AN ABSTRACT OF THE THESIS OF

Adisorn Tovanabootr for the degree of Masters of Science in Civil Engineering presented on April 23, 1997. Title: Aerobic Cometabolism of Chlorinated Aliphatic Hydrocarbons by Subsurface Microbes Grown on Methane, Propane and Butane from the McClellan Air Force Base.

Abstract approved: _____
Lewis Semprini

Subsurface microorganisms from the McClellan Air Force Base were grown in batch aquifer microcosms on methane, propane, and butane as gaseous cometabolic substrates. The potential for aerobic cometabolism of chlorinated aliphatic hydrocarbons including trichloroethylene (TCE), 1,1,1-trichloroethane (1,1,1-TCA), and chloroform (CF) was determined. Stimulation of microorganisms on all of the substrates tested indicates a diverse microbial community exists in the McClellan subsurface. Indigenous methane and propane-utilizers were capable of transforming TCE and CF. Propane-utilizers very effectively transformed TCA, while methane-utilizers could not transform 1,1,1-TCA. The butane-utilizers were not able to degrade any of the CAHs tested. TCE was transformed most rapidly during the period of active methane consumption, and continued at a slower rate for about 1 weeks after methane was consumed. The propane culture remained active for up to four weeks after propane was consumed, and the rate followed first order kinetics. Different TCE transformation yields (T_y) (mg CAH/mg substrate) developed in replicate microcosms with time. Changes in TCE transformation ability resulted from changes in TCE concentration or TCE product toxicity. Both methane and propane-utilizers showed a positive correlation between initial TCE transformation rates and primary substrate utilization rates. The ratio of the zero order TCE transformation rate to primary substrate utilization rate was directly proportional to

the ultimate transformation yield. For methane-utilizers, the ratio of transformation yield to the zero order rate ratio was about 0.5, while the ratio for propane-utilizers was about 0.2. Based on individual transformation yields, methane was the most effective substrate for TCE removal. Propane-utilizers exhibited the highest transformation yields for both CF and 1,1,1 TCA. Propane-utilizers were much more effective in transforming CAHs mixtures than methane-utilizers. The presence of CF and 1,1,1 TCA in the groundwater had a greater negative effect on ability of methane-utilizers to transform TCE. Methane and propane-utilizers remained activity toward TCE transformation after one year of exposure to increasing TCE concentration and the transformation of CAH mixtures. The results indicate long term cometabolic activity can be maintained under microcosm conditions when cometabolism occurs in the presence of ample growth substrate. The batch microcosms method tested appears to be a reliable method for evaluating the in situ cometabolic bioremediation potential of TCE and CAH mixtures.

Aerobic Cometabolism of Chlorinated Aliphatic Hydrocarbons by Subsurface Microbes Grown on Methane, Propane and Butane from the McClellan Air Force Base.

by

Adisorn Tovanabootr

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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PREFACE

This thesis is focused on aerobic cometabolic biodegradation of TCE and other chlorinated aliphatic hydrocarbons by McClellan's indigenous organisms grown on methane, propane, and butane. Chapters 1 and 2 provide the introduction of aerobic TCE cometabolism, background on the McClellan AFB, and the thesis literature review. Chapter 3 is written in the form of a manuscript to be submitted for publication. It will be condensed before submission to a journal. Chapter 4 presents the results from long term batch microcosms studies of TCE, 1,1,1-TCA and CF transformation by methane, propane, and butane utilizing microorganisms. Chapter 5 documents studies of nutrient requirements for TCE cometabolism. Chapter 6 contains suggestions for future research. The appendices document the relevant experimental protocols, data analysis, and experimental results not presented in the chapters.

Adisorn Tovanabootr

June 1997.

CHAPTER 1

Introduction and Thesis Overview

History of TCE Contamination at the McClellan Air Force Base

Many groundwater aquifers are contaminated with toxic and carcinogenic compounds such as chlorinated aliphatic hydrocