Compound	Estimated Half-life (years)
Tetrachloroethylene	0.7, 6*
Trichloroethylene	0.9, 2.5
Dichloromethane	1.5, 704
Trichloromethane	1.3, 3500
	* at 10 °C in sea water

(Vogel et al.,1987)

Oxidation-Reduction Reactions- The reactions of greatest importance in the degradation of chlorinated alkenes are oxidation-reduction (redox) reactions: both oxidative and reductive mechanisms are seen. Oxidation of TCE occurs when the alkene bond serves as an oxygen acceptor, resulting in epoxide formation:



Akyl-epoxides are generally unstable and break down into chlorinated and non-chlorinated derivatives depending on the parent compound (Vogel et al., 1987). In mammals epoxides are

mutagenic, even though short-lived. Breakdown products identified from the degradation of TCE by methane monooxygenase include formate, CO, glyoxylate, dichloroacetic acid, and trichloroacetaldehyde (chloral hydrate) (Ensley, 1991; Newman and Wackett, 1991). Newman and Wackett (1991) showed that the chloral hydrate decomposes into trichloroethanol and trichloroacetic acid. Enzymes commonly associated with epoxidation of TCE include the methane monooxygenase of methanotrophs and the mono- and dioxygenases of pseudomonads.

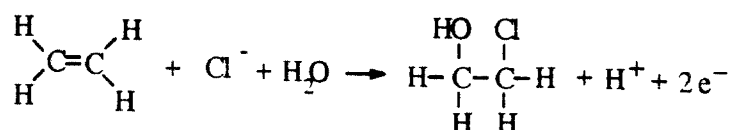
Frequently found enzyme systems catalyzing epoxide formation include methane and propane monooxygenase, and toluene mono- and dioxygenases, and cytochrome P-450 systems. In addition to epoxidation by bacterial enzymes, mammalian liver enzymes (cytochrome P-450) also convert TCE to epoxides. The reactivity of the epoxide with DNA can result in base deletions causing frame shift mutations. TCE is therefore classified as a mutagen and possible carcinogen in humans.

Biological catalysis of TCE oxidation results in the destruction of enzyme activity. In a study by Fox et al. (1990), complete inactivation of a purified three-subunit methane monooxygenase (MMO) was observed after approximately two hundred molecules of TCE had been oxidized per molecule of hydroxylase component. Inactivation of the enzyme was accompanied by the covalent modification of all the components of MMO.

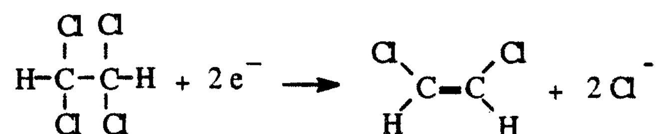
Similar enzyme damage may explain the cessation of linear TCE degradation by *Pseudomonads* used in bench-scale bioremediation units (Wackett and Gibson, 1988). Other reports also document the effects of toxic breakdown products on oxygenase systems and other cellular macromolecules (Alverez-Cohen and McCarty, 1991; Rasche et al., 1991; DeBruin et al., 1992; Pettigrew et al., 1991). Results presented by Winter et al. (1989) showed a 99.9% reduction (a decrease from 16 ppm to 2 ppb) of an aqueous TCE solution in 6 h by two recombinant *E. coli* strains constructed with toluene monooxygenase (TMO) genes from *P.*

mendocina KR-1. Recombinant *E. coli* strains pKY287/FM5 and pYM402/HB101 were grown on glucose to the desired cell mass at 30 °C, then at 42 °C to trigger expression the plasmid-borne lambda phage promoter PL that regulates tmo gene expression. Bioreactor use of these strains was at a non-growth temperature, meaning that TCE degradation resulted from presynthesized enzymes.

The biohalogenation of alkenes produces hydroxy-haloalkanes that are more susceptible to degradation than the parent alkene:



Reduction of haloalkanes is another source of chlorinated alkenes in the environment, as shown below (Vogel et al., 1987):



This reaction can lead to breakdown products that are of greater environmental concern than the parent compound.

Biological Degradation of Chlorinated Alkenes. Chlorinated alkenes (except VC) are considered untreatable by direct, aerobic biological treatment systems such as standard waste water treatment technology (trickling filters or activated sludge systems). Their persistence in the environment also indicates that the chloroalkenes are highly resistant to degradation by biological systems. While direct aerobic catabolism of chloroalkenes is apparently limited to vinyl chloride (VC) (Ensley, 1991, Hartmans and DeBont, 1992) these chemicals are microbially degraded by certain anaerobic bacterial processes and a limited number of aerobic bacterial cometabolic systems (Nelson et al., 1986; Wackett et al., 1989; Fathepure and Boyd, 1988; Kleopfer et al., 1985; Bagley and Gossett, 1990; Freedman and Gossett, 1989).

Cometabolism involves the oxidation of a chemical that is used for neither a carbon source nor electron source by the microorganism; this is discussed in greater detail later.

TCE Degradation by Anaerobic Systems- Anaerobic bacteria have been shown to mineralize PCE, TCE, DCE, and VC. Fathepure and Boyd (1988) showed that PCE was sequentially converted to TCE under methanogenic conditions. The reductive dechlorination of TCE to DCE in anaerobic environments had been previously demonstrated by Kleopfer et al. (1985). Reductive dechlorination of PCE to TCE and DCE was shown by Bagley and Gossett (1990) in sulfate-reducing environments. All these demonstrations indicated that environmental reductive dechlorination stopped short of complete mineralization. Freedman and Gossett, (1989), extended the work of Fathepure and Boyd, (1988), with methanogens by demonstrating that the sequential degradation of PCE continued on to vinyl chloride then to ethylene. DeBruin et al. (1992) confirmed that not only is PCE sequentially degraded to ethylene, but that ethylene is converted to ethane which is then converted to CO_2 and water. A later study by Fathepure and Vogel, (1991), summarizes much of the earlier work by stating that the reductive dechlorination is fairly rapid for highly chlorinated molecules, but decreases as the level of chlorination decreases. Less chlorinated compounds tend to persist longer in the environment because they are less susceptible to hydrolytic and oxidative processes.

The accumulation of VC was a cause of great concern to researchers working with methanogenic anaerobic systems prior to the demonstration that VC was metabolized to ethylene. Since VC is a more potent carcinogen than its parent compounds, the observation of its degradation to ethane was an important one which means that the anaerobic systems can possibly be utilized for in situ bioremediation purposes.

Kästner (1991) found that aerobic groundwater bacteria grown on a mixture of 2-butanone, butyl acetate, 2-chlorobenzene, ethyl acetate, isopropanol, and toluene could

catalyze the reductive dechlorination of PCE and TCE when their microcosm shifted from aerobic to anaerobic conditions and the redox potential was lowered from 0 to -150 mV by addition of sulfide. No methanogenesis was detected, and no transformation of PCE and TCE was detected under strictly anaerobic conditions. He concluded that PCE and TCE act as surrogate electron acceptors for electron carrying enzymes at appropriate redox potentials. In this case, the redox potentials for reduction of PCE/TCE and TCE/DCE correspond closely to the redox potentials for O_2/H_2O, and NO_3^-/NO_2^-, respectively.

Bryant et al. (1991) found that, at least for aromatic compounds, stereospecific effects can be seen in halogen removal during reductive dechlorination. Their work with chlorinated phenol derivatives showed the order of chlorine removal to be para > ortho > meta. In addition to questions concerning accumulation of toxic products during biotransformation, their work suggests the possibility of stereospecificity in the degradation of chloroalkenes. Certain compounds are known to accumulate during degradative processes (for instance cis-DCE in some reductive dechlorinations, Kästner, 1991). The implication is that degradation of some molecular conformations is favored over others.

Niels and Vogel, (1991), determined that reductive dechlorination of polychlorinated biphenyls occurs in a two step process. First, one electron is transferred to the halogen site on the aromatic ring, to form an anion radical, followed by expulsion of the halide ion, forming a carbon radical. In the second step, an electron is transferred to the carbon radical to form a carbanion, which is thought to abstract a proton from the solvent (water). The result of the two electron transfer is the replacement of a halogen ion on the ring with a hydrogen ion from the solvent.

Reductive dechlorination is usually thought of as occurring concurrently with methanogenesis. However, in their study of the transformation of PCE to ethene, DiStefano et

al. (1991) found that reductive dechlorination also occurred under strict anaerobic conditions, but in the absence of methanogenesis. Using anaerobic microcosms enriched with methanol and PCE, they found that at high PCE concentrations (550 µM), methanogenesis was inhibited but reductive dechlorination continued. Cultures of acetogenic bacteria were identified. The acetogenic bacteria, which possess the carbon monoxide-acetyl coenzyme A pathway and its variants apparently use the electron flow from the methanol to produce acetate and also dechlorinate PCE, instead of the electrons being used for methanogenesis. Whether the acetogenic bacteria were dechlorinating PCE as part of their inherent biochemical nature or they arose "simply...in response to the absence of methanogenic competition, while other organisms were responsible for the PCE dechlorination, either using methanol directly as an electron donor or perhaps using hydrogen produced by acetogens during methanol catabolism" was not determined (Thauer et al., 1989).

For reductive dechlorination to proceed, an electron source must be available. In the study by DiStefano (1991) methanol was used. Fathepure and Vogel (1991) compared the stimulatory effects of glucose, methanol, and acetate on an anaerobic biofilm-type reactor, and found acetate to be the best electron source of the three. A study by Gibson and Sewell (1992) measured the effects of five short-chain fatty acids (acetate, lactate, propionate, crotonate, butyrate), and three alcohols (methanol, ethanol, and isopropanol) on the reductive dechlorination of PCE in small microcosms. Using their system, lactate, propionate, crotonate, butyrate, and ethanol stimulated dechlorination, while acetate, methanol, and isopropanol did not. The differences between their work and that of DiStefano (1991) and Fathepure and Vogel (1991) may somehow lie in the fact Gibson and Sewell used a static system, instead of a continuous flow bioreactor. Henry and Grbic-Galic (1991) successfully used formate as an

electron source in their work. They also proposed that lipid storage granules within the cells acted as endogenous electron sources.

Degradation by Aerobic Cometabolic Microbial Systems- To date, only one report was found in the literature citing direct aerobic metabolic attack of TCE by naturally occurring microorganisms (Vandenberg and Kunka, 1988). However, as pointed out by Zylstra et al. (1989), no growth data are provided to show that *Pseudomonas fluorescens* PFL12 actually did use TCE as both a carbon and electron source. Therefore, at this time, there are no confirmed reports ofnaturally occurring organisms that can use TCE as a sole carbon source. Furthermore, all reports of DCE degradation are connected with cometabolic processes.[1]

The participation of cometabolic processes in the degradation of chlorinated alkenes has been well documented (Nelson et al., 1986, 1987, 1988; Shields et al., 1989; Folsom et al., 1990; Moore et al., 1989, 1991, Fogel et al., 1986; Vogel et al., 1987; Oldenhuis et al., 1989; Little et al., 1988; Fliermans et al., 1988; Tsien, 1988; Wackett et al., 1988, 1989; Wilson and Wilson, 1985; Perry, 1979, 1980). Cometabolism (cooxidation, originally described by Leadbetter and Foster, 1959, 1960) is defined as oxidation of non-growth hydrocarbons when they are present as co-substrates in a medium in which one or more different hydrocarbons are furnished for growth (Perry, 1979), or the degradation of chemicals that are used for neither growth nor cellular metabolism (Atlas, 1984). A wide variety of organic chemicals can induce the formation of the enzyme systems necessary for this phenomenon. In the present study, because of the composition of the leachate from which bacteria were isolated, and because of

[1]The suggested acrynyms for dichloroethylene and dichloroethane are DCE and DCA, respectively (Vogel et al., 1987). Some European authors refer to dichlorethane as DCE which is confusing because dichloroethane (DCA) can be directly degraded by microbial attack, while dichloroethylene (DCE) is still thought to be susceptable only to cometabolic degradation by aerobic microbial systems.

practical considerations for bioremediation, only toluene, methane and, propane were used in this work as substrates for induction of appropriate enzyme systems.

A variety of microorganisms are known to cometabolically degrade TCE and its chlorinated homologues (Table 2). The carbon metabolism of the major aerobic, cometabolic organisms falls into two categories: (1) aromatic metabolizing systems; and (2) gaseous alkane metabolizing systems. In all cases, the cometabolic degradation of TCE is catalyzed by oxygenases, all of which are thought to be inducible rather than constitutive. Both aromatic and alkane oxygenases work by incorporating atmospheric oxygen into the substrate molecule to form hydroxyl derivatives (Atlas, 1984, Ensley, 1991, Perry, 1979, 1980). In the case of TCE degrading microorganisms that metabolize aromatic compounds, catechols are formed (Pettigrew et al., 1991). The alkane oxygenases form primary or secondary alcohols (Perry, 1980, Patel et al., 1983b). However, the same oxygenases epoxidate alkenes, forming unstable molecules that decompose with relatively short half-lives (Vogel et al., 1987). Both PCE and VC are exceptions to this rule. PCE is reductively dechlorinated under anaerobic conditions (as discussed above), and is subjected to very little, if any, degradation by aerobic systems. VC is degraded by the oxygenases of methane- and propane-oxidizing bacteria, but not toluene oxidizing bacteria. Phelps et al. (1991) reported the isolation of a VC degrading actinomycete from a propane fed, TCE degrading consortium. Hartmans and DeBont, 1992, also reported aerobic vinyl chloride metabolism by *Mycobacterium aurum* L1.

Cometabolism by Aromatic-oxidizing Bacteria- Nelson et al. (1986) first reported the mineralization of TCE and DCE by a gram negative bacterium that used phenol as a carbon source. The organism, initially identified as *Acinetobacter* (Nelson et al., 1986), and later as *Pseudomonas cepacia* G4 (Folsom et al., 1990) was isolated from an industrial waste treatment facility, and degraded TCE when induced by phenol, toluene, o-cresol or m-cresol.

Table 2
Bacteria with Inducible Mechanisms for Chlorinated Alkene Degradation

Culture	Inducer	Comments
Pseudomonas sp. strain JS6	toluene or chlorobenzene	Pettigrew et al, 1991
Pseudomonas cepacia G4	phenol	Folsom et al, 1990 Folsom & Chapman, 1991; Nelson et al, 1987
Pseudomonas putida F1	L-arginine, toluene	Wackett & Gibson, 1988; PCE, VC, & ethylene not degraded
Mycobacterium aurum	None	Hartmans & deBont, 1992; very rapid inactivation of VC degradation seen
Nitrosomonas europaea	NH_4	Arciero et al., 1989
Acetogens or methanogens	Methane as e^- donor (no methanogenesis)	DiStefano et al, 1991
Rhine river sediment + anaerobic sludge	lactate as e^- source	deBruin et al, 1992

Table 2 (cont)
Bacteria with Inducible Mechanisms for Chlorinated Alkene Degradation

Culture	Inducer	Comments
Methylosinus trichosporium OB3b	methane	Oldenhuis et al. (1991); DeSpirito et al. (1990);
Methylamonas sp. A45	methane	Type I methanotroph; DeSpirito et al. (1990)
Methylamonas sp. MN	methane	Type I, DeSpirito et al. (1990)
Methylamonas sp. MM2	methane	Type I, DeSpirito et al. (1990)
Methylamonas sp. GD1	methane	Type I, DeSpirito et al. (1990)
Methylamonas sp. GD2	methane	Type I, DeSpirito et al. (1990)
Methylocystes parvus OBBP	methane	Type II, DeSpirito et al. (1990)
Methylococcus capsulatus (Bath)	methane	Type X, DeSpirito et al. (1990)

Table 2 (cont)
Bacteria with Inducible Mechanisms for Chlorinated Alkene Degradation

Culture	Inducer	Comments
Bacillus(?) and *Desulfoto-maculum*	Toluene + various organics	Kästner, 1991; degradation followed trans. from aerobic to anaerobic
Mycobacterium convolutum	propane	Wackett et al. (1989)
Mycobacterium rhodochrous W-21	propane	Wackett et al. (1989)
Mycobacterium rhodochrous W-24	propane	Wackett et al. (1989)
Mycobacterium rhodochrous W-25	propane	Wackett et al. (1989)
Mycobacterium vaccae JOB5*	propane	Wackett et al. (1989)

* *Mycobacterium vaccae* JOB5 TCE degradation assays run with 40 mM TCE in methanol stock solution. Methanol is known to inhibit TCE degradation (Wackett et al., 1989)

Bacteria capable of metabolizing aromatic compounds use mono- and dioxygenases for ring hydroxylation prior to ring cleavage (Nelson et al., 1987, 1988; Zylstra et al., 1989; Folsom et al., 1990). However, several different possibilities exist for oxygenation of benzene rings. In *Pseudomonas putidia* F1, the toluene dioxygenase system consists of three components, and results in the formation of cis-toluene dihydrodiol (Zylstra et al., 1989) which proceeds to methyl catechol (Rochkind et al., 1986). The system components (coded for by the todC1C2BADE genes of the tod operon) transfer electrons from NADH to toluene which acts as a terminal electron acceptor. The flavoprotein todA component (ReductaseTOL), transfers electrons from NADH to ferredoxinTOL (todB) which transfers electrons to an

iron-sulfur component (ISPTOL, todC1C2), which oxidizes toluene (Zylstra, 1989). A mutant strain of *P. putida* F1 lacking the toluene dioxygenase system was unable to degrade TCE. Oxygenation of toluene by *P. putida* mt-2, and *P. aeruginosa*, results in hydroxylation of the toluene methyl group. The toluene monooxygenase of *P. mendocina* has been shown to catalyze the p-hydroxylation of toluene to form p-cresol. *P. putida* mt-2 containing the TOL plasmid converts toluene to benzaldehyde (Shields et al., 1989) then to benzoic acid, which is converted to catechol. Hydroxylation by *P. aeruginosa* results in direct formation of benzoic acid which is converted to catechol (Rochkind et al., 1986). A novel mechanism of toluene oxidation was described by Shields et al., 1989, for *P. cepacia* G4. Data presented by this group indicated that the reaction was catalyzed by a toluene o-monooxygenase that resulted in the formation of o-cresol prior to the formation of 3-methyl catechol. Shields points out that this may be analogous to the production of 2,3-xylenol by cultures of *P. stutzeri*. The mechanism by which bacteria cleave catechol derivatives formed by mono- and dioxygenase systems apparently determines if TCE will be degraded. Organisms cleaving catechol derivatives in the meta-position degrade TCE, while those using the ortho-position do not (Nelson et al., 1988). The mono- and dioxygenases of *Pseudomonas* are capable of cometabolically degrading TCE and DCE, but not VC. A partial list of the aromatic metabolizing bacteria of interest in TCE degradation includes *Pseudomonas*, *Acinetobacter*, *Arthrobacter*, and *Beijerinkia*.

Cometabolism by Gaseous Alkane-oxidizing Bacteria- The gaseous alkane-oxidizing bacteria constitute the second major category of organisms capable of cometabolically mineralizing chlorinated alkenes. Although all the gaseous alkanes (C1-C4) can be metabolized by bacteria (Vestal, 1984), methane and propane have received the most attention because of the monooxygenase systems they induce, are responsible for cometabolic reactions

that degrade and mineralize TCE and DCE, as well as VC. The oxygenase systems of methane and propane metabolism hydroxylate alkanes, and form epoxides across alkene bonds (Patel et al., 1983a).

Methanotrophs- Bacteria that metabolize methane are classified as either facultative or obligate methylotrophs. Procaryotes unable to utilize methane are excluded from the family *Methylococcaceae*, even though they can utilize other single carbon compounds. Facultative methylotrophs are those organisms that can grow on a variety of carbon sources, including single-carbon compounds and those with carbon-carbon bonds such as glucose. Obligate methylotrophs require methane or methanol for growth and are designated methanotrophs. Some can use other carbon sources, if formaldehyde from the metabolism of methane or methanol is present or is added to the growth medium. In any case, for obligate strains, compounds such as carbon dioxide, acetate, formate, some amino acids, and cooxidizable substrates cannot be used as sole carbon sources (Whittenbury and Krieg, 1984; Higgins et al., 1981). Methanotrophs are divided into three groups, I, II, and X, based on the arrangement of intracellular membranes. Two metabolic systems are used, the ribulose monophosphate cycle by type I organisms, and the serine pathway by type II cultures. Methanotrophs are aerobic organisms, and should not be confused with the highly anaerobic methanogens which produce methane rather than consume it as a carbon source.

Methanotrophs are of interest for bioremediation processes because they produce methane monooxygenase (MMO), an enzyme that catalyzes epoxidation and subsequent degradation of TCE, DCE, and VC (Moore et al., 1989; DiSpirito, 1991; Cornish, 1984; Stirling, 1979; Green, 1989; Oldenhuis, 1989). Both soluble (sMMO) and membrane bound (particulate, pMMO) MMO are produced. While the sMMO is several orders of magnitude more efficient at cometabolically degrading TCE, it is not produced if copper is present in the

growth medium at a concentration of more than 4.8 μM. The pMMO is produced when copper is present in the medium. Historically, *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b have been used as bench-mark strains for evaluations of MMO degradative properties (Oldenhuis et al., 1989; Green et al., 1989; Cornish et al., 1984; Stirling and Dalton, 1979) because many researchers contend that pMMO does not degrade chlorinated alkenes. However, DiSpirito has provided data indicating that although pMMO is less efficient than sMMO, pMMO does degrade TCE (DiSpirito, 1991, 1992). Earlier work by Patel et al., 1983b, demonstrating epoxidation of gaseous n-alkenes by the pMMO from methane-utilizing bacteria supports DiSpirito's research.

Propane-oxidizing bacteria- The propane-oxidizing bacteria constitute the second major group of alkane-oxidizing organisms of importance to TCE degradation. The metabolism of propane as a sole carbon source is a common phenomenon, although the number of genera that can utilize it as a carbon source are limited. The recalcitrance of gaseous alkanes as growth substrates, as suggested by Perry (1980) is shown below:

C11 - C18 n-alkanes < C11 -C18 alkenes < gaseous alkanes < C5 - C9 n-alkanes.

In one culture collection, organisms with the ability to grow on propane made up about half of the cultures that utilized C10 - C20 n-alkanes (Perry, 1980). The propane-oxidizing bacteria are generally considered to be part of the *Mycobacterium- Nocardia-Corynebacterium* group (MNC), which is loosely associated with the genera *Rhodococcus, Brevibacterium, and Arthrobacter* (Woods and Murrell, 1989). More detailed description of microorganisms able to utilize propane was given by Perry (1980), and is listed in Table 3. The MNC group share several attributes that make these interesting microorganisms difficult to work with. They have long doubling times, low growth yields, stable mutants are difficult to obtain, and wild type cultures yield little or no extracellular metabolic products. *Rhodococcus*

rhodochrous PNKb1 is reported to have doubling times of 8 h and 3 h for propane and propionate, respectively (Woods and Murrell,1989). However, the organisms are ubiquitous, and can be isolated from soil and water by enrichment techniques.

Table 3 **Bacteria Associated With Propane-oxidation**
Arthrobacter
Brevibacter
Corynebacterium
Mycobacterium smegmatus
M. lacticolum
M. phlei
M. album strain 7E1B1W (ATCC 29676)
M. rhodococcus OFS (ATCC 29672)
M. rubrum var. *propionicum*
M. parafinicum
M. rhodococcus strain A-78 (ATCC 29670)
M. rhodococcus strain OC2A (ATCC 29675)
M. rhodochrous strain 7E1C (ATCC 19067)
M. vaccae strain JOB5 (ATCC 29678)
M. perrugosum var. *ethanicum*
M. convolutium strain R-22 (ATCC 29671)
M. convolutium strain NPA-1 (ATCC 29674)
Nocardia rubropertincta
N. caviae
N. brasiliensis
N. madurae
N. convoluta
N. asteroides
Pseudomonas liquifaciens
P. subluteum
P. caudatus
unclassified pseudomonads

(Perry, 1980)

One study yielded over eighty strains of Gram-positive propane utilizing bacteria, all of which fit the MNC group profile (Woods and Murrell, 1989).

The metabolism of propane is of interest because it is a three-carbon asymmetrical molecule, and as such has the potential for either terminal or subterminal oxidation. Lukins

and Foster (1962) state that terminal oxidation of alkanes is the most common mode of oxygen attack, leading to formation of a primary alcohol, then the corresponding fatty acid. For alkanes >C10, Vestal and Perry (1969) report (citing the work of Kester and Foster, 1963) that diterminal oxidation is possible, as a second oxidative alternative. Oxidation of the ß-carbon of propane represents a third possible site of attack.

Several lines of evidence indicate that the ß-carbon can be oxygenated. First, methyl ketones are produced as intermediates from the oxidation of normal C3-C6 alkanes by *Mycobacterium smegmatis* (Lukins and Foster, 1963). When *M. smegmatus* was grown on propane, significant amounts of acetone were produced, in comparison to less than 1% as much n-propanol. The same strain produced 2-butanone from n-butane, 2-pentanone from n-pentane, and 2-hexanone from n-hexane. The use of D_2O in their work showed that labeled acetone was not present in significant quantities, which suggests that propylene is not an intermediate of propane metabolism. In the same set of experiments, a methylotroph, *Pseudomonas methanica*[2] grown on methane, cooxidized acetone and 2-butanone to the corresponding primary alcohols. Second, metabolism of propane by *Mycobacterium vaccae* JOB5[3], yields isopropanol and acetone which are subterminal oxidation products. While not completely definitive by itself, fatty acid profiles from propane grown cells of *M. vaccae* JOB5 indicate metabolism of propane proceeds by synthesis of even-numbered intermediates, as opposed to odd-numbered intermediates with non-propane grown cells (Perry, 1980). Perry,

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[2] The name *Pseudomonas methanica* no longer has standing in bacterial taxonomy, according to Bergey's manual of Systematic Bacteriology, Volume 1. This microorganism may be *Methylomonas methanica* by current taxonomic designation.

[3] *M. vaccae* JOB5 has been used as a bench-mark strain in many studies, and a historical note on its origin is in order. The strain was isolated by Jiro Ooyama in the laboratory of the late Dr. J. W. Foster at the University of Texas, and was originally classified as *Brevibacterium*. The first published report of the organism was in 1965 by Ooyama and Foster. One assumes that JOB5 is an acronym for Jiro Ooyama *Brevibacterium* 5. (Vestal and Perry, 1980).

(1980), and Stephens and Dalton,, (1986), state that examination of fatty acid profiles alone can be deceptive, because some terminal oxidation of propane occurs along with the subterminal oxidation.

The role of terminal and subterminal oxidation in three strains of *Arthrobacter* was investigated by Stephens and Dalton, (1986). By examining oxygen uptake by cells of propane induced and non-induced cultures, they established that for these strains, both terminal and subterminal intermediates were formed. Woods and Murrell (1989) used the same approach to investigate the metabolism of propane by *Rhodococcus rhodochrous* PNKb1. This isolate uses propane as a sole alkane carbon source and therefore is a good test strain for studies of propane metabolism. The organism grew well on 1-propanol, propanal, propionate, 2-propanol, acetone, and acetol. The conclusion from these growth patterns is that *R. rhodochrous* PNKb1 can utilize intermediates of both terminal and subterminal oxidation. Substrate-dependent oxygen uptake was determined for propane, 1-propanol, isopropanol, and pyruvate grown cells. Data from these assays revealed that (1) propane oxidation was inducible only in propane-grown cells; and (2) that the enzymes for utilization of subterminal oxidation products are constitutively expressed in this organism. Together with the strain's low propionate oxidation level (which may indicate the lack of a propionate transport system) this suggests that PNKb1 has the metabolic potential to utilize both terminal and subterminal oxidation products of propane. Based on evidence from this work, the proposed pathway for propane metabolism in *R. rhodochrous* PNKb1 is shown in Figure 1.

Studies on methyl ketone production by alkane-grown cells (Lukins and Foster, 1963) led to proposals by several research teams concerning the nature and fate of intermediates of propane metabolism. By assaying for key enzymes in PMO induced and non-induced cells

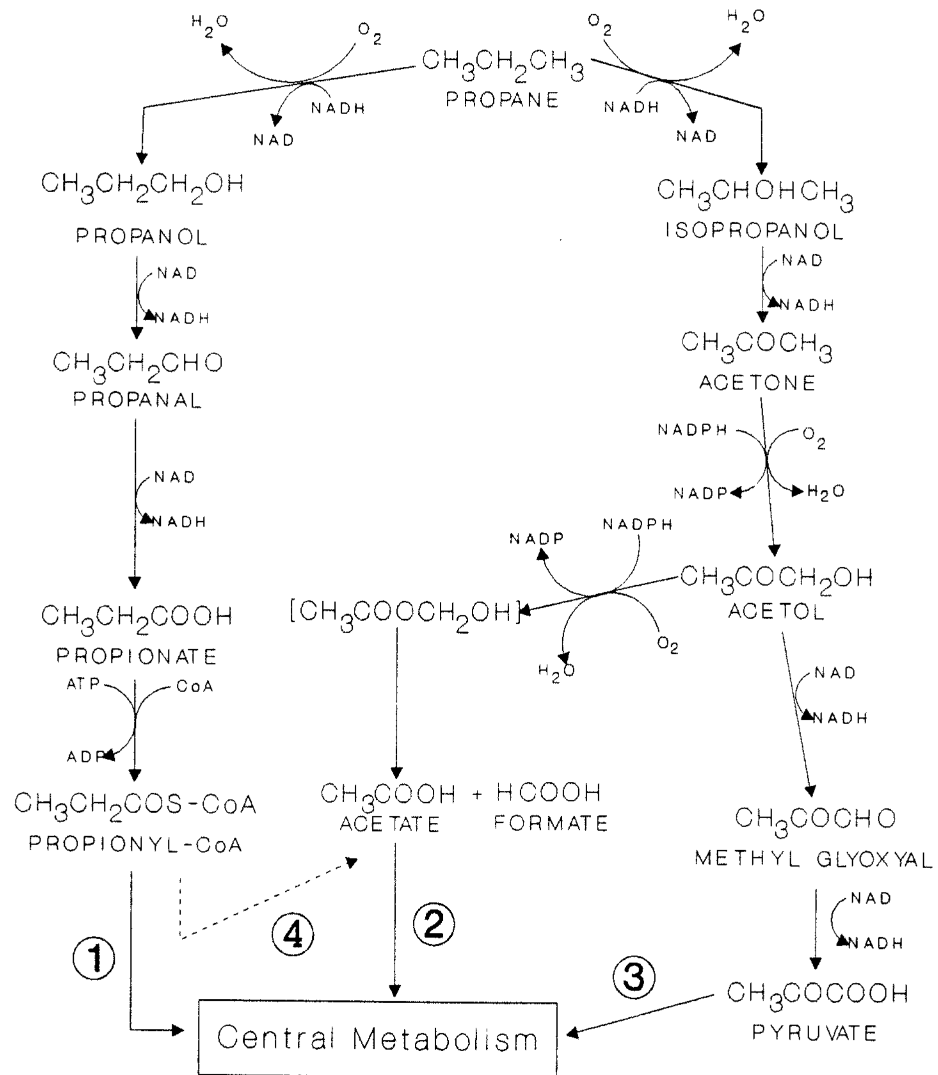

Figure 1. Proposed pathway for propane metabolism in *R. rhodochrous* PNKb1. 1. Terminal oxidation of propane. 2. Production of acetate and formate. 3. Pyruvate production by subterminal propane oxidation. 4. Acetate production from propionyl-CoA through the methylmalonate semialdehyde pathway.

(Figure 2), Vestal and Perry, (1969), suggested the existence of divergent pathways for propane and propionate by *Mycobacterium vaccae* JOB5. Further work in Perry's laboratory (1979, 1980) indicated that in *M. vaccae* JOB5, propane and propionate were utilized through different pathways. Vestal and Perry (1969) had noted that isocitrate lyase was present in propane-grown cells and absent in non-propane-grown cells. In their study, they measured the levels of the enzymes isocitrate lyase, malate synthetase, and methyl malonyl-CoA mutase in cells cultured on propane, propionate, n-propanol, isopropanol, acetate, and glucose.

Isocitrate lyase is a key enzyme in the glyoxylate shunt, an anapleurotic function of the TCA cycle that provides a source of C4 compounds, the lack of which would eventually drain the TCE cycle of reactants (Wegener et al., 1968). Following conversion of acetyl-CoA to citrate by citrate synthetase, isocitrate is cleaved to glyoxylate and succinate. The enzyme is inducible, and found in propane induced cells. Malate synthetase is a constitutive enzyme that converts acetyl-CoA to malate in the TCA cycle. Methyl malonyl-CoA mutase is instrumental in converting methyl malonyl-CoA to succinate and is constitutive. It is the second enzyme in the pathway that converts propionyl-CoA to methyl malonyl-CoA by way of methyl malonyl-CoA racemase.

Wegener's results showed that isocitrate lyase was induced in *Mycobacterium vaccae* JOB5 by growth on propane, acetone, or isopropanol. Growth on propionate or n-propanol resulted in formation of insignificant amounts of the enzyme. If α-oxidation utilized isocitate lyase, it should be induced in strain JOB5 by growth on propionate. They interpreted this to mean that propionate is metabolized through a terminal-oxidation pathway, and does not use isocitrate lyase, while propane is oxidized through sub-terminal oxidation using isocitrate lyase.

Corroborating evidence for this idea was obtained from the isolation of ^{14}C labeled intermediates. Vestal and Perry's (1969) proposed reaction scheme for propane and propionate

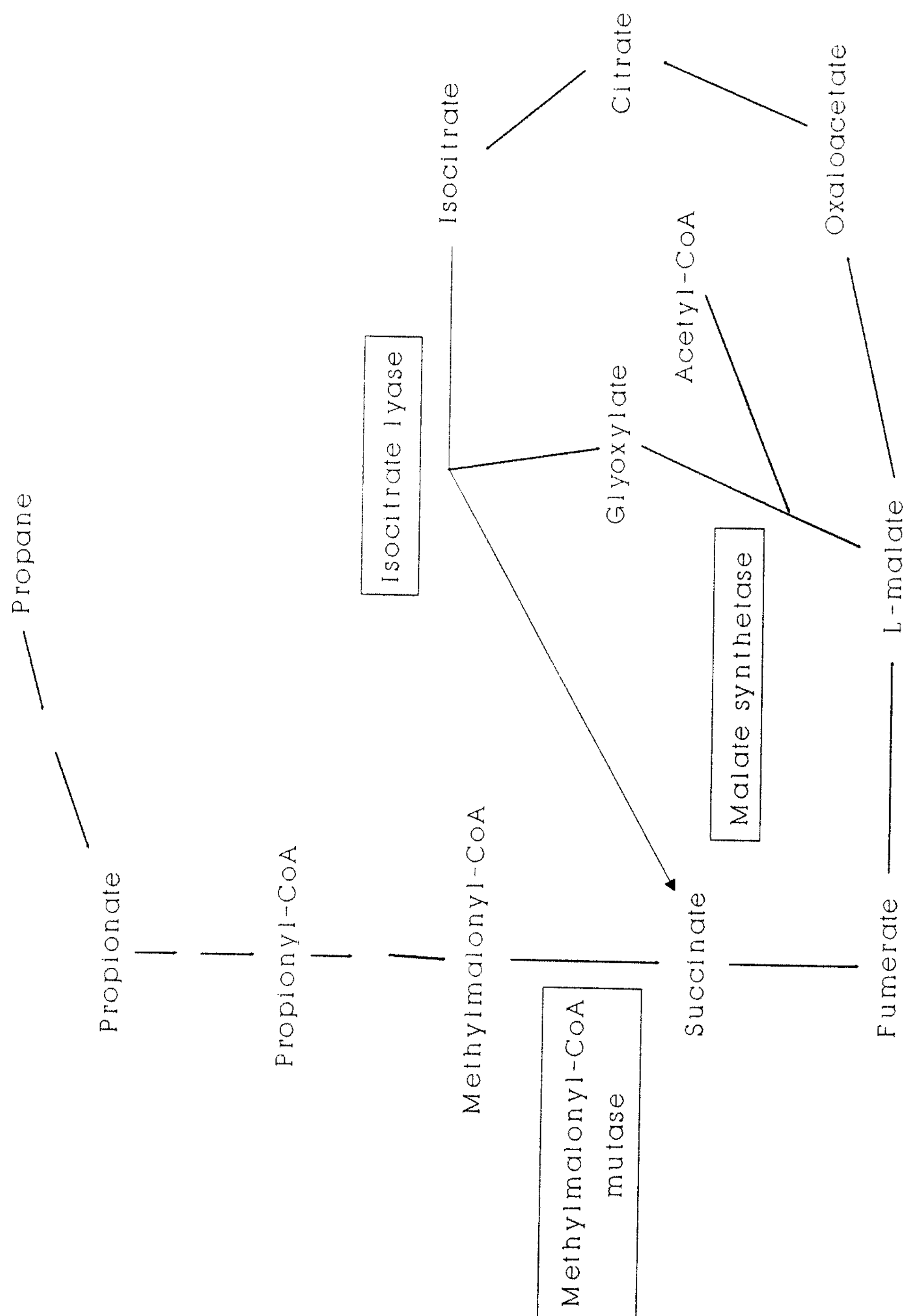

Figure 2. Enzymes indicitive of terminal- and subterminal-oxidation according to Vestal and Perry (1969).

metabolism is given in Figure 3.

Stephens and Dalton (1986) continued this line research by pointing out that in addition to the pathways determined by Vestal and Perry (1969) and Lukins and Foster (1963), it was also possible to convert propionyl-CoA to acetyl-CoA by the malonate semialdehyde or malonate semialdehyde-CoA pathways. Propionate is converted to propionyl adenylate by acetyl-CoA synthetase, then to propionyl-CoA by the same enzyme. At this point, the metabolic choices are for propionyl-CoA to proceed to succinate in the TCA cycle by the methyl malonyl-CoA path, or to acetyl-CoA by the malonate semialdehyde or malonate semialdehyde-CoA pathways. The benefit to the microorganism is that it could shift its metabolic output to supply the needs of the cell.

One implication of Stephens' and Dalton's work, (1986), is that it is not entirely accurate to predict the occurrence of terminal or subterminal oxidation of propane based solely on the presence or absence of isocitrate lyase. If the malonate semialdehyde or malonate semialdehyde-CoA pathways are used, isocitrate lyase will be induced to convert the resulting isocitrate to glyoxylate and succinate. For this reason both Stephens and Dalton (1986), and Woods and Murrell (1989) used specific oxygen uptake rate (SOUR) data from cells challenged with intermediates of propane metabolism as an indication of the oxidative pathway that was predominately expressed.

In work with oxygen uptake by *Arthrobacter*, Stephens and Dalton (1986) concluded that (1) the involved propane monooxygenases were nonspecific in their oxidation of propane since both n-propanol and isopropanol were produced; (2) that the nonspecific insertion of oxygen into alkanes, and formation of epoxides from alkenes was a matter of enzyme mechanics rather than specific adaptations to hydrocarbon metabolism; and (3) that not all alkane monooxygenases are mechanistically similar. They observed that growth of their

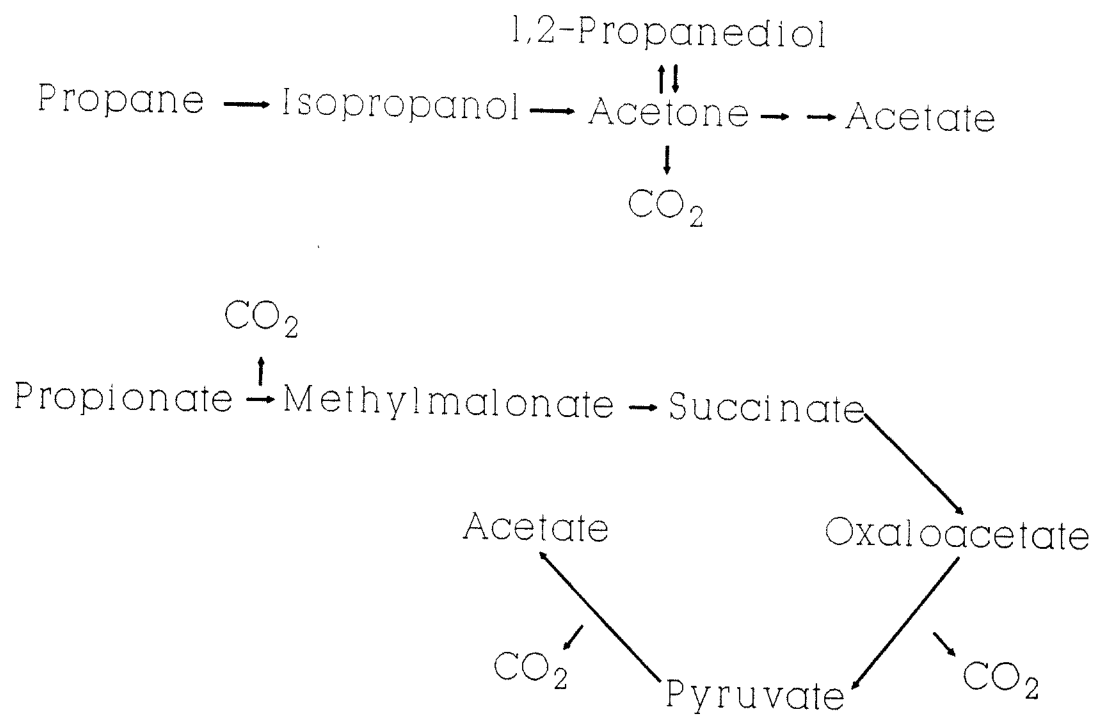

Figure 3. Proposed pathway of propane and propionate by *Mycobacterium vaccae* JOB5.

isolates on intermediates of terminal oxidation resulted in more rapid growth than when intermediates of subterminal oxidation were used. They interpreted this to mean that for these organisms, subterminal oxidation was a less efficient pathway than the terminal route. Their overall conclusion was that terminal oxidation of propane was the principle metabolic route, and was therefore more important in propane metabolism than previously thought. This work is summarized in Figure 4. Using the same oxygen uptake procedure, Woods and Murrell 1989) concluded that *Rhodococcus rhodochrous* PNK1b utilized both terminal and subterminal oxidation pathways.

The use of propane-oxidizing bacteria for degradation of TCE was first described by Wackett et al., in 1989. In this work, oxygenase production was induced in fourteen microorganisms by growth on propane. Following enzyme induction, all microorganisms were tested for their ability to degrade TCE. Data from the comparison (Table 4) indicates that the ability to degrade TCE is not a common property of broad-specificity oxygenases. Of the microorganisms tested, only the *Mycobacterium* propane monooxygenase (PMO) degraded TCE. Oxygenases assayed included both dioxygenases and monooxygenases, as well as cyclohexanone monooxygenase (a flavoprotein), nitropropane dioxygenase (an iron flavoprotein), cytochrome P-450$_{CAM}$ and cytochrome P-450$_{MEG}$ (heme iron proteins), alkane monooxygenases (a non-heme iron oxygenase), and propane monooxygenase, which constitutes an uncharacterized oxygenase group. Cumulative error in the quantitative procedures precluded accurate measurement of TCE at levels of degradation less than 8%. Reductions in TCE levels were well above the error limit of the experiment for all of the *Mycobacterium* species. It is interesting to note that the *Mycobacterium* species tested degraded TCE at different rates, indicating that species differences do occur with respect to this degradative ability. Like MMO, PMO hydroxylates propane to form an alcohol, and forms an epoxide

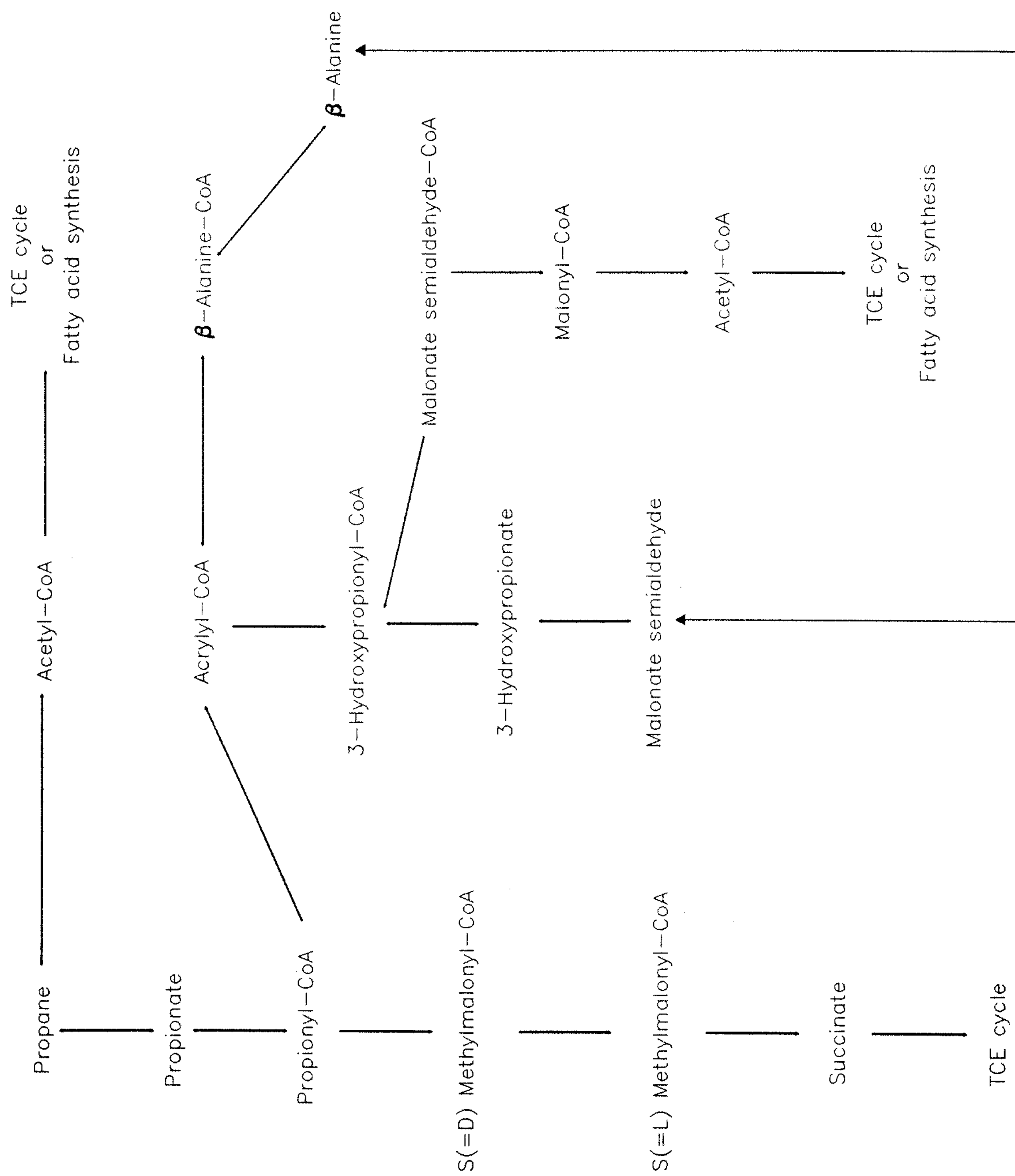

Figure 4. Biochemical pathways linking propane metabolism to central metabolism.

Table 4
Microorganisms, Their Catabolic Oxygenases, and TCE Degrading Ability

Microorganism	Oxygenase induced	%TCE degraded 1 h	%TCE degraded 24 h
Hansenula mraki	Nitropropane dioxygenase	<8	<8
Acinetobacter sp. strain NCIB 9871	Cyclohexanone monooxygenase	<8	<8
Bacillus megaterium ATCC 4581	Cytochrome P-450$_{MEG}$	<8	<8
Pseudomonas putida G786	Cytochrome P-450$_{CAM}$	<8	<8
P. putida 3400	4-Methoxybenzoate monooxygenase	<8	<8
Streptomyces griseus ATCC 13273	Preocene monooxygenase	<8	<8
S. griseus NRRL 8090	Preocene monooxygenase	<8	<8
Pseudomonas oleovorans PpG6	Hexane monooxygenase	<8	<8
P.aeruginosa 473	Hexane monooxygenase	<8	<8
Mycobacterium convolutum	Propane monooxygenase	20 ± 2	29 ± 5
M. rhodochrous W-21	Propane monooxygenase	23 ± 4	39 ± 2
M. rhodochrous W-25	Propane monooxygenase	15 ± 3	47 ± 2
M. rhodochrous W-24	Propane monooxygenase	16 ± 3	52 ± 3
M. vaccae JOB5	Propane monooxygenase	41 ± 3	99 ± 4

(Wackett et al., 1989)

linkage with alkenes (Patel et al., 1983a, b). The PMO catalyzed oxygenation of propane potentially yields a primary or secondary hydroxyl derivative.

In the preceding discussion, it has been shown that variations in alkane metabolism exist, with respect to the position of propane oxygenation. Some microorganisms such as the *Arthrobacter* species of Stephens and Dalton, show a preference for terminal oxidation of propane. Others, such as *M. vaccae* JOB5 seem to preferentially use the subterminal route, while *R. rhodococcus* PNK1b apparently is able to use both. Considering the results of the Wackett study (1989), and that the propane oxidative pathways for *Mycobacterium* and *Rhodococcus* are similar, it is possible that one of the oxidative pathways (terminal or subterminal oxidation) is preferentially expressed during cometabolic degradation of TCE. If TCE degradation by propane-oxidizing bacteria is dependent on a particular oxidative pathway that is not generally expressed, then the utility of these bacteria as bioremediation tools will be limited. If, on the other hand, TCE degradation is more nonspecific in nature, more study should be given to the possibility that one of the oxidative pathways (terminal or subterminal oxidation) is preferentially expressed during cometabolic degradation of TCE. This study proposes to provide data with which to answer this question.

CHAPTER II

MATERIALS AND METHODS

Gases and chemicals. CP-grade propane (99% purity) was used for enrichment of environmental isolates. Gases used for gas chromatographic analysis (helium carrier gas, and 95% argon-5% methane make-up gas) were all ultra-high purity grade (99.9995% purity). All chemicals used for media formulation and assays were of ACS grade or higher.

Growth media. Four types of growth media (Appendix) were used in these studies. General assays for viable cells in the leachate were done using the dilute peptone-glucose-yeast extract agar (DPTYG) of Balkwell and Ghiorse, 1985. A review of current literature shows that toluene-oxidizing bacteria are routinely identified as pseudomonads, therefore, Stanier's minimal salt medium (SMSA, Stanier et al., 1966) was used for isolation and characterization of these isolates. Tom Phelps, Oak Ridge National Laboratory, noted in his work with indigenous TCE degrading microflora, that the addition of small amounts (50 mg per liter) of yeast extract and trypticase peptone accelerated growth and degradative efficiency (Fliermans et al., 1988). These compounds were added as supplements to standard Stanier's medium. Propane- and methane-oxidizing isolates were isolated and maintained on the nitrate-mineral salts media of Higgins (Cornish et al., 1984), or Whittenbury, 1984. Both Higgins, and Whittenbury media gave rise to the same colony types, the only difference being that colonies on Whittenbury were more mucoid. Therefore, the medium yielding the most concise

colonies, i.e., Higgins medium, was selected for routine use (see Bacterial Isolation Methods below).

Soil and Groundwater Samples.

French-drain leachate- Bacterial isolates for TCE degradation studies were obtained from an industrial groundwater below about 25 feet (nominal depth) leachate collected from a French-drain system. The French-drain system is down gradient from a contaminated area that was used as a disposal pit for industrial wastes during the 1950's and 1960's (Figure 5). Material deposited included aircraft paint, and a variety of hydrocarbons and solvents, including perchloroethylene and trichloroethylene. Once full, the pit was covered, and paved for use as a parking lot. Downward flow of the groundwater is prevented by a limestone aquatard, while lateral motion (down gradient) toward the French-drain is accelerated by a natural groundwater flow and leaks in the fire mains of the company in charge of the area. From an engineering standpoint, these leaks are rather inconsequential, but terms of groundwater movement a significant volume of water is throughput. This artificial groundwater charge significantly increases the volume of water traversing the dump area, and possibly limits the areas of anaerobiosis. The liquid level in the French-drain was approximately eight feet below ground level and approximately six feet deep.

Leachate composition- Gas chromatographic analysis of the French-drain leachate was done by Hargis and Associates, Thousand Oaks, CA., as part of a bioremediation study for the company exercising custodianship of the contaminated area. In addition to chemical constituents listed, a large number of non-priority, in semiquantified chemicals, as well as substantial amounts of oil and grease were present in the leachate (Table 5).

Leachate sample collection- Sterile, one-liter, screw capped Erlenmeyer flasks were used to collect leachate samples from the French-drain. Bottom sediment in the drain was

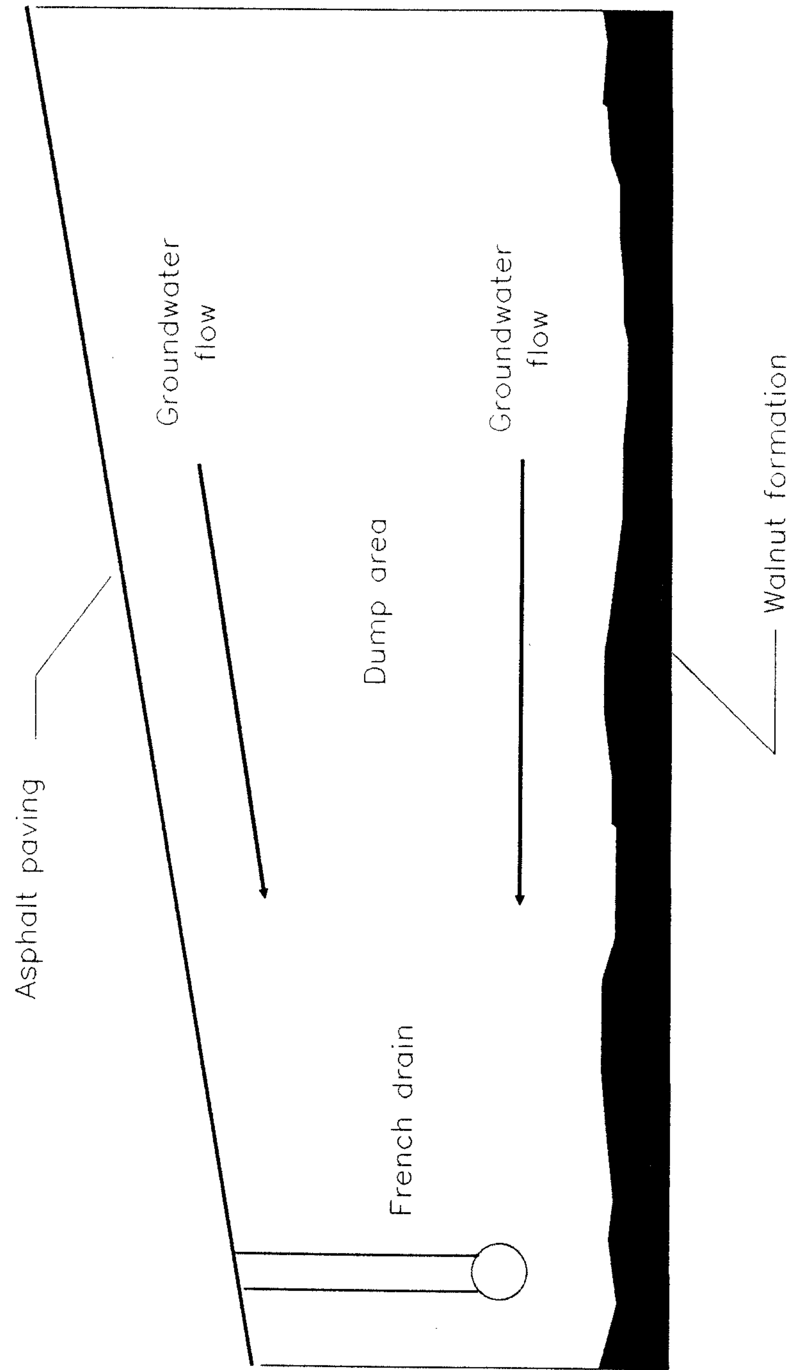

Figure 5. Cross section of French drain area.

agitated with the lead-bottomed sample holder as sample collection proceeded and was

Table 5
Organic Contaminants in Leachate Stream

Contaminant	Concentration (ppm)
Tetrachloroethylene	0.80
Trichloroethylene	3.30
cis-Dichloroethylene	46.25
Vinyl chloride	3.00
Toluene	0.63
Copper	0.20

collected in the Erlenmeyer flask along with the leachate. Samples were capped and stored on ice for transport back to the laboratory, where they were stored at 4 ^{0}C until processing. Time from sample collection to processing did not exceed 4 hours.

pH and temperature measurements- pH measurements for water samples were made by conventional techniques, using an Orion 720 pH meter and temperature compensated electrode. For soil samples, 10 g of dry soil was added to 20 ml of distilled water and stirred for 30 minutes before the pH was determined. Temperature measurements were made on site with a standard laboratory thermometer. A one year chronological history of pH and temperature measurements was obtained.

Soil Samples- To determine the number of viable cells in soil surrounding the drainage area for the French drain system, soil samples were assayed using pour plates of dilute peptone-yeast extract-glucose agar (DPTYG). The samples were collected from several areas at the corporate facility involved, with sample depths ranging from the surface to a depth of 22.5 feet. Samples were taken from split-spoon core borings and selected sections of the soil cores were placed in plastic bags, then stored at 4 ^{0}C for no longer than 4 h prior to processing. The sections of soil core for assay were placed in 150 X 15 mm petri dish bottoms

in a laminar flow hood and aseptically split open with sterile knives. Ten-gram portions from the interior of the soil cores were collected and transferred to 100 ml aliquots of filter sterilized 0.1% sodium pyrophosphate (PP) (adjusted to pH 7 with HCl), then sonicated 6 min. Ten-fold serial dilutions were made using PP blanks, then plated. Plates were incubated at 25 ^{0}C and counted after 48-72 h, and again after nine days of incubation. Some of the core-samples were notable because of the presence of jet fuel (JP-4), which could be detected by odor. In addition two samples came from an area several acres in size that had been covered with a 10-inch thick cement covering for about 30 years.

Acridine orange epifluorescent direct cell counts (AODC) - Acridine orange epifluorescent direct cell counts were conducted by the technique of Balkwell and Ghiorse et al. (1985), on the groundwater used for isolations in order to determine total numbers of cells present. This technique identifies all cells, both viable and nonviable, because the acridine orange dye (AO) absorbs to all organic material. The dye also can absorb to extraneous organic material in the sample such as humus. Noncellular absorption yields either objects that are sometimes difficult to distinguish from cells (especially submicron size particles), or a background "glow" that reduces the contrast of the preparation. Soil samples were prepared by adding 2.5 g of soil to 20 ml of filter sterilized PP in a 125 ml Erlenmeyer flask. Samples were placed on a rotary shaker at 160 rpm for 15 min to separate microbial cells from soil particles.

Specimens were prepared by spreading 5µl aliquots of supernatant solution or groundwater in 1 cm diameter circular areas on glass slides. The water was allowed to dry, then mildly heat fixed, before addition of 0.01% aqueous, filter sterilized AO. The dye was allowed to remain on the slide 3 minutes before being washed off with distilled water. While still wet, preparations were covered with a cover slip, and edges sealed with clear lacquer to

prevent drying. Slides were then examined with a 100X objective. The microscope was fitted with a calibrated, 1.0 mm X 1.0 mm ocular reticle with 100 individual areas. The number of areas counted depended on the number of cells in the preparation, as shown in Table 6.

Table 6
Cells/Field of View Versus Fields of View Counted

Cells/Field of View	Fields of View Counted
>10	10
5-10	15
<5	25

Isolation and identification procedures.

Toluene-oxidizing isolates- Primary enrichment of toluene-oxidizing bacteria was accomplished through addition of toluene to leachate samples incubated in an electrolytic respirometer (see Electrolytic Respirometry section below). For this procedure, two pH values, 6.0 and 7.2 were used. Standard Stanier's 1 M phosphate buffer was used to adjust to pH 7.2. To obtain pH 6.0, 1 M Na_2HPO_4 was titrated with 1 M KH_2PO_4. Initial viable cell counts for this procedure were determined using serial dilutions of the leachate in 0.1% Na pyrophosphate buffer (pH 7, filter sterilized). Two liters of leachate were centrifuged at 10,000 g for 20 minutes, and the resulting pellet washed three times with sterile 0.1% Na pyrophosphate buffer (pH 7). The final pellet was resuspended in 90 ml of buffer and used as inoculum for the electrolytic respirometer flasks. Ten ml portions of this suspension were used as inoculum for the respirometer flasks. The flasks contained 1 liter each of Stanier's mineral salts medium supplemented with 50 mg each of yeast extract and trypticase peptone.

Final pH values of duplicate samples were 6.0 and 7.2, respectively. Water bath temperature was maintained at 25 ^{0}C. Toluene volumes of 58 µl (equals 50 ppm) were added at T_0=85, 117.2, and 144.2 hours of incubation. Although respirometer incubation continued for an extended period of time (220 h), samples were withdrawn from the reactor vessels for viable cell counts at 63 h. SMSA spread plates were used for the enumeration. Appropriate colonies were transferred for purification and further work.

After several preliminary experiments to determine an acceptable method of culturing soil and groundwater isolates with volatile hydrocarbons, a sealed cylinder with side-port and solvent reservoir was designed and constructed (Volatile hydrocarbon incubation chamber, VHIC, Figure 6)[4]. Toluene was added in vapor phase by allowing it to diffuse into the incubation tube from a side arm test tube connected to the plexiglass chamber by a piece of teflon tubing. Plexiglass plates were sealed to the top and bottom of the incubation tube with high-vacuum silicon grease, thereby maintaining a toluene-air atmosphere. An alternative procedure utilized Durham fermentation tubes taped to the under-side of petri dish lids. The tubes contained a few µl of toluene and were plugged with sterile cotton plugs. This technique also works well for cultivation of phenol degrading organisms. For isolation of individual microorganisms, 100 µl aliquots of the primary enrichment solution were spread on SMSA plates, which were incubated the VHIC at 25 ^{0}C. Colonies resulting from spread plate incubation were picked and transferred to fresh plates for purification.

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[4]Origination of this method was partially the result of conversations with Mr. R.L. Raymond of the duPont Corporation. According to Raymond, early German petroleum microbiologists tried to culture bacteria by placing a beaker of the hydrocarbon in the closed container with the culture dishes. This led to evaporation of an excess amount of hydrocarbon, which displaced the oxygen at the bottom. The resulting anoxic conditions were unfavorable to the growth of aerobic bacteria. Introduction of the hydrocarbon through a side port (such as the vacuum port of a large vacuum desiccator) limits the amount the amount of hydrocarbon that defuses into the growth chamber.

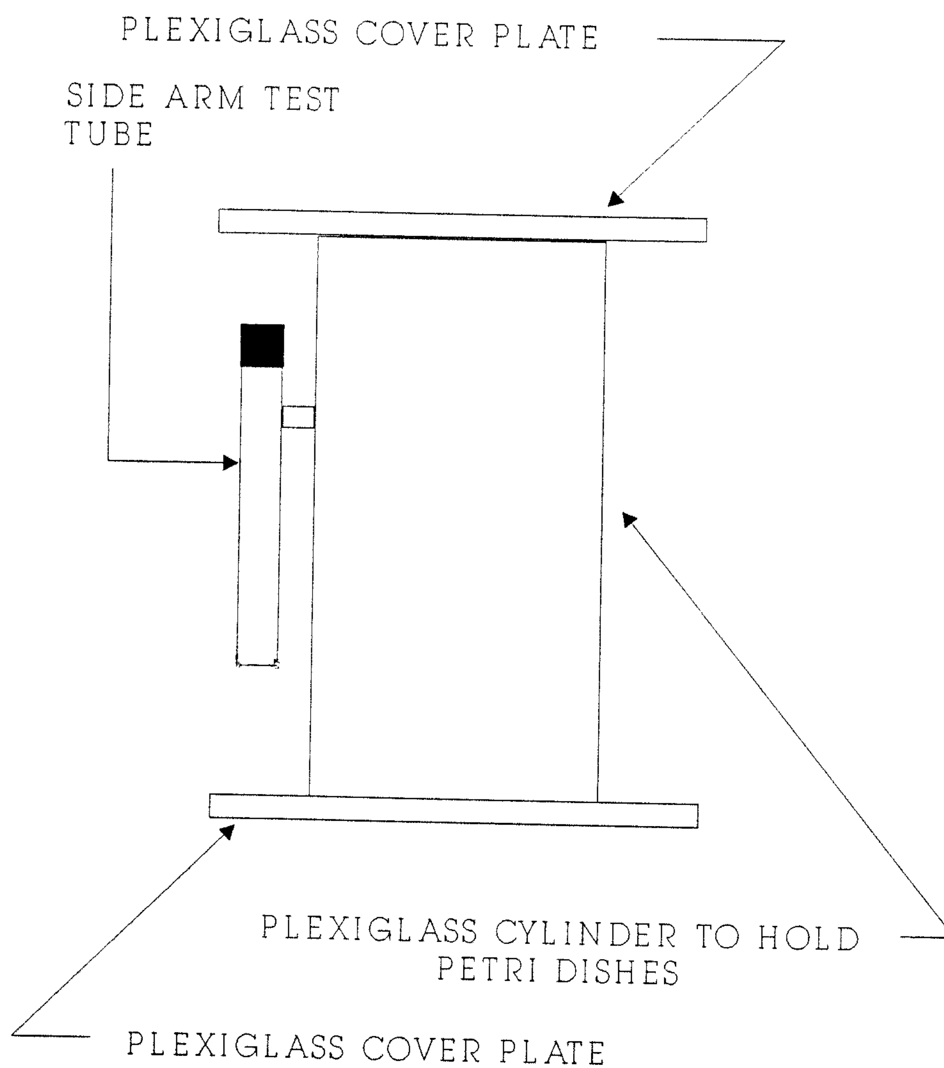


Figure 6. Volitile hydrocarbon incubation chamber.

To determine the relative oxygen use contributions of representative isolates, two cultures (T2-1-1, and T2-4-1) were selected for further respirometric work. These isolates were selected because of their growth characteristics and prominence among representative colonies on isolation plates. Both isolates were grown for 48 hours in liquid medium and 1 ml aliquots transferred to respirometer flasks containing medium at pH 6.0 or 7.2.

Propane-oxidizing isolates- One liter of leachate was centrifuged for 20 minutes at 10,000 g at 4 ^{0}C. The pellet was washed three times in 0.1 M phosphate buffer and the final pellet resuspended in 25 ml of phosphate buffer. Five-ml aliquots were used as inoculum for 250 ml portions of Higgins, and Whittenbury media (pH7) in 1 liter flasks closed with neoprene stoppers. Hypodermic needles (18 gage) with removable end plugs were inserted through the neoprene stoppers and used as gas ports. The head space of these flasks were purged with a 1:1 mixture of propane and air at a rate of approximately 1.5 ml/minute. Gas mixtures were passed through 0.2 μm porosity syringe-tip filters before entry into flasks. Incubation was at 30 ^{0}C in a rotary water bath shaker at approximately 200 rpm for one week, after which the head space purged again. After 9 days of incubation, 75 ml portions of liquid primary cultures were added to 75 ml double strength solid media and used for triplicate pour plates. Both Higgins and Whittenbury media were used and solidified with Difco Purified Agar. After 12 days of incubation, phase contrast microscopy showed higher cell densities in the Higgins medium. At this point, 1 ml aliquots of primary cultures were transferred to 250 ml stoppered Erlenmeyer flasks containing 40 ml of Higgins or Whittenbury medium. Head spaces of flasks were purged as in the primary isolations and incubated at 30 ^{0}C in a rotary incubator shaker. Head spaces of secondary enrichments were purged at 7 day intervals. Secondary enrichments were transferred to solid media after 15 days incubation. All plates were incubated in closed, purgable containers containing a 1:1 mixture of propane-air. A petri

dish of desiccant was placed in the container with the plates to absorb moisture. Colonies were picked from plates after approximately 9 days incubation. Colony selection was based on overall morphology including color and texture. Isolates were able to grow interchangeably on either medium, but seemed to be more mucoid on Whittenbury medium. Therefore, Higgins medium was used exclusively to facilitate the purification process. As a result of the incubation time on solid media and inherent growth characteristics of the isolates, most of the colonies picked were mixed and had to be restreaked numerous times to obtain pure cultures. Stock cultures were made from isolates and maintained on Higgins slants under propane-air.

Methanotrophs- Methane-oxidizing cultures were isolated using the same procedure as used for propane-oxidizing bacteria, except that the headspaces of isolation flasks were purged with a 70:30 air-methane mixture.

Bacterial strain selection criteria- Bacterial isolates used in this study were selected on the basis of differences in colonial morphology and substrate utilization, or growth conditions. After initial selection and purification, isolates were tested for their ability to use a specific substrate as a sole carbon source (as with the propane-oxidizing bacteria), as well as their metabolic capabilities (such as the ring-cleavage mechanism for toluene-oxidizing isolates). The final selection of propane-oxidizing isolates was based almost solely on ability to accumulate significant amounts of biomass in a reasonable amount of time, i.e., four to six weeks under the culture conditions used. Because of previous studies conducted in other laboratories on *Mycobacterium vaccae* strain JOB5, and because the metabolic pathways of interest for the strain are known (Pettigrew, 1991, Reineke, 1988), it was used as a reference strain for TCE degradation studies of the new isolates.

Bacterial identification procedures.

Toluene-oxidizing isolates- Toluene oxidizing isolates were characterized by selected

cellular morphology, Gram stain, motility, and biochemical techniques: catalase, oxidase, oxidative/fermentative reactions with glucose, ring cleavage mechanism, and with a Hewlett-Packard 5898A Microbial Identification System (MIS). For conventional biochemical tests, cultures were grown to mid-log phase before being subjected to identification procedures (Gerherdt, 1981).

Principal isolates from toluene enrichments were subjected to total cellular fatty acid analysis with the MIS. In this procedure, fatty acids are extracted from bacterial cells, saponified, and converted to their methyl ester derivatives. Cluster analysis techniques are used to generate dendrograms from the data, making it possible to separate isolates on the basis of difference in Euclidian distances (Moss, 1990). The Hewlett-Packard 5898A system consists of a 25 m x 0.2 mm methyl phenyl silicon fused capillary column, an auto sampler, a reporting integrator, a computer, and a printer.

Before analysis, toluene-oxidizing cultures were transferred to BBL trypticase soy broth solidified with 1.5% Difco Bacto agar (TSBA) and incubated at 25 ^{0}C for 24 hours before being harvested. Cells were scraped from the medium surface, transferred to screw cap tubes and stored at -20 ^{0}C. Microbial samples were prepared for analysis by first lysing and saponifying the cells at 100 ^{0}C for 30 min after the addition of 15% NaOH in 50% aqueous methanol. After cooling to ambient temperature, 1.5 ml of ether:hexane (1:1) was added, mixed, and the phases allowed to separate. The bottom aqueous phase was removed, and 1 ml phosphate buffer (pH 11) was added. After mixing and subsequent phase separation, the organic phase containing the fatty acid methyl esters (FAME) was removed for analysis. The chromatographic analysis was conducted at 170-280 ^{0}C (5 ^{0}C/min increase) in conjunction with a flame ionization detector.

Aromatic ring fission- Determination of *ortho* and *meta*-ring cleavage mechanisms was accomplished by the procedures of Stanier et al. (1966) and Nelson et al. (1987). *Meta*-fission positive colonies were bright yellow after the test; *ortho*-fission tests were considered positive when a purple band formed in the test solution.

Propane-oxidizing isolates- Principle propane-oxidizing isolates were characterized morphologically, with Gram and acid fast stains, and observations for motility. The propane-oxidizing isolates were then subjected to MIS identification procedures. Cultures for gas chromatographic analysis were grown at 30 ^{0}C on trypticase soy broth agar (TSBA) for 4 days before harvest. This length of time was required for sufficient cell mass to accumulate for the analyses. Data bases used in the identification were the standard Hewlett-Packard library, an FDA library consisting of data input by FDA personnel, and a *Mycobacterium* library. The latter data base (from the instrument supplier) consisted of data from mycobacterium of medical significance, and grown at 35 ^{0}C, on Middlebrook 7H10 medium in an atmosphere of 5-10% CO_2.

Electrolytic respirometry (oxygen and mixed gas). Electrolytic respirometry is a technique described as the electronic equivalent of conventional biological oxygen demand (BOD) measurements. The apparatus consists of magnetically stirred 1.075-liter culture vessels fitted with CO_2 traps (5 ml 30% KOH in a ventilated finger-trap), and an oxygen generation systems (Figure 7). The oxygen generation system contains an electrolyte (1.0 N H_2SO_4) and can produce 128 mg O_2/h (maximum), with a precision of ± 0.5%. The generation of oxygen by

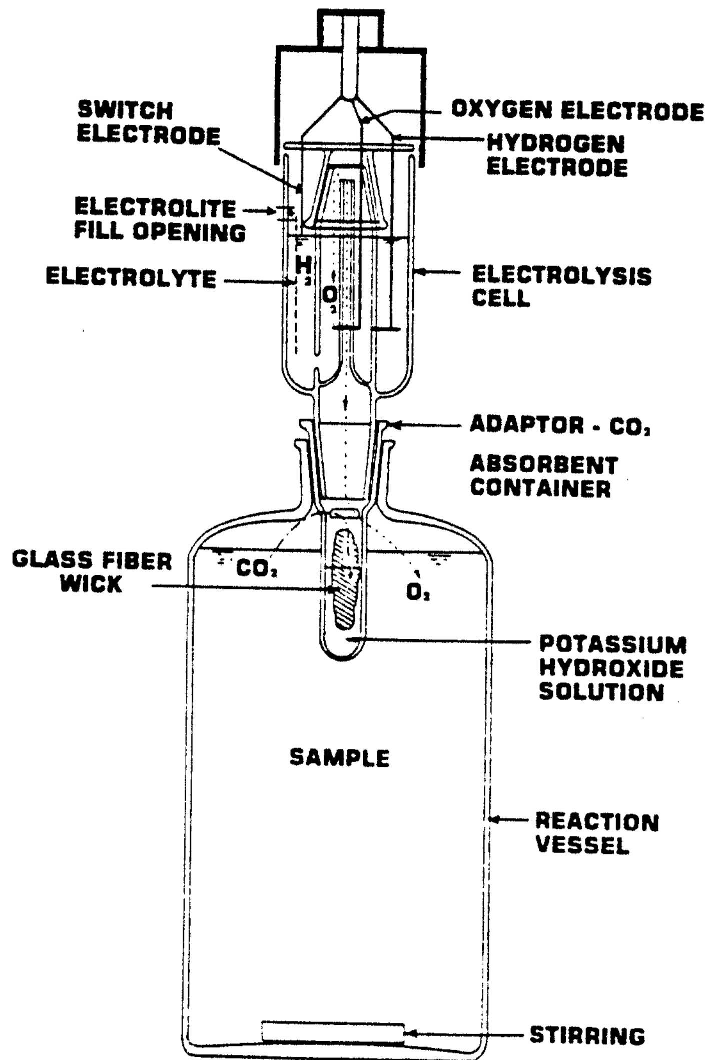


Figure 7. Schematic representation of the electrolytic respirometer apparatus.

ER obeys Faraday's first law of electrolysis which states that

"the weights of substances formed at an electrode during electrolysis are directly proportional to the quantity of electricity that passes through the solution" (Quagliano, 1964). Accordingly,

$$H_2O + 2\ e^- \rightarrow H_2 + ½\ O_2.$$

Oxygen generation is reported as mg oxygen consumed per hour. Temperature was controlled at ± 1 ^{0}C. As the bacteria grow in the culture vessel, the oxygen supply is depleted and resulting CO_2 is absorbed by the KOH, thereby creating a reduced pressure in the vessel. The reduced pressure results in a raise of the electrolyte level in the inner chamber of the O_2-generation system. Because the inner chamber of this vessel communicates with the outer chamber by a hole near the bottom of the dividing wall, the electrolyte level in the outer chamber is lowered. Contact is then broken with a switch electrode, which turns on an electrical current to the hydrolysis electrodes. Oxygen is generated from the center electrode; hydrogen from the outer one. The reduced pressure in the growth vessel pulls the oxygen down into it, where it is mixed by the vortex. Hydrogen generated by the hydrolysis escapes through a port in the exterior wall of the oxygen generation system.

An attempt was made to use the electrolytic respirometer for a multiparameter experiment that plotted bacterial growth curves, established the stoichiometric relation between oxygen and propane consumption, and tracked degradation of TCE. Following inoculation, the head space of a respirometry vessel and one liter of growth medium were saturated with propane. It was theorized that by knowing the initial amount of propane present (based on Henry's law), the amount of oxygen used, and by following the consumption of propane by GC/TCD and cell mass increase, that growth curves could be plotted and stoichiometric relationships developed. TCE degradation was to be monitored by periodic withdrawal of

liquid sample for analysis with GC/ECD. One consortium of propane-oxidizing bacteria, HPL-III was used to generate a growth curve over a 25+ day period. (See "Electrolytic respirometry", Appendix B)

Biomass accumulation procedures and cell storage techniques for propane-oxidizing isolates.

Although a large number of isolates were obtained by primary and secondary enrichment with propane, a significant amount of difficulty was encountered in obtaining enough cell mass with which to work. Several procedures were tried, including growth in shake flasks, on silica gel plates, in the electrolytic respirometer, and on Higgins medium solidified with electrophoresis grade agarose. It was important that only the selected carbon source be available for growth, so that induction of desired enzyme systems was maximized. Descriptions of the silica gell plate, and ER techniques are given in the Appendix.

Shake flask technique- One-liter aliquots of Higgins medium were placed in 2.8 liter Fernbach flasks, in a rotary incubator shaker at 30 ^{0}C and 200 rpm for 4-6 weeks. Inoculum for the flasks was obtained by harvesting growth from the surface of 150 x 15 mm Higgins agar plates incubated in a propane/air atmosphere. Flasks were closed as previously described. Headspaces were flushed with a 1:1 propane/air mixture.

Higgins agarose plates- In this study, Higgins medium was originally solidified with Difco Purified agar, but tests revealed that in the agar supported limited growth of the propane isolates when no propane was present. Therefore, a series of platings were done using two brands of electrophoresis grade agarose and purified agar. Growth occurred on all plates, indicating that the agarose either contained enough utilizable carbohydrate to support growth or broke down during autoclaving. The net affect was that great difficulty was encountered in finding a solid medium that allowed propane-dependant growth. The agarose plate method provided a reliable source of inoculum and was therefore used extensively. Higgins-agarose

plates were sometimes fortified with 50 mg/l each of trypticase peptone and yeast extract to encourage growth (Fliermans, 1988).

Gas Chromatographic Analyses.

Operating procedures- TCE degradation studies utilized 1.8 ml amber crimp sealed vials and teflon-lined rubber septa obtained from Hewlett-Packard. All chromatographic analyses were done of a Hewlett-Packard 5890 Series II gas chromatograph with a cryogenically cooled oven. Data was collected and processed by a Hewlett-Packard 3396A Integrator. For analysis of chlorinated alkenes, an electron capture detector (GC/ECD) was used. Analyses of TCE solutions utilized a 50-meter Hewlett-Packard Ultra II capillary column (5% cross-linked phenyl-methyl-silicon). Operational parameters for the gas chromatograph were as follows: initial oven temperature 15 ^{0}C for 1 minute, increased at 15 ^{0}C/minute to 125 ^{0}C; injector temperature, 200 ^{0}C; ECD, 250 ^{0}C, carrier gas, helium, 6 ml/min. (total flow), 1 ml/min through the column. The injector was run in split-mode with a split ratio of 0.17. Make-up gas flow through the detector was 25 ml/minute. Sample volumes of 1µl were used for TCE analyses.

Analysis of propane, propene, propylene oxide and its possible breakdown products utilized a thermal conductivity detector (GC/TCD) and a 30-meter capillary PoraPlot-Q column. An on-column injection port was used for analysis of all non-chlorinated alkanes. For propane, the oven was run isothermally at 30 ^{0}C, injector temperature was 100 ^{0}C, and the detector temperature was 250 ^{0}C. For propene, propylene oxide and its possible breakdown products, the oven was operated isothermally at 180 ^{0}C, the TCD at 250 ^{0}C, high sensitivity setting, and the injection port at 150 ^{0}C. Helium was used for both a carrier gas and make-up gas. Sample volumes of 1 µl were used for propane, propene, and propylene oxide analyses.

Preparation of TCE calibration curves- For each TCE concentration, triplicate sample vials were prepared using Higgins salt solution as a diluent. Aliquots of 500 µl were used in place of cell suspensions. Ten-µl portions of aqueous TCE solutions of 1, 2, 3, 4, and 5 mM were used to give final concentrations of 20, 40, 60, 80, and 100 nMol per vial. Vials were immediately crimp sealed with teflon-lined rubber stoppers after each TCE addition, and were incubated at 30 ^{0}C in a rotary incubator at approximately 200 rpm. After 12 hours, TCE was extracted from the aqueous phase by addition of 500 µl of *n*-pentane through the septum with a gas tight syringe. The pentane contained 0.5 mM dibromoethane (DBA) as an internal standard. Following centrifugation at 4500 g (4 ^{0}C) for 20 min., vials were inverted and 1 µl volumes withdrawn from the pentane phase for injection into the GC. k-factors were generated by dividing peak areas for TCE by peak areas for the DBA for each sample analyzed. k-factors were averaged to give a mean for each TCE concentration. A linear best-fit line was computer generated for the resulting graph showing k-factors versus TCE concentration. A correlation coefficient (r^2) for the standard curve was then calculated. While not used in degradation studies, cis-, and trans-Dichloroethylene (DCE) and vinyl chloride (VC) were analyzed to determine their relative retention times (RT).

Sample preparation- For TCE degradation experiments, cell suspension aliquots of 500 µl were placed in the vials, and 10 µl of the aqueous 5mM TCE solution were added to give an concentration of 100 mM. Vials were immediately sealed and placed on a rotary shaker at 200 rpm. Incubation of the reaction mixture was continued at 30 ^{0}C for 12 hours. The analysis procedure assumes that TCE partitions into the liquid as predicted by Henry's Law, as degradation proceeds (Wackett et al., 1988).

For each isolate, three preparations were assayed in triplicate: pentane extractions at T_0; heat killed cells; and live cells. Protein contents were determined for all cell suspensions,

allowing degradation results to be expressed as micromoles of TCE degraded per mg protein per hour.

Specific oxygen uptake rate (SOUR) experiments. A YSI Model 53 Biological Oxygen Monitor was used to measure oxygen consumption by whole-cell suspensions challenged with intermediates of propane metabolism. Assays were conducted at 30 ^{0}C. Baseline oxygen consumption and drift of the apparatus was determined by measurements using 2.7 ml aliquots of air saturated Higgins buffer. Water bath temperatures were maintained at +/- 0.1 ^{0}C. Barometric pressures were measured and recorded with a Taylor recording barograph. Henry's law (for oxygen, K_2=3.30 x 10^7, partial pressure, p_{O2} =0.2) was used to determine the quantity of oxygen in the buffer solution, according to the following formula:

$$K = p/X = 3.3 \text{ X } 10^7 = (p/n)(1000/18),$$

where K is the Henry's law constant, n is the mole fraction in moles/liter, p is the partial pressure of the gas, X is the mole fraction of the solvent.

In conjunction with the standard paper-tape record (100 mV full-scale output) used with the oxygen monitor, digital multivolt meter was connected to monitor's output jacks. More accurate starting and ending mV values were obtained from the volt meter than from the tape alone. The tape-recording was used primarily to show the pattern of oxygen consumption.

Oxygen consumption rates were determined in triplicate for the aerated buffer alone, buffer + substrate, buffer + bacterial cells, and buffer + substrate + cells. Aliquots of buffer (2.7 ml) were placed in the test chamber and the temperature equilibrated for 3 minutes. The oxygen probe was inserted into the test chamber and allowed to equilibrate, then the analog meter output of the monitor was adjusted to 100%. For measurement of baseline respiration, 200 µl of cell suspension was added and oxygen consumption measured for 10 minutes. To

measure oxygen consumption of cells with substrate, 100 µl of a 20 mM intermediate were injected into the chamber with Hamilton gas-tight syringes. All measurements were done in triplicate.

Differences between initial and final mV readings represented the percentage decrease in oxygen concentration in the test solution. The calculated oxygen concentration in the solution (nmol, as estimated by Henry's law) was multiplied by the percent decrease in oxygen; the resulting figure was then divided by the run time to give nanomoles of oxygen consumed/minute as shown below:

$$(\text{nMol } O_2 \text{ X \% Decrease } O_2)/\text{minutes}$$

These data were divided by protein values to give nanomoles of oxygen consumed/minute/mg protein. Measurements were made of cultures grown on propane, and propionate. Cells used for oxygen uptake studies were from the same batch cultures as cells used for TCE degradation studies.

Protein Determinations. Cellular protein was used as a reference point for expressing both TCE degradation (nM TCE/h/mg protein) and oxygen consumption (nM O_2/min/mg protein). Cells were disrupted with a Virtis Virsonic 300W ultrasonication apparatus, equipped with a 4 mm diameter microtip. To determine optimum sonication time, cell suspensions were diluted 1:1 and 1:4 (cell suspension to DI water) and 4 ml placed on ice. Aliquots of 100 µl were withdrawn at T=0 and at three 10 minute intervals of sonications. The Bradford protein assay method (BioRad protein assay kit) was used for protein quantitation. Absorbance was read at 595 nm, using a Gilman spectrophotometer. Unbroken cells were not removed prior to protein analysis. Data were plotted as protein released per minute. For standard protein concentration curves, Sigma RIA grade serum albumin was used as a standard at concentrations of 0.175, 0.35, 0.70, and 1.40 mg/ml. Data (absorbance versus concentration) was plotted using Grapher

(Version 1.75, Golden Software, Inc., Golden, CO). Linear best-line fits of the calibration curves were prepared with the same software package. Generally, 30 minutes proved to be an adequate sonication time for a 1:4 dilution. This dilution was chosen because in several cases, cell mass was limited and sufficient water had to be added for the sonication process.

CHAPTER III

RESULTS

This study was conducted to examine the organic contaminants in an industrial leachate stream, to determine the presence of indigenous degradative microflora and to gain specific insight into the metabolism of a particular subset of the microbial population. The leachate stream from this site is unusual because of the comparatively large amount of *cis*-DCE in relation to PCE, VC, and toluene which are also present. The question posed by the site custodian pertained to the ability of indigenous microbial populations to eliminate these compounds, i. e., could the existing microbial populations transform these compounds to nonhazardous materials or mineralize them to Cl_2, CO_2, and H_2O? Toward this end, soil and groundwater analyses for selected microbial populations were initiated.

pH and Temperature of French drain leachate. As seen in Table 7, annual conditions of leachate from the French drain were relatively constant with respect to temperature and pH. Temperature varied somewhat seasonally; the minimum occurred in February and the maximum in November. pH levels appear to stay close to neutrality year round, varying by 0.65 pH units during the time of the monitoring. No correlation was seen between pH and leachate temperature (r^2=-0.01). Variations in pH are probably related to variations in leach rates for pockets of contamination up-gradient from the French drain system. The consequences of these variations may translate into variations of selected carbon sources, or increases in acidic material that could inhibit growth.

Table 7
Chronological History of pH and Temperature for the French-drain Leachate

Date	pH	Temperature (°C)
2/11	6.93	18.0
3/11	6.75	21.0
6/11	6.85	24.0
7/7	n.d.	25.5
7/9	6.62	25.5
11/3	7.11	27.0
11/6	7.25	26.0
11/18	6.80	23.0
1/12	7.23	19.5
2/3	n.d.	17.0
2/29	6.98	17.0

Soil and groundwater population estimates. Prior to isolation and characterization of specific microbial subpopulations, the background microflora of soil and groundwater in the area was determined. Using DPTYG medium, plate count assays were done on split-spoon core samples from test wells in the area surrounding the French drain (Table 8). From these data, it is obvious that the soil in the drainage area surrounding the leachate source contains viable populations to a depth of >20 ft. The low cell counts from the 25 ft depth[5] of sample F215-4 are possibly an artifact. Soil in this area is high in clay, which sometimes hinders dissociation of cells from soil particles, causing the cell counts to appear low. Also, microbial cells are restricted in their movement into areas of high clay concentration (LEK, unpublished data from assays of Trident submarine basin clay, Cape Canaveral, Florida). All core samples tested

[5] Soil borings were stopped at this depth because a limestone layer (geologically designated as the Walnut formation) underlies the entire area at varying depths. The Walnut formation generally retards the movement of surface water into the deeper Paluxi aquifers, from which some of the surrounding area obtains its drinking water.

were either in or close to areas designated as fuel saturation areas. This indicates that at some time, a major fuel leak (probably jet fuel) occurred in the immediate area, either because of an overt spill or pipe line leak. The presence of jet fuel was obvious in some samples by its

Table 8
Viable Cell Counts for Area Adjacent to French Drain

Sample	Depth (ft)	Average Counts per g soil
F215-1	0.5	$190 \pm 6 \times 10^4$
F215-2	4.0	$5.0 \pm 0^* \times 10^2$
F215-3	13.0	$11.0 \pm 1.3 \times 10^4$
F215-4	25.0	$<10^1$
F222-1	5.0	$33 \pm 6 \times 10^4$
F222-2	8.0	$36 \pm 2.8 \times 10^4$
F222-3	10.0	$38 \pm 1.4 \times 10^4$
F222-4	14.4	$\approx 7.3 \times 10^5$ (n=2)
F223-1	3.5	$240 \pm 21 \times 10^3$
F223-2	10.0	$150 \pm 18 \times 10^2$
F223-3	18.0	$39 \pm 5.1 \times 10^4$
F223-4	21.5	$80 \pm 10 \times 10^3$

* Estimate- plates too low to be statistically valid

odor. If the jet fuel had accumulated in significant amounts at the upper surface of the Walnut formation at the F215 site, it could account for the observed low cell numbers for sample 215-4. Viable cell counts from the 21.5 ft level of F223-4 indicated counts of 10^4 cells per gram of soil. Assays of a core sample from a site approximately 0.25 miles south of F215 also gave counts of 10^4 cells per gram of soil at the 17.5 ft. level, showing that viable cells could be recovered from that depth. Test sites F215 and F223 had been covered with a concrete slab approximately 10 in. thick, for about 30 years prior to the drilling, restricting

ready access to the ambient environment. This resulted in limited flow of both ambient air and water to the surface areas in well drilling locations.

Total microbial population levels in the groundwater were determined by the AODC procedure. Data compiled for the French drain leachate over a 5 month period showed total microbial cell levels of 10^6-10^8 cells per ml of water (Table 9). However, limited INT-dye reduction data (Appendix) places the viable cell counts in the 10^5-10^6 cell per ml range, which is approximately 10% of the total number of cells as determined by AODC.

Table 9
AODC Total Cell Counts for the French Drain Leachate

Date	Average cell count per ml
5/11	4.3×10^6
6/11	2.6×10^8
7/7	2.6×10^6
7/9	1.9×10^6
11/18	3.4×10^7

Isolation of specific bacterial subpopulations. To investigate the potential of bioremediation for the French drain leachate, subpopulations of toluene-, methane-, and propane-oxidizing bacteria were isolated by enrichment processes. An extensive amount of research (worldwide) has been done on cometabolic degradation of TCE by toluene-oxidizing microorganisms. Because toluene is present at a concentration of 0.63 ppm, initial investigations targeted toluene-oxidizing bacteria.

Toluene-oxidizing isolates- Toluene enrichment at acidic and neutral pH levels revealed that different toluene-oxidizing subpopulations (based on morphology and biochemical reactions) could be separated by pH preference. Triplicate plate counts (using SMSA) of inoculum from the French drain indicated T_0 viable cell levels of $140 \pm 12 \times 10^3$

cells per ml. Sixty-three h after the first 58 µl addition of toluene to the ER enrichment cultures, plate counts of reactor contents indicated viable cell levels of 37 ± 3.7 x 10^6 cells per ml for the pH 6.0 reactor and 20 ± 2 x 10^6 cells per ml for the pH 7.2 reactor. T_0 isolates were more highly pigmented (red, orange, and yellow colonies) than were the isolates following toluene enrichment. Colonies from toluene enrichments were generally not pigmented. The noticeable change in pigmentation of the isolates, together with the increase in numbers of viable cells indicates that a population shift had occurred.

Oxygen consumption increased upon addition of toluene in both the primary and secondary reactors at pH 6.0 and pH 7.2 (Figures 8 and 9). Biomass increases were assumed to be proportional to oxygen consumption with non-leachate organisms. Early in the experiment, the pH 6.0 control reactor became contaminated. This contamination accounts for the rise in oxygen consumption in the pH 6.0 reactor as seen in Figure 8. Following the original T_0 toluene additions, injections of 58 µl were made at 85, 117.2, and 144.2 h. As can be seen from data presented in Figure 10, the lag periods under both pH conditions were approximately 5 h. Log phase growth at pH 7.2 continued for approximately 17 h, while what appears to be total log phase growth at pH 6.0 continued approximately 50 h. The oxygen consumption curve for the pH 6.0 reactor parallels the pH 7.2 reactor through the first 10 h of log phase growth but the pH 6.0 respiration rate seems to slow at about 25 h into the procedure. Following a 10 h period of declining respiration, the reactor showed renewed log phase growth for about 15 h before again declining. Both reactors responded to further additions of toluene and at the end of approximately 122 h of incubation, cumulative oxygen consumption by growth at pH 7.2 began to exceeded that of growth at pH 6.0.

In spite of differences in oxygen consumption of the primary enrichment consortia, oxygen consumption by monoculture of the primary isolates at each pH approximately parallel

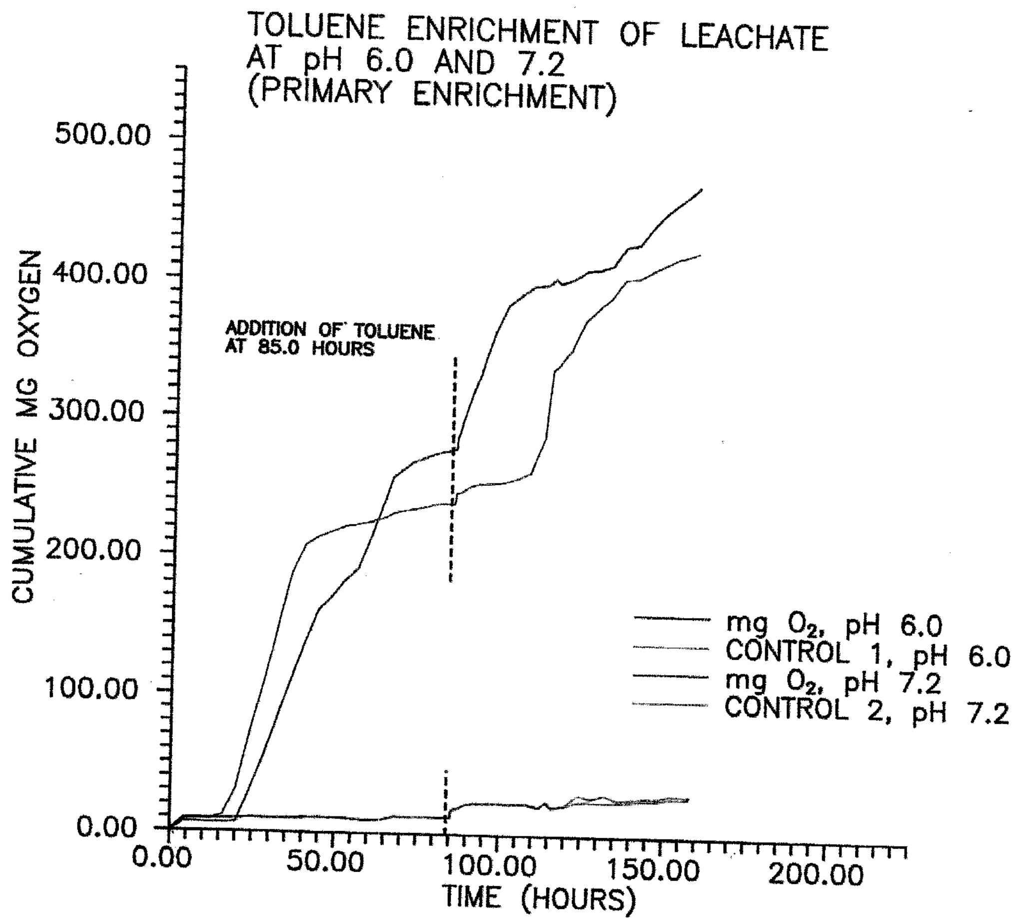


Figure 8. Oxygen consumption by primary enrichment of French drain leachate with toluene.

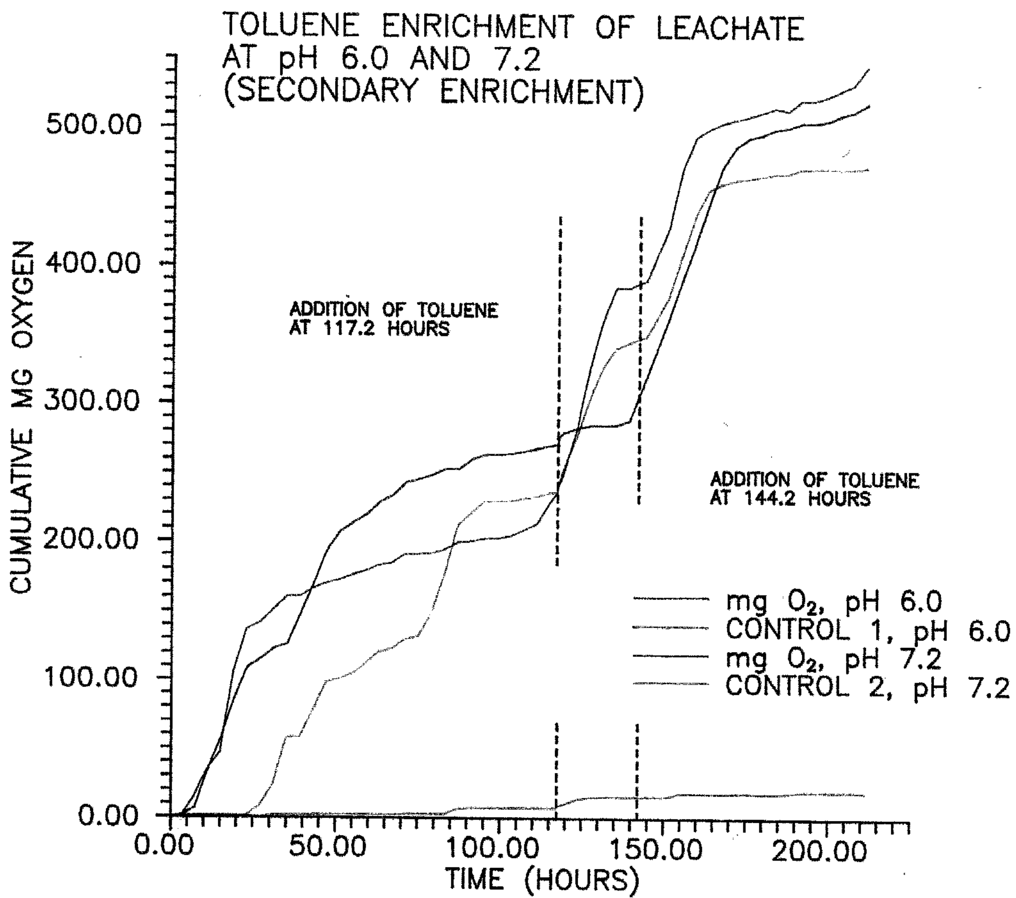


Figure 9. Oxygen consumption by secondary enrichment of French drain leachate with toluene.

each other (Figure 10), with growth at pH 7.2 slightly exceeding that for growth at pH 6.0.

Portions of the liquid from both primary reactors were withdrawn and plated on SMSA for isolation and characterization of enriched organisms. Twenty-two toluene-oxidizing isolates were obtained from the enrichment procedures. A summary of the results of morphological and biochemical characterization are shown in Table 10. Gram stain, morphology, and motility data indicate that at pH 7.2, the growth of Gram negative, motile rods was favored, while conditions at pH 6.0 selected for Gram negative, nonmotile coccobacilli.

Table 10
Summary of Toluene Enrichment Isolates*

pH	Oxidase - Catalase + Motility +	Oxidase + Catalase +	Oxidase + Catalase - Motility +	Oxidase + Catalase - Motility -
6.0	6 Gram - cocci	1 Gram - cocci 4 Gram - rods	2 Gram - rods	2 Gram - rods
7.2	1 Gram - rod	3 Gram - rods	3 Gram - rods	None

* Isolates can be grouped into the major groups of *Pseudomonas*, *Alcaligenes*, and *Acinetobactor*.

Cultures identified with the Hewlett-Packard 5898A Microbial Identification System included *Pseudomonas aureofacians*, *P. cichorii*, *P. putida* (biovar A), *P. stutzeri*, *Arthrobacter* sp., and *Acinetobacter*

Methane-oxidizing isolates- Methane-oxidizing isolates were not characterized in detail, but their presence in the French drain leachate and Persian Gulf sea water (Appendix) was demonstrated. Phase contrast examination of Higgins medium in the primary enrichment flasks of the leachate and seawater showed coccobacilli in both stationary and highly motile

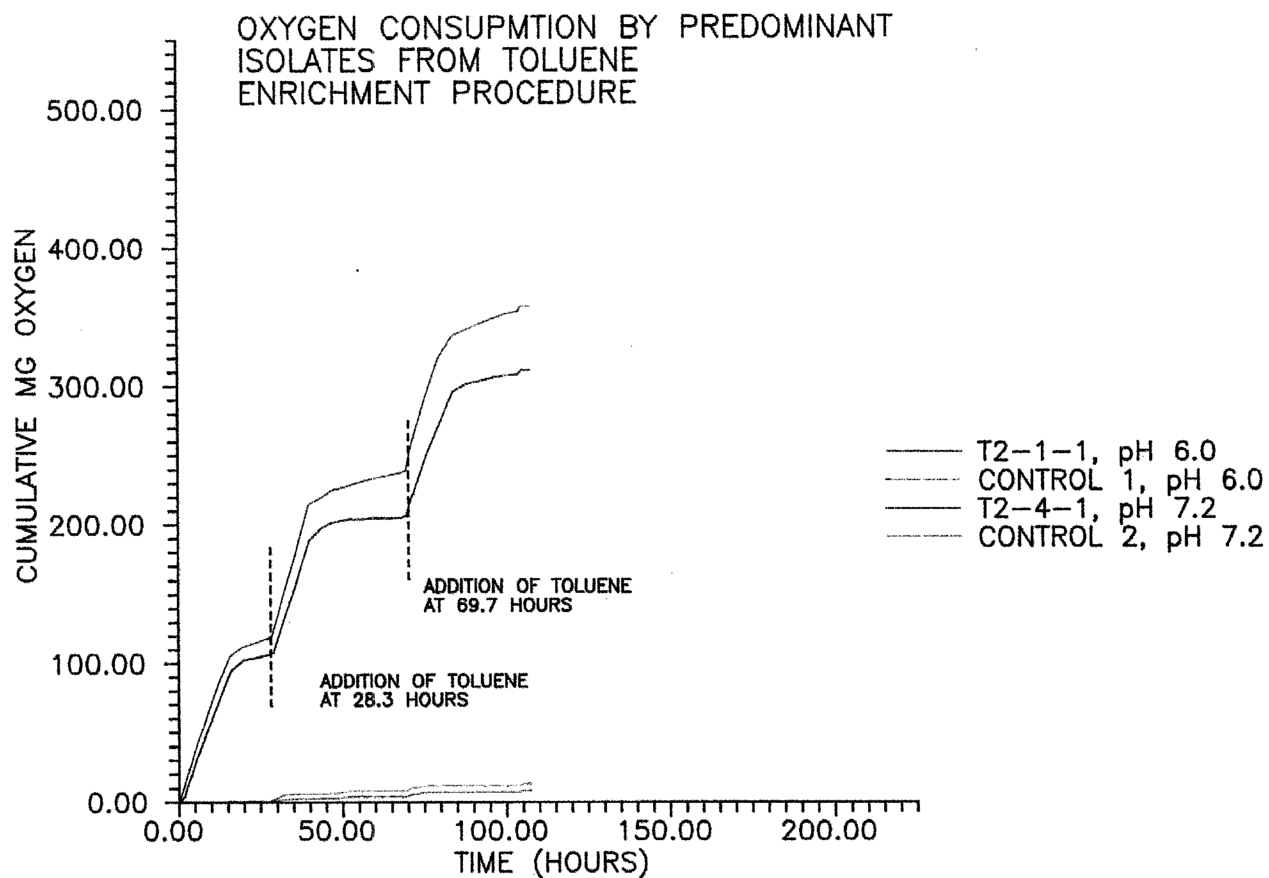


Figure 10. Oxygen consumption by two isolates from toluene enrichment of French drain leachate.

phases after 5 days incubation. As the incubation of the leachate progressed, enrichment flasks became progressively more pink, and secondary enrichment flasks had a very definite pink color. Streak plate isolations of primary enrichments yielded pink, and brownish-yellow mucoid colonies. Colonies from secondary enrichments showed predominately pink, mucoid colonies. Leadbetter and Foster (1958) isolated similar pink cultures and designated them *Pseudomonas methanica* (see footnote 2). Plate count assays of primary enrichment medium indicated 86 ± 14 x 10^1 cfu/ml after 6 days incubation. A large amount of cellular exopolymer accumulated in both primary and secondary enrichment flasks causing the bacteria to adhere to flask surfaces. Methane enriched cultures of both leachate and seawater yielded pure culture growth. An electron micrograph of one isolate from the leachate is shown in Figure 11. Internal membranes within the cell indicate this isolate to be a Type I methanotroph.

Maintenance of the methane-oxidizing cultures, and determination of their degradation spectrum was by passed in favor of the propane-oxidizing isolates. However, samples of the leachate were used in a parallel study by Alan DeSpirito, formerly of the University of Texas at Arlington. A publication detailing the TCE degradation capabilities of isolates is found elsewhere (DeSpirito et al., 1990).

Protozoan grazing- Phase contrast microscopy of primary and secondary enrichments (leachate and seawater) of toluene-, propane- and methane-oxidizing bacteria revealed ciliated protozoans grazing on bacterial growth. This was observed for both the leachate and the Persian Gulf seawater. The protozoans grazed on the edges of areas of extracellular polymeric masses, and actively consumed free motile and stationary cells. Free swimming cells were more easily consumed than cells trapped in masses of exopolymer. Such mixed populations have since been noted by Gurijala and Alexander, 1990.

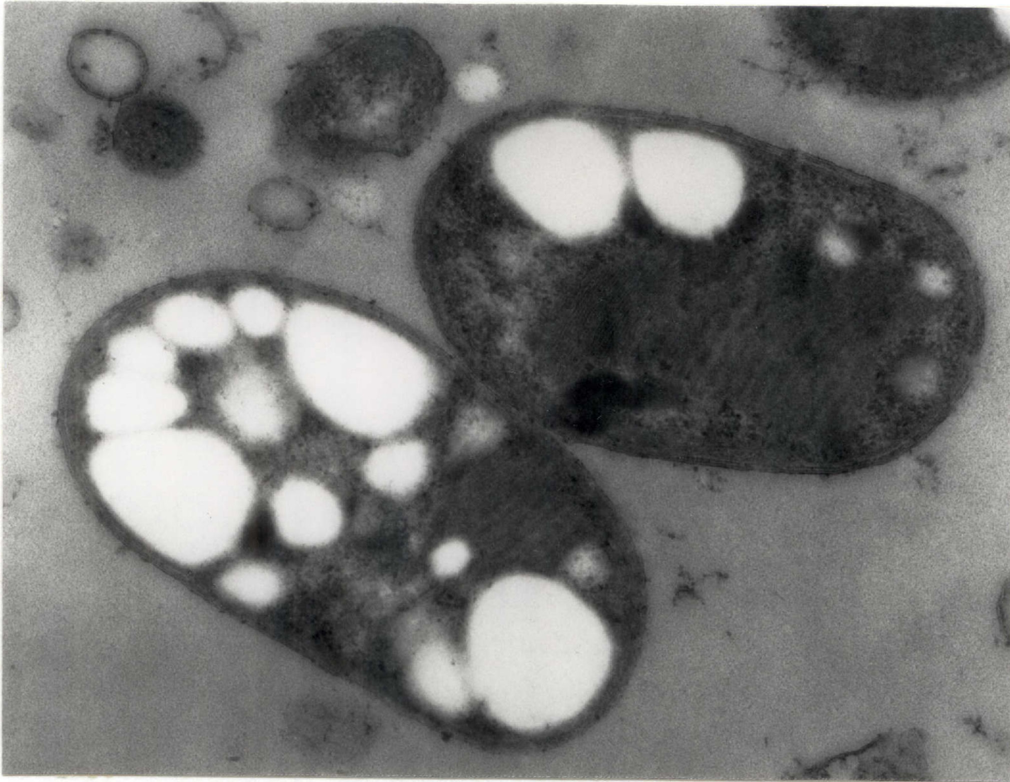


Figure 11. Type I methanotroph isolated from the French drain leachate (electron micrograph by Ann Foster, UNT).

Propane-oxidizing isolates- Growth of the propane-oxidizing isolates was demonstrated by mixed gas ER as shown in Figure 12. The consortium used for this experiment, HPL-III, was incubated approximately 640 h (26.7 days) before the experiment was concluded. The original plan was to use the HPL-III consortium for a proof-of-concept run prior to the use of pure cultures and analysis of propane concentrations, cell count assays, and TCE degradation analyses. However, at the conclusion of the HPL-III-mixed gas ER evaluation, growth was deemed too slow, and the respirometer was returned to its original use.

Propane induced cultures were used as inoculum (10 ml) for the mixed gas ER experiment. About 70 h into the incubation, oxygen use by the consortium triggered the electrolytic cell on the respirometer. Growth of the consortium declined slightly after approximately 240 h, but resumed after addition of propane. The same pattern was seen again at 551 h. A visual increase in turbidity indicated that growth was occurring.

Colony selection for the propane-oxidizing bacterial isolates was made on the basis of differences in colonial morphology of growth on Higgins agar plates. Generally, the propane-oxidizing bacteria were highly pigmented, ranging in color from various shades of yellow to orange to coral. One group of isolates produced translucent colonies. Distinctions in colonial morphology were readily obvious, but purification of the isolates was difficult. Two major factors, slow growth rates and variable pigmentation of colonies contributed to this problem.

Slow growth rates of the propane-oxidizing isolates were an obstacle to this study. Isolates streaked on Higgins-glucose medium usually required about 1 week to reach an apparent maximum biomass level. Isolates on Higgins agar in a propane atmosphere required 3-4 weeks to reach the same cell density; the same isolates in Higgins liquid medium with a 1:1 mixture of propane and air in the headspace required 4-5 weeks to reach acceptable cell

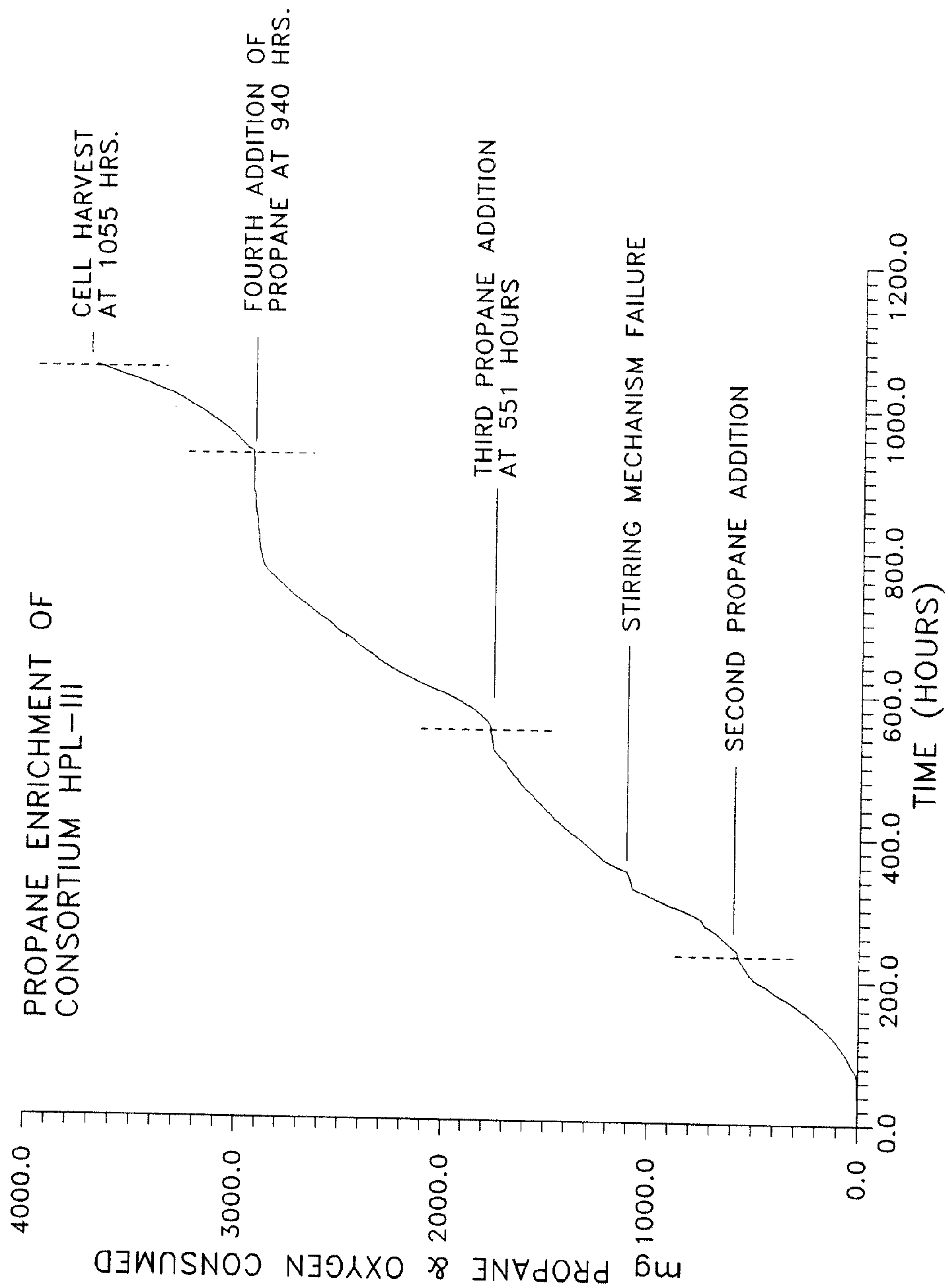


Figure 12. Total gas consumption by propane-oxidizing consortium HPL-III. Incubation temperature=25 ^{0}C; head space gas concentrations were 50% air, 50% propane.

densities. Isolates grown on glucose require an extensive incubation period with propane for full conversion to growth on propane (J. J. Perry, personal communication).

Growth of the isolates on solid media usually yielded faster growth, but it was found that extraneous material (presumably sugars) in the purified agar and agarose supported a low but consistent and observable level of growth. For this reason, as well as the desire to induce production of only the specific enzyme systems related to propane-oxidation, cultivation of the isolates on silica gel plates was attempted. However, the isolates grew only sparely, if at all on the medium. Time required for high cell densities of the cultures that grew exceeded 3 months.

A second obstacle to the study was the variability of pigmentation in the colonies. At one point, the isolates were grouped into seven major categories based on colonial morphology and color. But it was found that as the purification process continued, these groupings changed. Different combinations of isolates produced different colony colorations and textures. The long incubation times sometimes obscured this point. Colonies exposed to light during their growth developed pigment more quickly than those incubated for long periods without light. The young, unpigmented colonies had to be distinguished from new colonial types.

Overall, the isolates showed mixed response to growth on 0.1% propionate. Of the isolates selected for further study, three, including *M. vaccae* JOB5, grew moderately well on propionate, while two isolates did not grow on 0.1% nor 0.05% propionate.

Once isolated and purified, the cultures maintained stable cellular, morphological, and growth characteristics. Sufficient cell mass could not be obtained by shake flask techniques to allow the majority of the isolates to be used for the SOUR and TCE degradation assays. Of

the original 27 propane-oxidizing isolates, 4 were used for oxygen uptake and TCE degradation studies. Characteristics of the major isolates are given in Table 11.

Table 11
Major Characteristics of the Propane-Oxidizing Isolates

Culture	Gram-stain	Acid-fast stain	Morphology
M. vaccae JOB5	+	+	Rods
Mycobacterium sp. II	+	+	Small rods
Mycobacterium sp. 5	+	+	Pleo. rods
Rhodococcus sp. C	+	-	Cocci
Nocardia brasiliensis strain IIIP	+	-	Coccobacilli
2	+	+	Coccobacilli
4W	weak +	+	Small rods
4Y	+	+	Pleo. rods
18	+	+	Cuneiform rods
22B4-3W	+	+	Pleo. rods
22B4-2	variable	+	Blunt-ended rods
1	+	+	Blunt-ended rods
3C	+	+	Coccobacilli
3P	-	-	Rods
7P	+	-	Cocci
10	+	-	Cocci/cocci-bacilli

Table 11 (cont)
Major Characteristics of the Propane-oxidizing Isolates

Culture	Gram-stain	Acid-fast Stain	Morphology
11A-2	-	-	Cocci/cocci-bacilli
11B	+	-	Cocci/cocci-bacilli
12R	+	-	Tetrads?
12S	-	-	Coccobacilli
20A-1	+	-	Rods
20B-1	+	±	Pleo. rods
22B-2	+	±	Pleo. rods
22AP	+	-	Blunt ended rods
22BW	+	-	Coccobacilli
IIIW	+	-	Cocci/cocci-bacilli
IIIY	+	-	Cocci/cocci-bacilli

Tentative taxonomic assignments of the 4 isolates used for SOUR and TCE degradation studies were made on the basis of cellular morphology, Gram- and acid-fast staining, and suggestive evidence by the MIS. The MIS was unable to positively identify any of the isolates, even though three data bases were searched. It did, however, indicate that isolates C and IIIP were nocardioforms and could be either *Nocardia* or *Rhodococcus*. Both were Gram-positive and non-acid-fast, and catalase positive.

Isolate C was non-motile, aerobic, and no conidia, substrate or aerial mycelia were seen. It was observed as a Gram-positive coccus forming coral colored butyrous, circular, entire, convex colonies when grown on both Higgins-glucose and Higgins-propane media. No

budding was observed during multiple microscopic examinations. Because of the lack of branching and evidence from the MIS, isolate C is tentatively considered as a *Rhodococcus* species.

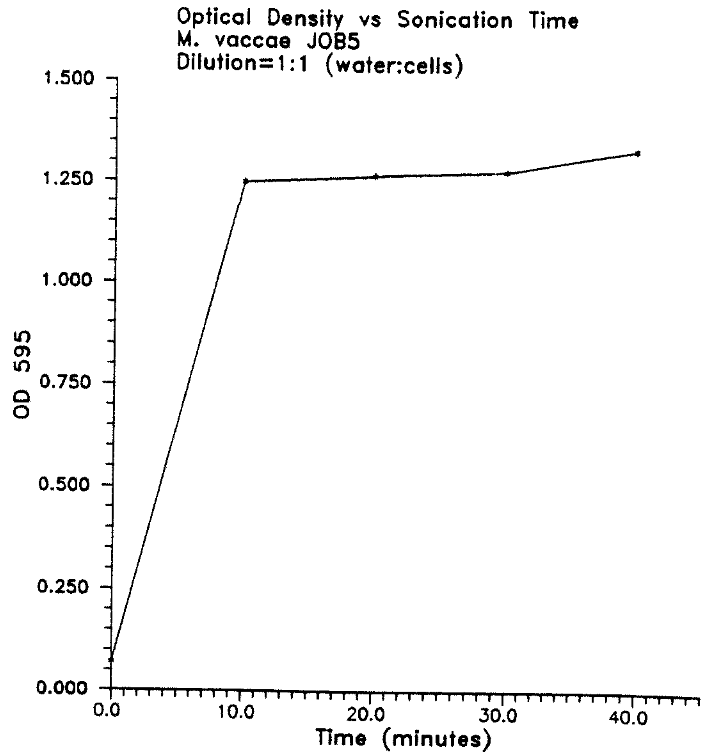
Isolate IIIP displayed club-shaped rods, exhibited neither conidia nor mycelia, and grew in pink irregular, umbonate colonies. Data from the MIS favors *Nocardia brasiliensis* over *Rhodococcus rhodochrous* as a taxonomic classification.

Isolates II and 5 were assigned to the genus *Mycobacterium* because of their cellular morphology (short, unbranched Gram positive, strongly acid fast rods). Both isolates produced smooth, creamy yellow, round entire colonies when grown on Higgins-glucose, and Higgins-propane media. The color intensity of isolate II increased with the age of the colony. MIS analysis of isolates II and 5 showed them to be separate strains and different from *M. vaccae* JOB5.

The reference strain, *M. vaccae* JOB5, grew as Gram positive, acid fast rods. Colonies were smooth, yellow and entire.

Protein determinations. Because the volume of each frozen cell suspension aliquot was not large enough to complete an entire TCE degradation experiment or specific oxygen uptake study (SOUR analyses) multiple tubes were used. Protein determinations were done for each tube.

Protein release curves were generated to estimate the optimum sonication time for cell disruption. *Mycobacterium* and *Rhodococcus* have very strong cell walls and consequently, it was important to know the efficiency of the protein liberation process. Protein assays for the two dilutions of cells are shown in Figure 13, A and B. A dilution of 1:1 (cell suspension: water) yielded the most rapid protein release, as opposed to the 1:4 dilution. But cell



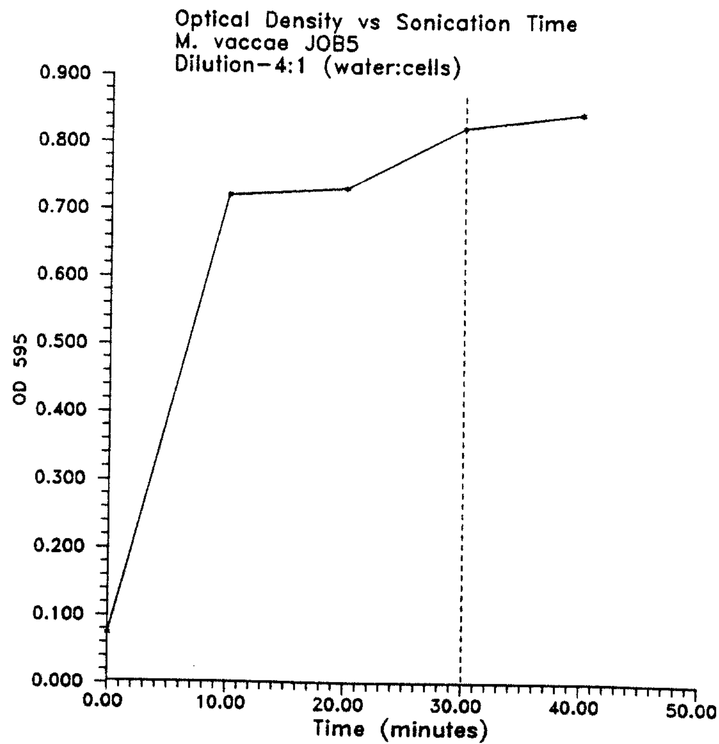


Figure 13. Protein liberation curves. A. 1:1 dilution (cell suspension:water), B. 1:4 (cell suspension:water).

suspension volumes were limited so the 1:4 dilution was used with a 30 min sonication time.

TCE degradation experiments.

TCE calibration curves- A calibration curve plotting nmol of TCE in standard solutions versus k-values is shown in Figure 14. The correlation coefficient for the data indicates good correlation (r^2=0.995) between TCE concentrations in standard solutions and measured k-values. For the operating parameters used with the gas chromatograph and electron capture detector, retention times (RT) for TCE and DBA were 5.33-5.34, and 7.02-7.05 min, respectively. The lower homologs of TCE were analyzed by GC/ECD under the same operating conditions. RT values for cis- and trans- DCE, and VC were 3.92 and 4.44 min, respectively. A review of chromatograms generated by these analyses show no RT values corresponding to the RT's of the lower homologs, indicating they were not by-products of TCE degradation by these cultures. Representative chromatograms for *M. vaccae* JOB5 are shown in Figures 15 A, B, C, and D to illustrate patterns typical for the study. The GC/ECD recordings for different treatments are shown to demonstrate qualitative consistency rather than quantitative values. Quantitative consistency is indicated by standard deviations of the k-values.

TCE degradation by propane and propionate grown isolates- As shown in Table 12, propane grown cultures of *M. vaccae* JOB5, and *Mycobacterium* species II and 5 are much more efficient TCE-degrading strains than are *Rhodococcus* species C and *Nocardia brasiliensis* strain IIIP. The actual amounts of TCE degraded by *Rhodococcus* sp. C is obscured by large experimental error factors (poor TCE recovery), never the less, low

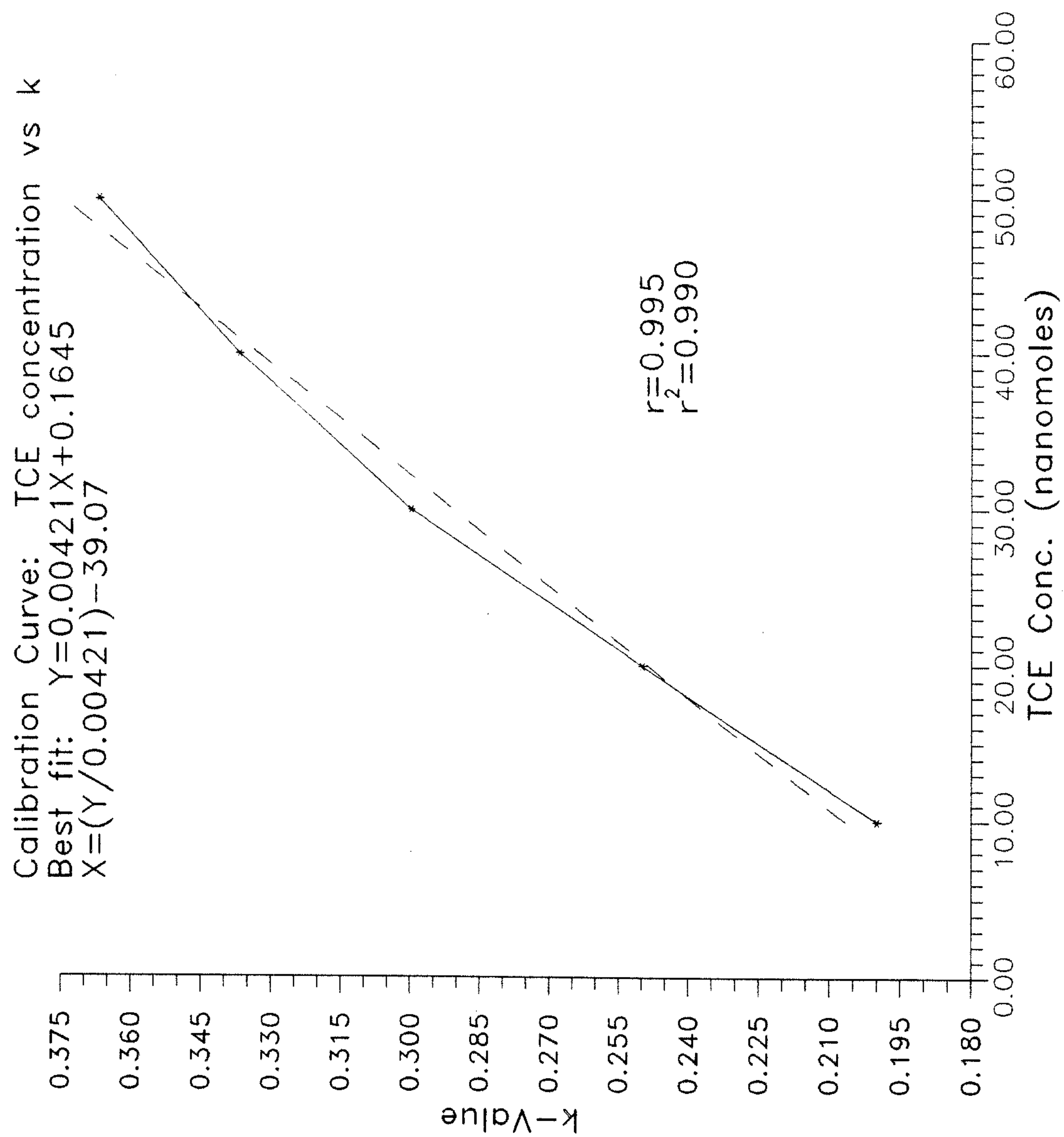


Figure 14. TCE calibration curve (nmol TCE versus k).

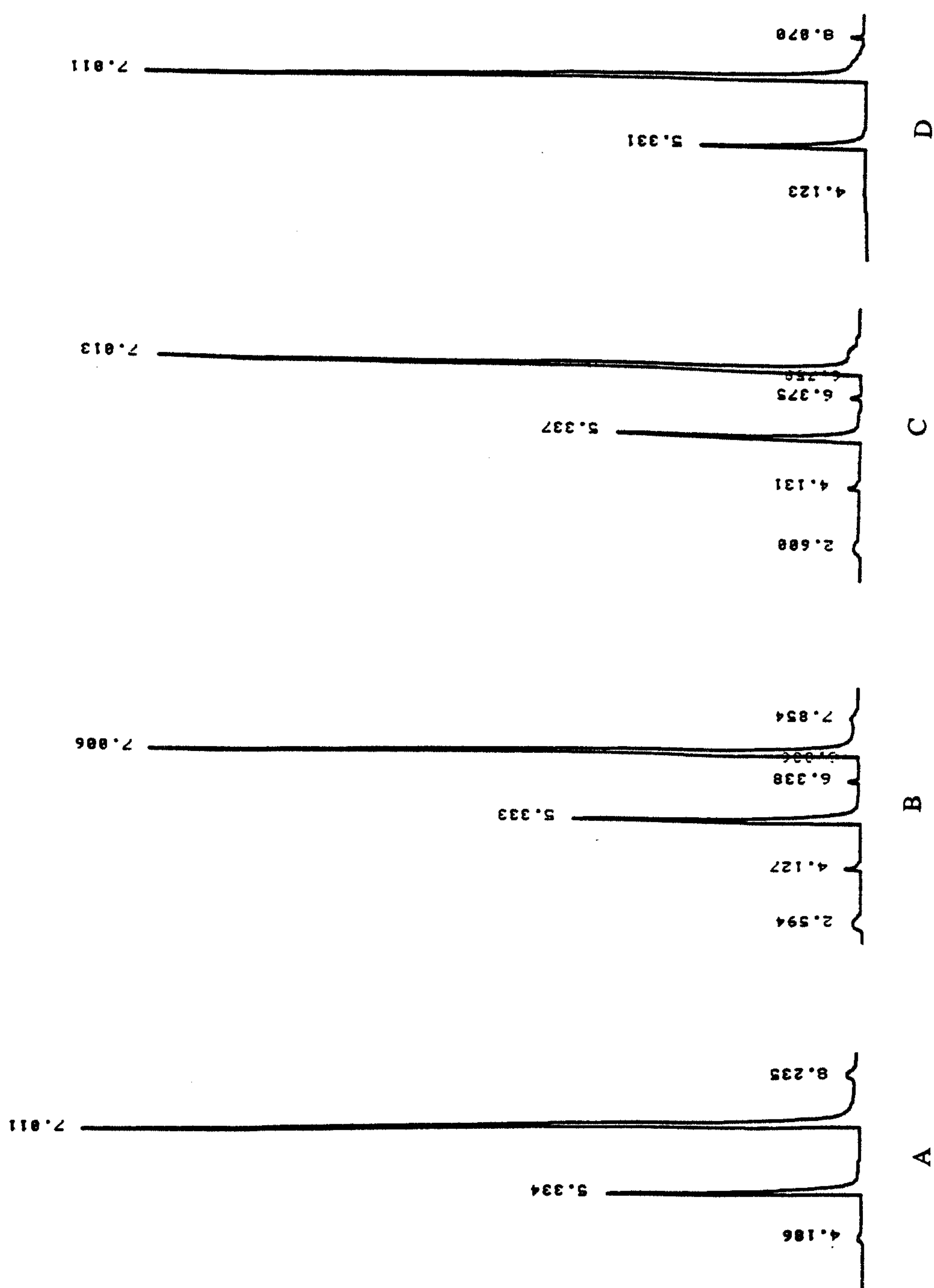


Figure 15. Representative chromatograms for TCE analysis. A. 5 mM stock TCE solution in water, B. T_0 preparation, *M. vaccae* JOB5, C. heat killed control, *M. vaccae* JOB5, D. active cells, *M. vaccae* JOB5.

Table 12
TCE Degradation by Selected Propane-oxidizing Isolates

Isolate	Inducing Substrate	nmol TCE degraded $min^{-1}mg\ protein^{-1}$ (% TCE degraded)	%TCE Recovered⁺
M. vaccae JOB5	Propane Propionate	66.5 (50.5) 21.9 (13.2)	69.1 57.5
Mycobacterium sp. II	Propane Propionate	>69.4* (100) no growth	96.2 ---
Mycobacterium sp.5	Propane Propionate	>97.6* (100) no growth	105.6 ---
Nocardia brasiliensis strain IIIP	Propane Propionate	17.1 (17.1) 5.2 (8.5)	107.0 99.0
Rhodococcus strain C	Propane Propionate	2.5 (2.8) 6.1 (2.8)	55.4 68.4

* Indicates total consumption of available TCE, therefore maximum degradation rates could not be obtained; ⁺ (TCE $conc._{killed\ control}$)/(TCE $conc._{T0\ control}$)

degradation rates were manifest. The quantity of TCE degraded by *Nocardia brasiliensis* strain IIIP approaches 20% but is still far less than the amounts degraded by *M. vaccae* JOB5, and *Mycobacterium* sp II and 5. Experimental data indicates that TCE degradation is not efficiently carried out by propionate grown cells; all TCE degradation values for propionate grown cells fall within or close to the range of experimental error for this series of assays.

Since this study represents the first reported use of a "miniaturized" TCE assay system in which the reaction vial contained a headspace, it was important to determine if leakage occurred. TCE concentrations recovered from heat-killed controls ranged between 68 and 106%, indicating that leakage occurred during the assay process (low recovery values) or erroneous measurements during initial sample preparation. Possible error sources for the low

values were volatilization at the initial loading , or during the pentane extraction process. The technique generally reported in the literature uses 10-15 ml vials and 2-3 ml of cell suspension.

Specific oxygen uptake rate studies. Specific oxygen uptake rate (SOUR) analyses, in general, showed trends rather than absolute results. In the case of propane grown *M. vaccae* JOB5 (Table 13), comparatively high oxygen consumption values were observed for isopropanol and the intermediates of terminal oxidation, indicating that the terminal oxidation

Table 13
Oxygen Consumption by *Mycobacterium vaccae* JOB5

Metabolic Intermediate	Growth substrate	
	Propane	Propionate
Subterminal Intermediates		
Isopropanol	144.7*	<ER**
Acetone	21.2	<ER
Acetol	19.5	<ER
Methyl glyoxal	0.6	<ER
Acetate	12.4	<ER
Pyruvate	14.7	<ER
Terminal Intermediates		
n-Propanol	81.8	3.7*
Propionaldehyde	167.1	<ER
Propionate	175.9	<ER
Endogenous Respiration	11.2	12.6

* data reported as nmol O_2 $min^{-1}mg$ $protein^{-1}$ in excess of endogenous respiration rate;
** <ER=respiration less that endogenous respiration rate.

pathway was expressed more strongly than the subterminal pathway. Oxygen consumption for subterminal intermediates was in excess of endogenous respiration, but less than the observed values for subterminal oxidation. Propionate grown cells of *M. vaccae* JOB5 displayed depressed respiration rates when challenged with subterminal intermediates and all terminal intermediates except *n*-propanol. Respiration occurred when the propionate grown cells were challenged by the terminal and subterminal intermediates, but observed values were less than those for endogenous respiration. A similar pattern was seen for propane grown cultures of *Mycobacterium* sp. II (Table 14). Respiration values for the terminal oxidative intermediates

Table 14
Oxygen Consumption by *Mycobacterium* sp. II

Metabolic Intermediate	Growth Substrate	
	Propane	Propionate
<u>Subterminal Intermediates</u>		No growth on propionate
Isopropanol	14.1*	
Acetone	17.4	
Acetol	36.2	
Methyl glyoxal	12.9	
Acetate	18.5	
Pyruvate	2.6	
<u>Terminal Intermediates</u>		
n-Propanol	43.1	
Propionaldehyde	39.4	
Propionate	9.4	
Endogenous Respiration	21.3	

* data reported as nmol O_2 $min^{-1}mg$ $protein^{-1}$ in excess of endogenous respiration rate

n-propanol and propionaldehyde were elevated as was the value for acetol, a subterminal intermediate. The average oxygen consumption for these terminal intermediates was about

three times that of the average for the subterminal intermediates (excluding the value for acetol). *Mycobacterium* sp. II did not grow on propionate, therefore no oxygen uptake data is available for this substrate/organism combination.

Mycobacterium species 5 cells cultured on Higgins-propane medium (Table 15) showed fairly even respiration rates when challenged with both subterminal and terminal intermediates, meaning that no single intermediate is shown a preference in the respiratory process. The conclusion is that both the terminal and subterminal oxidative pathways are being expressed in this organism. As with *Mycobacterium* species II, *Mycobacterium* species 5 could not be cultured on propionate.

Table 15
Oxygen consumption by *Mycobacterium* sp. 5

Metabolic Intermediate	Growth Substrate	
	Propane	Propionate
Subterminal Intermediates		
Isopropanol	14.3*	No growth on Propionate
Acetone	8.6	
Acetol	5.0	
Methyl glyoxal	11.3	
Acetate	6.4	
Pyruvate	6.7	
Terminal Intermediates		
n-Propanol	7.3	
Propionaldehyde	12.0	
Propionate	8.0	
Endogenous Respiration	34.7	

* data reported as nmol O_2 $min^{-1} mg$ $protein^{-1}$ in excess of endogenous respiration rate

The respiratory values for *Rhodococcus* species C (Table 16) seem to indicate that again, the terminal oxidative pathway is preferentially expressed. Conclusions for this

organism are obscured by several points. First, data are missing for acetate and pyruvate. Second, a high oxygen consumption value was observed for isopropanol, but a very low value was seen for *n*-propanol. Respiration rates of the culture when challenged with propionaldehyde and propionate were about twice that for acetone, acetol, and methyl glyoxal. Data from propionate grown cells indicates a very strong preference for the terminal pathway. Observed values for intermediates of subterminal oxidation were depressed below endogenous respiration rates, with the exception of isopropanol and acetone. This leads to the guarded speculation that the terminal oxidative pathway is preferentially expressed in this organism.

Table 16
Oxygen Consumption by *Rhodococcus* strain C

Metabolic Intermediate	**Growth Substrate**	
	Propane	**Propionate**
Subterminal Intermediates		
Isopropanol	43.2*	13.7*
Acetone	6.6	18.8
Acetol	7.6	<ER**
Methyl glyoxal	10.4	<ER
Acetate	ND+	<ER
Pyruvate	ND	<ER
Terminal Intermediates		
n-Propanol	3.0	50.0
Propionaldehyde	15.9	44.4
Propionate	17.4	66.2
Endogenous Respiration	28.0, 24.2	103.0

* data reported as nmol O_2 $min^{-1}mg$ $protein^{-1}$ in excess of endogenous respiration rate;
** <ER=respiration less than the endogenous respiration rate.
\+ ND= not done

Propane grown cells of *Nocardia brasiliensis* strain IIIP (Table 17), showed a pattern similar to, but perhaps more distinct than that of *Rhodococcus* sp. C. Oxygen uptake for *n*-propanol was over twice that of isopropanol. Respiration values for the subterminal

intermediates acetol and methyl glyoxal were elevated, as was the oxygen uptake for the terminal intermediate propionaldehyde. Respiratory values for acetone, acetate and propionate were depressed. The respiration rate for propionate in the propane grown cells is curious since the oxygen uptake data, in general, for terminal products of propionate metabolism in propionate grown cells is high. The intermediates of terminal oxidation are unquestionably used preferentially in the propionate grown cells. Comparing average data for terminal and subterminal oxidation in propionate grown cells of *Nocardia brasiliensis* strain IIIP and *Rhodococcus* species C shows that the ratios subterminal to terminal values are about the same. This suggests that, as one would suspect, the terminal oxidation pathway is used in preference to the subterminal pathway in cells grown on propionate.

Table 17
Oxygen Consumption by *Nocardia brasiliensis* strain IIIP

Metabolic Intermediate	Growth Substrate	
	Propane	Propionate
Subterminal Intermediates		
Isopropanol	3.5*	1.1*
Acetone	1.0	0.0
Acetol	10.9	<E.R.**
Methyl glyoxal	8.6	1.0
Acetate	1.0	0.3
Pyruvate	ND+	ND+
Terminal Intermediates		
n-Propanol	4.6	0.4
Propionaldehyde	<E.R.	<E.R.
Propionate	6.3	2.6
Endogenous Respiration	2.8	2.8

* data reported as nmol O_2 $min^{-1}mg$ $protein^{-1}$ in excess of endogenous respiration rate.
** <E.R.= respiration less than the endogenous respiration rate
\+ ND= not done

CHAPTER IV

DISCUSSION

Initial planning of this project included selection and growth of appropriate bacteria from the contaminated site and design and implementation of an operational pilot-scale bioremediation unit. However, it was soon realized that insufficient information existed for satisfactory selection and operation of such a unit. Questions pertaining to the presence of applicable indigenous microorganisms, their nutritional needs, metabolic pathways, degradation spectra, breakdown products, overall reaction rates, and leachate toxicity should be addressed prior to any field application or pilot reactor. Even though the "aerobic oxidative processes for TCE and the other chlorinated alkenes contain a considerable amount of promise for the eventual biological treatment of groundwater... the characteristics of these processes present formidable challenges" which are not yet solved... "Practical use applications and in situ treatment of these four compounds by aerobic oxidation may never be simple and is currently a long way from exploitation" (Ensley, 1991). Problems encountered in aerobic cometabolic systems are known to include enzyme destruction and competitive inhibition by inducing substrates with resulting trivial levels of chlorinated alkene degradation. Plans to build a bioreactor to treat the leachate were therefore abandoned until more of the basic scientific questions were answered.

Leachate Composition. A question about leachate composition that has persisted since the beginning of the study concerns the amount of DCE present (Table 7) in relation to the other chlorinated alkenes. A possible explanation is found in the work of Kästner (1991) in which

he evaluated the degradation of PCE and TCE in a system that went from aerobic oxidation of selected organic chemicals to an anaerobic state with a low redox potential. He noted that the chlorinated alkenes acted as electron acceptors for oxidative enzymes when the redox potential was sufficiently low (-150 mV). He further noted that the degradation was limited and often stopped at DCE. The dissolved oxygen content of groundwater passing through contaminated soil is probably relatively high initially because of constant recharge from nearby fire mains. As it passes down gradient, the dissolved oxygen is used in the mineralization of hydrocarbon contaminants, and it is possible that reducing compounds such as sulfide are leached from the soil, further lowering the redox potential and thus allowing for amethanogenic reductive dechlorination that results in DCE accumulation. It is also possible that the soil up-gradient from the French drain collection area is anaerobic and the transit time of the water through the anaerobic zone is too short to allow complete degradation of PCE and TCE to VC or ethylene.

Documentation of indigenous microflora levels. Before beginning work to isolate specific microbial subpopulations from the industrial waste site, an assessment of inherent leachate toxicity and assays to demonstrate the presence of a viable microflora were conducted. Leachate toxicity studies (data not presented) indicated that while the leachate was not inherently toxic to its microflora, it was limited with respect to utilizable carbon sources and growth cofactors. Although the presence of specific subpopulations is generally an *a prior* proposition, previous contamination of the study area with jet fuel, heavy metal containing aircraft paint and a variety of hydrocarbons and solvents necessitated the quantitation of cell numbers. Because of the contamination, it is possible that large areas of land could have greatly diminished microbial populations. Studies such as the one by Fliermans et al. (1988), who showed that TCE concentrations over 300 ppm inhibited growth of an indigenous

microflora illustrate that anthropogenic contamination can reduce natural populations. Consequently, quantitation of viable populations was needed as a base-line for further work.

In fuel saturation areas, which contained widely varying amounts of jet fuel, microbial populations fluctuated with depth, but viable population numbers did not necessarily decrease. Variations in cell numbers at different depths could have resulted from at least two causes. Much of the soil in the area of the study was fill-dirt and therefore heterogenous in composition. If a selected soil sample had a high clay content, as some did, organic chemical contaminants would be retained in higher concentrations, resulting in reduction of viable bacterial cells. Areas of high clay content also are less penetrable by microbial populations; a condition that results in lower viable populations per gram of soil.

Examination of 26 isolates randomly picked from core sample assay plates showed the majority of the isolates to be Gram positive rods. Gram negative rods were also seen, but in smaller numbers. The morphology of the rods ranged from symmetrical to pleomorphic, budding and non-budding, and probably represented (at a minimum) the major expected genera of soil organisms: *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Brevibacter*, *Mycobacterium*, *Nocardia*, *Corynebacter*, *Actinomyces*, as well as a variety of fungi.

For the leachate, acridine orange direct cell counts (AODC) techniques were used to estimate the total number of microbial cells per ml, followed by INT-dye reduction, and plate count assays to estimate viable cell numbers. Total cell counts for the French drain leachate were in the 10^6-10^8 cells per ml range, while viable cells numbered in the 10^5-10^6 cells per ml range. Characteristically, about 1%-10% of the total cell count will be viable, according to a study by Webster et al. (1985). Overall, the viable cell counts are within an expected range if compared with Webster's data.

The microorganisms in the leachate probably originated from contaminated soil areas, since the leachate is collected down gradient from the former dump area. The postulated origination of the leachate microflora from contaminated soil means that these microbial populations were exposed to a variety of organic soil contaminants prior to entry of the original populations into the French drain. The results of this and continued exposure was the selection of microbial strains that were capable of using some of the organic constituents as carbon sources, in addition to withstanding the solvent properties of the chemicals and toxic effects of the heavy metals. The organic constituents of the leachate require that surviving microorganisms be able to remain in either a dormant state in the presence of the contaminants or be able to utilize selected compounds as carbon sources for growth (Britton, 1984).

Toluene-oxidizing subpopulations. Since much of the early research on TCE degradation is tied to oxidation of aromatic compounds, and toluene is one of the principle organic chemicals in the leachate, it was natural to first target these organisms for enrichment and isolation from the leachate. The presence of several different toluene-oxidizing bacterial species reflects differing niches within the ecosystem. Both the non-motile *Acinetobacter* and the motile *Pseudomonas* are aerobic, but in natural systems the *Acinetobacter* relies on its capsular exopolymer to maintain a sessile position where aeration is adequate for growth. The motile *Pseudomonas* is free to migrate toward more favorable growth conditions. The separation of the organisms on the basis of pH reflects the lower pH growth optimum of *Acinetobacter* as also reported by Juni, 1984.

The isolation of toluene-oxidizing bacteria from the leachate (Table 10) shows that appropriate enzymatic machinery is present to elicit aromatic ring cleavage. This result is significant, since the first reported pure culture to cometabolically degrade TCE (Nelson et al., 1986) was determined to be a phenol-oxidizing *Pseudomonas.* Later studies by Nelson and

others showed that toluene also induced production of dioxygenases capable of ring hydroxylation and subsequent ring cleavage. Since that time, the cometabolic degradation of TCE by pseudomonads has been shown to result in production of toxic compounds that react with the toluene mono- and dioxygenases, ultimately destroying the enzymes (Ensley, 1991). Enzyme destruction may account for published reports of non-linear degradation of TCE by toluene-oxidizing bacteria (Wackett et al., 1988; Nelson et al., 1987, 1988). Additionally, the inability of toluene-oxidizers to degrade VC prompted a search for a more cometabolically comprehensive group of microorganisms.

Methane-oxidizing subpopulations. Methanotrophic bacteria offer a potential alternative microbial system that does mineralize VC. Parallel studies of the methylotrophic leachate isolates from this study by DeSpirito (1991, 1992) showed the organisms to be the type I methanotroph, *Methylomonas*. Since the methanotrophs are considered ubiquitous aerobic organisms that occur where methane is present (Whittenbury and Krieg, 1984), their presence indicates that some portion of the French drain system is maintained at highly anaerobic conditions that are methanogenic, otherwise no methane would be present to sustain their growth.

The isolation of the pink type I methanotroph is interesting because Strandberg et al. (1989) and Leadbetter and Foster (1958) also reported the isolation of similarly pigmented organisms as the predominant cultures in methane enrichments. One of Söhngen's main methylotrophic isolates in 1906 was also pink (Leadbetter and Foster, 1958). It was Leadbetter and Foster's (1958) contention that pigmentation is one of the chief differences in methanotrophs (there being four basic color groups), but they concluded that the pink isolates were preferentially obtained during laboratory enrichment with methane, but did not constitute the most abundant species in nature. While speciation cannot be based solely on pigmentation,

it is tempting to speculate that (also using criteria of cell morphology and motility) their cultures were the same genus as isolated in this study.

The cometabolic enzymes of the methanotrophs (sMMO and pMMO) differ from the toluene mono- and dioxygenases, in that MMO can mineralize VC, as well as TCE and DCE, making the methanotrophs a somewhat better candidate for bioremediation of TCE and its lower homologs than the toluene-oxidizing bacteria. Even so, the methanotrophs are susceptible to inactivation by toxic breakdown products of TCE degradation similar to toluene-oxidizers. Fox et al. (1990) calculated that MMO is destroyed after about 200 TCE molecules have been degraded.

Propane-oxidizing bacteria. Viewed in its entirety, data from this study presented in Tables 13-17 indicates that both the terminal and subterminal pathways are expressed in propane grown cultures of *M. vaccae* JOB5, *Mycobacterium* sp. II and 5, *Rhodococcus* sp. C, and *N. Brasiliensis* strain IIIP. The data further suggest that when some cultures (*M. vaccae* JOB5, *Mycobacterium* sp. II, and *Rhodococcus* sp. C) are grown on propane, larger incremental uptake of oxygen occurs when they are challenged with terminal propane intermediates than with subterminal intermediates.

Specific oxygen uptake rate studies- The propane-oxidizing bacteria constitute the third group of microorganisms examined in this study. The major alkane metabolizing characteristics of this group of organisms (made up of *Mycobacterium-Nocardia-Coryneform* and related organisms) have been known for years, but the organisms have only recently been reported to degrade TCE (Wackett et al., 1989). Consequently, this group of microorganisms has received little attention with respect to its potential for bioremediation.

According to a number of authors (Vestal and Perry, 1979; Perry, 1979, 1980; Stephens and Dalton, 1986; Woods and Murrell, 1989; Taylor et al., 1980; Vestal, 1984;

Wackett et al., 1989) growth of bacteria on propane induces production of propane monooxygenase (PMO) and a series of NAD/NADH linked dehydrogenases and oxygenases that sequentially oxidize propane subterminally to acetate and pyruvate, products that directly feed the central metabolic cycles. A similar series of oxidative reactions occurs as a result of constitutive enzymes that mediate terminal oxidation following the initial oxidation of propane by PMO. Pathways for both terminal and subterminal pathways have been delineated for *Mycobacterium* (Perry, 1980), *Rhodococcus* (Woods and Murrell, 1989) and *Arthrobacter* (Stephens and Dalton, 1986) in work dealing with the metabolism of propane. Although Wackett et al. (1989) showed that propane grown cells of several *Mycobacterium* species cometabolically degrade TCE, the question of which oxidative pathway (terminal or subterminal) is favored for the cometabolic process has not previously been addressed. Regarding the question of preferential oxidative pathway expression, Lukins and Foster (1963 b) stated that terminal oxidation "is the most common means by which microorganisms utilize aliphatic, saturated hydrocarbons..." even though they demonstrated methyl ketone production in mycobacteria, a process that mechanistically requires subterminal oxidation. Subterminal oxidation has also been documented by other authors (Vestal and Perry, 1979; Perry, 1979, 1980; Stephens and Dalton, 1986; Woods and Murrell, 1989; Taylor et al., 1980; Vestal, 1984). Woods and Murrell (1989) showed that *Rhodococcus rhodochrous* PNKb1 utilized both terminal and subterminal oxidation when grown on propane.

Oxygen uptake data from these studies warrants more in depth discussion. In these experiments, isolates, including *M. vaccae* JOB5, obtained from Jerome Perry and used as a reference strain, were shown to utilize both terminal and subterminal oxidative pathways when grown on propane, while propionate grown cells (when growth occurred) showed poor respiratory response to intermediates of subterminal oxidation. The results of oxygen uptake

studies are summarized in Table 18. The interspecies differences in specific oxygen uptake rates for the same metabolic intermediate suggests that differences exist with respect to expression of oxidative pathways. Work by Stevens and Dalton (1986) supports this for

Table 18
Summary of responses to intermediates of terminal- and subterminal-oxidation by isolates from this study.

A. Response of Mycobacterial Species

	Growth substrate	
Oxidation path	**Propane**	**Propionate**
Subterminal	Good	Poor
Terminal	Good	Poor

B. Response of Non-mycobacterial Isolates

	Growth substrate	
Oxidation path	**Propane**	**Propionate**
Subterminal	Moderate to good	Poor
Terminal	Good	Good

A, response of mycobacterial isolates *Mycobacterium vaccae* JOB5, species II, and 5; B, response of non-mycobacterial isolates *Rhodococcus* species C, and *Nocardia brasiliensis* strain IIIP.

Arthrobacter species. While data obtained from the current study indicate that the differences exist, the magnitude and exact scope of the differences must await more definitive techniques. The relatively high oxygen consumption by propane grown *Nocardia brasilinesis* sp. IIIP (Table 17) suggests that while both oxidative pathways are expressed, the subterminal intermediates acetone, acetate, and the terminal intermediate, propionate, are used in much

smaller amounts than are the other intermediates. *Mycobacterium* sp. II (Table 14) also showed diminished usage of acetone and propionate, although the ratio of oxygen uptake for acetone/acetol in sp. II is 0.48, while in IIIP the ratio is 0.09. This suggests that in both isolates, catabolism of acetone is a rate limiting step, with a more extreme limitation seen in *N. brasiliensis* IIIP.

The inability of *Mycobacterium* sp. II and 5 (Tables 14 and 15) to utilize propionate as a carbon source, and the lower respiration rate of propane-grown cells on propionate can be interpreted as either a problem with transport of propionate into the cells, or that a rate limiting condition exists. Data showing a relatively high respiration rate for propionaldehyde indicates that the necessary constitutive enzymes of terminal oxidation are functional, since propionaldehyde is the last step in the oxidative pathway prior to formation of propionate and propionyl-CoA.

The lack of growth of the isolates II and 5 on propionate may be due to insufficient quantities of propionate entering the cell to support growth. Data in Table 14 and 15 show that oxygen is consumed by the cells when presented with intermediates of terminal metabolism.

By comparison, propane grown *M. vaccae* JOB5 (Table 13) showed high respiration rates for all terminal oxidation intermediates, with very little difference seen between oxidation of propionate and propionaldehyde. However, data in Table 13 show a depression of the oxygen uptake below the endogenous respiration rate for all intermediates except n-propanol for propionate grown *M. vaccae* JOB5. A suitable explanation for this depressed respiration rate has not been found.

Propionate grown cells of *Rhodococcus* sp. C (Table 16) also showed depression of respiration when challenged with the subterminal intermediates acetol, methyl glyoxal, acetate,

and pyruvate. This observation could indicate a lack of required enzymes for these compounds. The fact that acetone was used in an apparently disproportionate rate could mean that the acetone was being oxidized by an unrelated, non-enzymatic oxidation of acetone. Since oxygen uptake by propane grown cells of this isolate suggests that subterminal oxidation does occur, the depressed respiration data for propionate grown cells challenged with subterminal intermediates indicates that subterminal oxidative enzymes are not induced by growth on propionate. This explanation agrees with the idea of inductive subterminal enzymes as proposed by Vestal and Perry (1969), Perry (1980), and Vestal (1984). In this case high oxygen uptake by the same cell suspensions challenged with terminal intermediates seems to show a clear demarkation between the constitutive terminal and the inducible subterminal enzyme systems.

Propane grown cells of *Nocardia brasiliensis* strain IIIP (Table 17) generally displayed weak response to both subterminal and terminal intermediates for both propane and propionate grown cells. However, within the confines of these data it appears that propane grown cells are best able to utilize both terminal and subterminal intermediates, suggesting that enzymes for both the terminal and subterminal oxidative pathways are present.

Based on the approximately equal oxygen consumption for isopropanol and n-propanol by propane grown cells of isolate IIIP, it is conceivable that the same alcohol dehydrogenase was used for both. Respiration values for acetone are depressed indicating a possible rate limiting step in the oxidative pathway. Oxygen uptake values for acetol and methyl glyoxal are the highest in the series. Taylor et al. (1980) reported on the conversion of acetol to methyl glyoxal to pyruvate by a acetone catabolizing Gram-positive isolate from soil. In their study, the enzyme methyl glyoxal dehydrogenase was implicated in the conversion of methyl

glyoxal to pyruvate. High activity of this enzyme in strain IIIP could explain the higher respiration rate when the culture was exposed to methyl glyoxal.

Considering the rate of propionate oxidation by propane grown cells of isolate IIIP, it is perplexing that propionate grown cells do not utilize propionate and the other terminal intermediates more readily. According to these data, propane grown cells utilized propionate about three times faster than did the propionate grown cells. The propionate grown cells also showed much lower respiratory values for subterminal intermediates than did the propane grown cells. A comparison of oxygen consumption values for the propionate grown *Rhodococcus* strain C culture and the *Nocardia brasiliensis* strain IIIP indicates a much better oxidative response to propionate for the *Rhodococcus* strain.

Overall, these data for *Nocardia brasiliensis* stain IIIP suggest that the isolate utilizes both terminal and subterminal oxidation of propane, but responds poorly to propionate as a growth substrate. TCE degradation data (Table 12) shows that strain IIIP does degrade small amounts of TCE which is consistent with the idea that both the terminal and subterminal pathways can be involved, but is far from the best isolate for the task.

Examination of comparative respiration rates for isopropanol and n-propanol for both propane and propionate grown cells show mixed results. Generally, the alcohol dehydrogenases of alkane grown bacteria are diverse, sometimes showing multiple isozymes with overlapping functions. Oxygen uptake data for propane grown cultures used in this study showed that three of the five (*M. vaccae* JOB5, *Mycobacterium* sp. 5, and *Rhodococcus* sp. C) preferentially oxidized isopropanol, while the remaining isolates (*Mycobacterium* sp. 2, and *Nocardia brasiliensis* strain IIIP) preferentially oxidized n-propanol. This could mean that separate alcohol dehydrogenase existed for each alcohol or that the alcohol dehydrogenase in each isolate was not altogether specific in its actions.

TCE degradation by propane-oxidizing isolates- All of the *Mycobacterium* isolates obtained from the French drain leachate degraded TCE well, while the *Nocardia brasiliensis* strain IIIP and *Rhodococcus* sp. C demonstrated insignificant degradative capabilities (Table 16) . Since data from SOUR studies showed oxygen uptake by all isolates challenged with propane metabolism intermediates, the poor TCE degradation rates of *Nocardia brasiliensis* strain IIIP and *Rhodococcus* sp. C could result from low PMO production levels or a PMO of a different type from that found in the *Mycobacterium* species. Wackett et al. (1989) observed that not all propane oxygenase producing bacteria degraded significant amounts of TCE. Data in Table 12 seem to support his findings. The idea of different PMO's is further substantiated by Cardini and Jurtshuk (1970) who showed that *Corynebacterium* sp. Strain 7E1C produced a PMO different from any others found to that date.

Comparison of the TCE degradation rates of the isolates from this study with rates reported in the literature is difficult because of differences in reporting methods used by various authors (see Table 19). Generally, degradation results were expressed in most studies as nmol TCE degraded per unit time per cell concentration or as a percentage reduction in TCE levels during the assay. Some authors list their data as specific rates, other as V_{max} and K_m. Data obtained in this study were for specific time periods and TCE concentrations and are not V_{max} calculations. Data for V_{max} and K_m calculations were not collected because of the limited cell mass available.

Based on a comparison of data in Table 19, it appears the *Mycobacterium* isolates in this study degraded TCE in a range comparable to the overall body of collected results. *Mycobacterium* isolates II and 5 and *M. vaccae* JOB5 are superior to the pseudomonads for

Table 19
Chlorinated Alkane Degrading Microorganisms and Rates of Degradation

Culture	Inducer	TCE Degradation Rate	Other compounds Degraded	Comments
Pseudomonas sp. strain JS6	toluene or chlorobenzene	1nm/min/mg protein	Cl- and methyl-aromatics	Pettigrew et al, 1991
Pseudomonas cepacia G4	phenol	apparent K_s = 8.5, apparent V_{max}=466 nmol $min^{-1}mg^{-1}$; .7g/24h/g cells;		Folsom et al, 1990 Folsom & Chapman, 1991; Nelson et al, 1987
Pseudomonas putida F1	L-arginine, toluene	1.8 nmol min^{-1}mg $protein^{-1}$ (initial velocity)		Wackett & Gibson, 1988; PCE, VC, & ethylene not degraded
Mycobacterium aurum	None	55 nmol $min^{-1}mg^{-1}$ (dry wt)	VC, DCE	Hartmans & deBont, 1992; very rapid inactivation of VC degradation seen
Nitrosomonas europaea	NH_4	1 nmol $min^{-1}mg^{-1}$ protein	VC (approx. 5nmol $min^{-1}mg^{-1}$)	Arciero et al., 1989
Acetogens or methanogens	Methane as e^- donor (no methanogenesis)	----	PCE- 550 mol reduced to 80% ethane & 20% VC in 2 days @ 35 ^{0}C; almost complete reduction in 4 days	DiStefano et al, 1991

Table 19 (con't)
Chlorinated Alkane Degrading Microorganisms and Rates of Degradation

Culture	Inducer	TCE Degradation Rate	Other Compounds Degraded	Comments
Rhine river sediment + anaerobic sludge	lactate as e^- source	3.7 $molL^{-1}h^{-1}$	PCE	deBruin et al, 1992
Methylosinus trichosporium OB3b	methane	290 nmol min^{-1} mg $cells^{-1}$	t-1,2-DCE, V_{max}= 330 nmol min^{-1}mg cells; chloroform, V_{max}= 550 $nmol^{-1}$mg cells	Oldenhaus et al. (1991); no Cu in medium
Methylosinus trichosporium OB3b	methane	5.8 nmol min^{-1}mg cell protein		DeSpirito et al. (1990); Cu in medium; type II
Methylamonas sp. A45	methane	29.6 nmol min^{-1}mg $protein^{-1}$		Type I methanotroph; DeSpirito et al. (1990)
Methylamonas sp. MN	methane	23.2 nmol min^{-1}mg $protein^{-1}$		Type I, DeSpirito et al. (1990)
Methylamonas sp. MM2	methane	0.9 nmol min^{-1}mg $protein^{-1}$		Type I, DeSpirito et al. (1990)
Methylamonas sp. GD1	methane	<0.1 nmol min^{-1}mg $protein^{-1}$		Type I, DeSpirito et al. (1990)
Methylamonas sp. GD2	methane	<0.1 nmol min^{-1}mg $protein^{-1}$		Type I, DeSpirito et al. (1990)

Table 19 (cont)
Chlorinated Alkane Degrading Microorganisms and Rates of Degradation

Culture	Inducer	TCE Degradation Rate	Other Compounds Degraded	Comments
Methylocystes parvus OBBP	methane	67.7 nmol $min^{-1}mg$ $protein^{-1}$		Type II, DeSpirito et al. (1990)
Methylococcus capsulatus (Bath)	methane	20.2 nmol $min^{-1}mg$ $protein^{-1}$		Type X, DeSpirito et al. (1990)
Bacillus(?) and *Desulfoto-maculum*	toluene + various organics	14.6 mg l^{-1}	16.2 mg l^{-1} PCE	Kästner, 1991; degradation followed trans. from aerobic to anaerobic
Mycobacterium convolutum	propane	0.5 nmol h^{-1}		Wackett et al. (1989)
Mycobacterium rhodochrous W-21	propane	0.8 nmol h^{-1}		Wackett et al. (1989)
Mycobacterium rhodochrous W-24	propane	0.9 nmol h^{-1}		Wackett et al. (1989)
Mycobacterium rhodochrous W-25	propane	0.7 nmol h^{-1}		Wackett et al. (1989)
Mycobacterium vaccae JOB5*	propane	1.7 nmol h^{-1}		Wackett et al. (1989)

* *Mycobacterium vaccae* JOB5 TCE degradation assays run with 40 mM TCE in methanol stock solution.

Table 19 (cont)
Chlorinated Alkane Degrading Microorganisms and Rates of Degradation

Culture	Inducer	TCE Degradation Rate	Other Compounds Degraded	Comments
Mycobacterium vaccae JOB5⁺	propane	40 nmol/24 h	PCE, 1.0 nmol/2 h; cis-1,2-DCE, 22.0 nmol/2 h; t-1,2-DCE, 3.0 nmol/2 h; 1,1-DCE 20.0 nmol/2 h; VC, 40.0 nmol/2 h	Wackett et al. (1989)
Mycobacterium vaccae JOB5	propane	66.5 nmol $min^{-1} mg^{-1}$ protein		This study
Mycobacterium sp. II	propane	>69.4 nmol $min^{-1} mg^{-1}$ protein		This study
Mycobacterium sp. 5	propane	>97.6 nmol $min^{-1} mg^{-1}$ protein		This study

⁺ *Mycobacterium vaccae* JOB5 TCE degradation assays were run with a 4 mM stock solution.

which results are listed, as well as reported results for *Nitrosomonas europaea.*

Methylosinus trichosporium OB3b (in a study by Oldenhuis et al., 1991) showed the highest TCE degradation rate at 290 nmol min^{-1}mg $cells^{-1}$ or approximately 160 nmol min^{-1} mg $protein^{-1}$ (assuming 55% protein, wet cell weight). These results were obtained from cultures grown on medium lacking copper and therefore reflect TCE degradation by sMMO. In comparison, data obtained by DeSpirito et al. (1991) for the same organism grown in medium containing copper shows degradation rate of 5.8 nmol min^{-1}mg $protein^{-1}$, or 3.6% of the sMMO degradation rate. The presence of copper allowed induction of only pMMO. Another study by Oldenhuis et al. study (1989) showed higher degradation rates of chlorinated alkanes by sMMO, as opposed to pMMO.[6] A degradation rate of 160 nmol min^{-1}mg $protein^{-1}$ by *Methylosinus trichosporium* OB3b appears to be roughly 2.6 times greater than *Mycobacterium* sp. 5, used in the current study. The TCE degradation rates for both *Mycobacterium* sp. 5 and *Mycobacterium* sp. II are somewhat artificial and probably do not represent the upper limits of degradation since both cultures obtained a 100% reduction of the TCE presented during the 12 h period of the assay. Assuming comparability of technique with DeSpirito et al. (1991), it appears the *Mycobacterium* of this study had greater TCE degradation rates than the isolates reported in the methanotroph study.

The analysis system used for measurements of TCE disappearance was a scaled-down version of what has become a more-or-less standard method for monitoring TCE levels in assay samples. Data indicate poor to good TCE recovery, as shown by the 68-106% recoveries when comparing T_0

[6]According to Oldenhuis et al. (1989), a 4.8 µM (approximately 0.3 ppm) copper concentration appears to be the upper threshold limit for sMMO production. At this concentration or above pMMO is thought to be the only MMO produced. For purposes of in-situ bioremediation, it appears that sMMO would be active in soil or groundwater streams below this concentrations, above which the pMMO would assume the degradative task. Copper concentrations of 0.0-0.2 ppm are considered low, while concentrations >0.2 ppm are considered as high (TAMU soil Testing Procedures). For ex-situ bioremediation projects in which mineral concentrations can be controlled and the copper concentration limited, the higher rate could be used as a degradation-rate estimation factor.

samples with heat killed controls. Further trials of this system should be conducted before wholesale endorsement is given. The system offers advantages if the inconsistencies can be overcome. The use of the 1.8 ml vials for this type of analysis means that much smaller cell volumes can be used, which is a major factor in some cases. Two key points apply to the assay system: (1) even though a smaller volume is involved, Henry's law still applies to the solubilization of the headspace TCE into the buffer solution; and (2) any system of this nature must have a closure that re-forms a seal adequate to contain the target compounds (TCE and DBA) and the extractant (pentane). Otherwise, volatile chemicals are lost while samples are held for assay. The same point applies to vessels in which standard solutions are prepared and stored.

If the gas chromatic analysis for propylene oxide production had proven to be functional, valuable data could have been obtained for the relative quantities of PMO produced by the test organisms. Because of its reactivity, propylene oxide is a difficult compound to analyze. This reactivity may have caused the difficulties encountered in this study, although no identifiable reaction products were seen in test chromatograms.

Correlation of TCE degradation and apparent expression of specific oxidative pathways. The isolates characterized as *Mycobacterium* had good TCE degradation rates. In comparison, the isolates characterized as *Rhodococcus* and *Nocardia* species had insignificant TCE degradation rates. Propane grown cells of all isolates showed some degree of oxygen utilization when challenged with both terminal and subterminal intermediates, indicating that not only was subterminal oxidation induced in all isolates grown on propane, but that both pathways were expressed during TCE degradation. That only the *Mycobacteria* examined in the study degraded TCE, suggests that the inherent characteristics of the PMO are more closely associated with efficient TCE degradation than are expression of oxidative pathways per se.

The possible advantage to the organism of being able to utilize both pathways is metabolic flexibility, i.e. greater capabilities of producing needed metabolites for cell activities. For example, a cell with the ability to utilize only subterminal enzymes during propane metabolism would be limited to production of acetate and pyruvate as end products. Microorganisms can exist using these metabolites as carbon sources, but greater metabolic flexibility is achieved if the terminal-oxidation pathway is used, since the TCA cycle can be fed via succinate or acetate produced for fatty acid synthesis, etc.

Use of propane-oxidizing bacteria as bioremediation tool. The use of propane-oxidizing bacteria as a bioremediation tool appears to be a possibility based on (1) measured degradation rates for TCE, and (2) the ubiquity of these microorganisms. However, the use of any single group or cometabolic substrate as a panacea for in-situ bioremediation applications may not be possible (Ensley, 1991).

Several potential problems exit for in-situ bioremediation. TCE oxygenases are known to be destroyed by toxic breakdown product of TCE degradation. Wackett et al. (1989) showed that the time course for TCE degradation by *Mycobacterium vaccae* JOB5 is nonlinear and it is assumed that PMO is also subject to enzyme breakdown as with the toluene-oxygenases and MMO. Since cometabolic degradation of TCE is a competitive and finally lethal function, a selective shift away from TCE degrading organisms may occur because of macromolecular damage to oxygenase producing populations. Such a population shift would select for microorganisms that utilize propane as a carbon source but do not degrade TCE. If a large and more diverse population of TCE degrading organisms was used, such as a combination of propane- and methane-oxidizing bacteria as suggested by Fliermans et al. (1988), the dynamics of growth and survival for the individual populations would conceivably be different enough to allow remediation the TCE problem without significant detrimental impact on the microflora. A second possible problem with in situ bioremediation, whether cometabolic or direct metabolism, is plugging of the soil formation in which the remediation is being

conducted. If microbial growth in the contaminated soil occurs too rapidly or too great an extent, it is possible to prevent the movement of water through the soil thereby preventing the distribution of further nutrient supplies (Dick Raymond, personal communication). Plugging of formations by propane-oxidizing *Mycobacterial* populations could be inconsequential because of their doubling times, however, other bacteria with faster reproduction times could also cause other problems.

Ex situ remediation utilizing bioreactors to facilitate pump-and-treat operations for groundwater such as the French drain leachate or industrial waste water streams are a possibility because better control of bioreactor populations can be obtained. For in situ treatment, the numbers of degradative organisms must be preferentially enhanced, while for ex situ applications, seeded cultures must remain in the reactor, ideally proliferating during the treatment. The generation time of the microorganisms used in ex situ reactors, in combination with degradation rates for the specific compound will determine the type of reactor used. Chemicals that are rapidly mineralized and require only a short residence time could possibly be treated with a chemostat type design as used by Winter et al. (1989) and Folsom and Chapman (1991) for degradation of TCE by *Pseudomonas cepacia* G4. Chemostat type reactors of this type show the feasibility of bench-scale units, but their utility may be limited for field use because of wash out effects. Since the first-order degradation rates for chlorinated alkenes mean that low concentrations require higher residence times (Janssen et al., 1991), the overall size of chemostat-type reactors may preclude their use. Both the problem of wash out and excessive retention time are conceivably solved or reduced by packed-bed or fixed-film reactors.

Strandberg et al. (1989) used a fixed-film, packed-bed reactor to treat a synthetic mixture of TCE and DCE (1 mg/L each) and obtained a 90% removal rate with a 50 minute retention time. Some of the original experimental data on cometabolic degradation of TCE came from a packed column reactor that used sandy loam as a support for a methanotrophic microflora (Wilson and White, 1986). Reactors of this type are susceptible to upsets due to excess or detrimental chemical loading as

are chemostat types. However, because the microbial growth has grown into the packing material (as is the case when diatomacious earth pellets are used as packing) the reactor is more resilient and should regain operational status following an upset.

Multiple stage reactors such as the one built and operated by Fathapure and Vogel (1991) offer the most promise for ex situ treatment of chlorinated alkenes. This design utilized an anaerobic primary reactor to degrade the higher homolog chlorinated alkenes PCE, TCE, and DCE, and an anaerobic secondary reactor for VC and degradation products from the primary stage. This concept can conceivably be extended to in situ treatment of contaminated soil by inducing anaerobic growth followed by placing aerobic interdiction wells in the path of groundwater flow.

The reactor that was constructed for this project was to have operated as a combination of the two major reactor types. The fixed-film, packed column portion was intended to immobilize the selected microbial consortia. The chemostat part of the reactor was to allow measurement of oxygen consumption and facilitate sampling of the growth medium. From a mechanical standpoint, the reactor failed because these problems prevented full implementation. Whether or not the total reactor concept was valid could therefore not be determined.

Conclusions- Data obtained from this study suggest that for the study area and the French drain leachate, viable subpopulations exist, meaning that previous chemical contamination has not eliminated a complex microflora. Examination of the propane-oxidizing bacteria indicates that a good subpopulation of propane-oxidizing bacteria exist, but not all can degrade TCE. These results suggest that only specific microorganisms within a community are able to cometabolically degrade chlorinated solvents. The results further suggest that intrinsic properties of the PMO control chlorinated alkane degradation, and that the organism regulates the use of the terminal and subterminal oxidative pathways, which can potentially end in different products, according to its metabolic state at the time.

APPENDIX A

GROWTH MEDIA

Formula for growth media used in this study are given in this appendix.

Table 20
Medium for isolation and growth:
peptone-glucose-yeast extract agar (DPYTG)

Per liter:
10 g glucose
10 g yeast extract
5.0 g trypticase peptone
5.0 g phytone peptone
0.6 g $MgSO_4$ X $7H_2O$
0.07 g $CaCl_2$ X $2H_2O$
15.0 g Bacto agar
Dilute to a 5% concentration for use.

Table 21
Medium for isolation and growth:
Stanier's mineral salts medium (SMSA)

Stanier's Mineral Salts Medium	**Stock Salt Solution** (grams/liter)
Standard mineral base 40 ml Na_2HPO_4 + KH_2PO_4 (1 M, Ph 6.8)	2.5 g EDTA
20 ml Hutner's vitamin free mineral base	10.95 g $ZnSO_4$-$7H_2O$
1 g $(NH_4)_2SO_4$	1.54 g $MnSO_4$-H_2O
Dilute to 1 liter	0.392 g $CuSO_4$-$5H_2O$
	0.248 g $Co(NO_3)_2$-$6H_2O$
Hutner's mineral base (grams/liter)	0.177 g $Na_2B_4O_7$-$10H_2O$
10 g nitrilotriacetic acid	1-liter distilled water
14.45 g $MgSO_4$	
3.335 g $CaCl_2$-$2H_2O$	Stanier's medium was supplemented with 50 mg each of trypticase peptone and yeast extract.
0.00925 g $(NH_4)_6Mo_7O_{24}$-$4H_2O$	
0.099 g $FeSO_4$	
50 ml stock salt solution	
950 ml distilled water	

Table 22
Formula for Higgins medium

Per liter:	**Vitamin stock solution**
0.85 g $NaNO_3$ 0.53 g KH_2PO_4 0.86 g Na_2HPO_4 0.17 g K_2SO_4 0.37 g $MgSO_4$ x $7H_2O$ 1 ml stock 1 M $FeSO_4$ solution (add after autoclaving mineral solution)	Per liter: 0.02 g biotin 0.02 g folic acid 0.05 g thiamine-HCL 0.05 g calcium pantothenate 0.0001 g vitamin B_{12} 0.05 g riboflavin 0.05 g nicotinamide

Dilute 100 ml of 10X stock in 900 ml of tap distilled water for 1X solution; use 10 ml filter sterilized 1X vitamin solution/liter of 1X medium

Table 23
Formula for Whittenbury medium

Whittenbury medium
10X stock solution

Dissolve in 900 ml tap distilled water:
4.89 g $MgSO_4$,
10.0 g KNO_3,
2.0 g $CaCl_2$.

Add 10 ml Whittenbury trace element solution, 5.0 ml 0.1% sodium molybdate solution, 1.0 ml 3.8% FeEDTA solution, 0.25 ml 100 Mm $CuSO_4$ solution. Bring volume to 1 liter.

Use 100 ml 10X solution per liter of tap distilled water; add 1 ml Whittenbury trace element solution, 10 ml vitamin mix, and 10 ml sterile phosphate buffer.
2 ml trace element solution (add after autoclaving mineral solution),
10 ml of 10X vitamin stock solution.

Whittenbury trace element solution
Per liter:
0.5 g $FeSO_4$
0.4 g $ZnSO_4$
0.02 g $MnCl_2$
0.05 g $CoCl_2 \times 6\ H_2O$
0.01 g $NiCl_2 \times 6\ H_2O$
0.015 g H_3BO_3 (Boric acid)
0.025 g NaEDTA
Use 1 ml/liter

APPENDIX B

MISCELLANEOUS PROCEDURES

While completing the research for this study several experimental procedures were attempted that either failed altogether or proved less than desirable. For the sake of completeness, these procedures are given below. Likewise, analysis of a seawater sample was initiated but later abandoned.

INT-dye reduction for direct viable cell counts. In an attempt to count respiring bacteria in leachate samples, the INT-dye reduction method was used. Water samples (10 ml) were incubated at room temperature for 20 min. with 1 ml of a 0.2% aqueous INT-dye (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) solution. The reaction was stopped by addition of 0.1% formaldehyde, and solutions stored at 4 ^{0}C until processing. Solutions were filtered through 0.2 μm porosity filter membranes. After drying, the membranes were viewed under bright field conditions with oil immersion optics. Viable bacteria reduce the tetrazolium dye to intercellular formazan crystals that appear red when viewed with a light microscope. The technique can be combined with the AODC technique by using filter membranes presoaked in sudan black. Combination of the methods allows one to switch between preparations for viable and nonviable cells for the same sample. Several problems were encountered with the INT-dye method. First, distribution of the dye did not seem uniform between different types of cells. Some cells had two or more formazan crystals (generally at the poles of bacillary cells), while coccoid cells had one, making it difficult to distinguish between cells with multiple crystals and multiple cells. If the distinction was improperly made, the resulting count was incorrect. Second, crystals were the most visible with a wide aperture, high intensity light setting which rendered counting extremely fatiguing. In addition, for water samples, the high light conditions of the INT-dye technique made it difficult to go immediately to AODC preparations which are done in low ambient/low

background light conditions. This increased the time necessary for microscopic examination of samples. Because the procedure was judged to be imprecise for the samples being examined and because of the time required per sample, use of the technique was discontinued.

Biomass accumulation procedures and cell storage techniques for propane-oxidizing isolates. Although a large number of isolates were obtained by primary and secondary enrichment with propane, a significant amount of difficulty was encountered in obtaining enough cell mass with which to work. Several procedures were tried, including growth in shake flasks, on silica gel plates, in the electrolytic respirometer, and on Higgins medium solidified with electrophoresis grade agarose. It was important that only the selected carbon source be available for growth, so that induction of desired enzyme systems was maximized.

Silica gel plates- Purified bacterial isolates were grown on Higgins-silica gel plates. Equal volumes of 2X Higgins medium and silica gel dissolved in 7% KOH were combined. Solidification was achieved by addition of 1 ml of 20% phosphoric acid per 10 ml of medium. Both 100 X 15 mm and 150 X 15 mm plates were used. Plates were stored inverted to drain off water of reaction. Propane, and 0.1% propionate were used as carbon sources and incubations were carried out in sealed containers to prevent excessive drying of the plates. Some isolates grew better than others, but overall, this combination of medium and organisms was unsuccessful. Growth was slow, requiring up to six months incubation for significant growth of the best cultures. Cells were harvested from plates by washing with Higgins salt solution and centrifuged, but insignificant biomass was obtained.

Electrolytic respirometry. Electrolytic respirometry vessels were inoculated as in the shake flask technique, and the headspace of the vessels purged with propane as described above. As

stated, the growth rates of the isolates were so slow that the technique offered no practical advantage.

Propylene oxide analyses. In an attempt to demonstrate the presence of propane oxygenase, 250 µl cell suspensions were placed in 1.8 ml vials and capped as in the TCE degradation experiments. The vials were prewarmed for 30 seconds in a 30 ^{0}C water bath, after which 900 µl of air were withdrawn from the vial. Propene in 900 µl aliquots were injected into the vials, which were then reincubated with shaking at 30 ^{0}C. After 5 minutes, 2 µl volumes were withdrawn and analyzed by GC/TCD. This procedure resulted in inconsistent retention times for propylene, propylene oxide, and possible breakdown products of propylene oxide, and therefore could not be used.

Construction of a bioreactor for TCE degradation. An attempt was made to incorporate a bench-scale packed-bed bioreactor with the electrolytic respirometer, operating in mixed gas mode. The reactor portion of the apparatus was roughly based on a design from Oak Ridge National Laboratories (Garland et al., 1989). A schematic diagram of the reactor is shown in Figure 7. This design included a closed loop gas flow for maintaining constant gas concentrations and to eliminate stripping losses of TCE, while maintaining a constant non-recycled liquid flow through the chemostat section. The oxygen generation unit of the electrolytic respirometer was fitted on a specially fabricated 1.5 liter, three-neck flask (fabricated by Mr. Dick Lemuix, Texas Christian University) that served as the chemostat. By monitoring the carbon source (methane) concentration in the gas stream, using electrolytic respirometric data for oxygen consumption values, and TCE concentrations of chemostat effluent (known volumes introduced into the system), one could calculate material balances for

all the major constituents. The column consisted of a 38 inch X 2 inch OD acrylic pipe, capped at both ends. Gas flow was counter-current to the liquid flow and controlled by a pinch clamp for gross control and a mercury-filled Cartesian diver valve for fine control. For test purposes, gas was supplied by a pressure regulated compressed air tank. Gas mixtures were circulated through the column and respirometer vessel with a small piston-type aquarium pump, and a Ranin peristaltic pump was used to maintain constant liquid flow. The reactor was to use Higgins mineral salts medium. Liquid flow was controlled by varying the pump speed. Control of liquid flow through the system proved to be no problem; however, attempts to regulate gas flows resulted in small, but cumulative uncontrollable oscillations of the Cartesian diver valve. This resulted in a positive imbalance of pressure within the system, causing the electrolytic respirometer to remain in an oxygen generation mode, thereby bioreactor schematic indicating the system was using far more oxygen than it actually was. All attempts to damp the harmonics of the gas recycle system failed, and the reactor phase of this project was abandoned. The reactor project was also abandoned because of microbiological considerations.

Persian Gulf Seawater. Sample of sea water purportedly from the Persian Gulf was assayed for the presence of propane-, methane-, and toluene-oxidizing bacteria. The only history given for the water sample was that it was from the "Persian Gulf". It was assumed that it came from the area near Iraq, due to the time frame in which it was collected (post-Persian Gulf War), but details were unavailable. Since alkane and aromatic degrading procaryotes are ubiquitous in distribution, and since the sterility and storage conditions of the sample were

unknown, it was impossible to say that isolates actually came from the Persian Gulf. Therefore, isolates were considered merely as salt tolerant (1.5% NaCl) organotrophic bacteria.

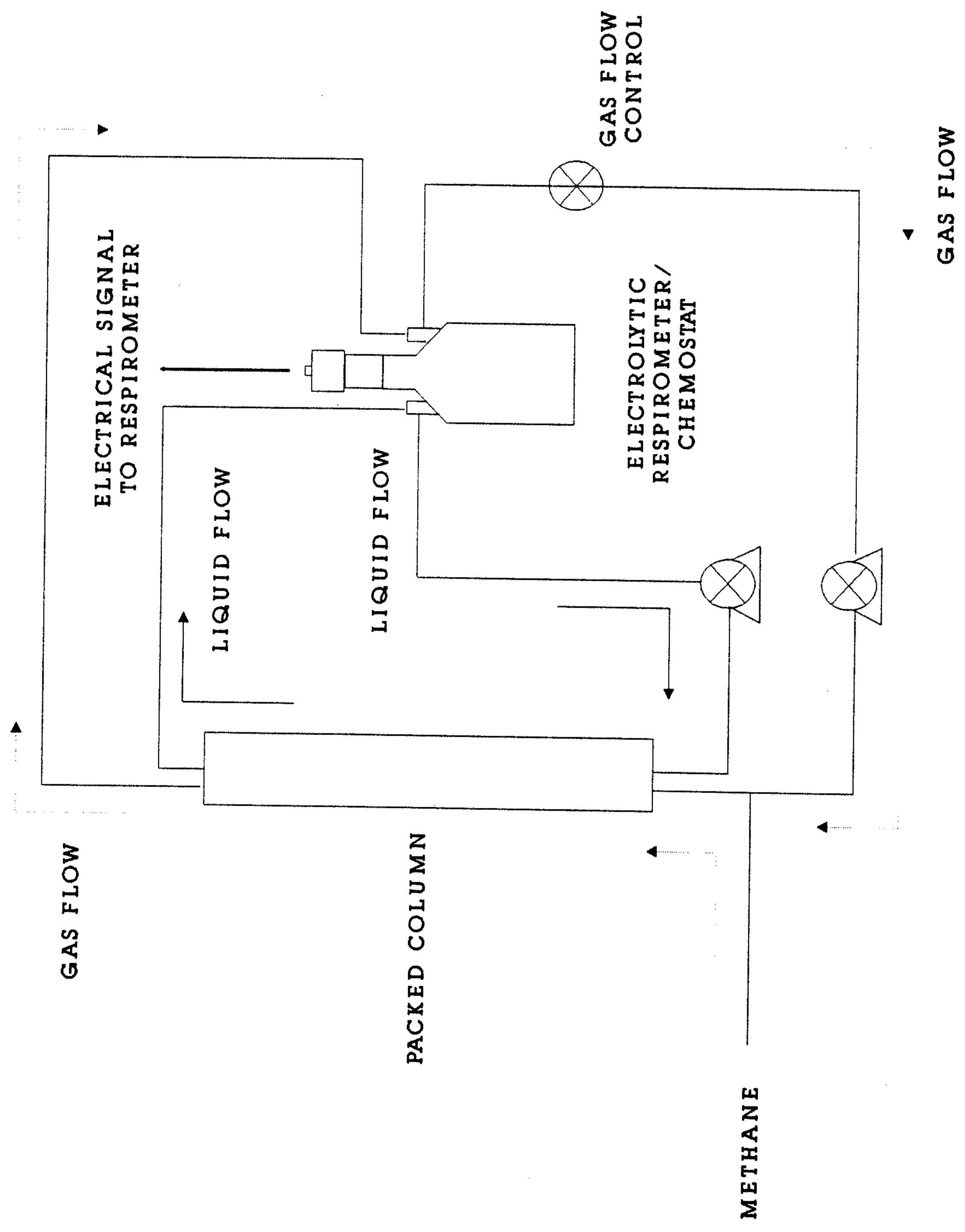


Figure 16. Schematic representation of bioreactor.

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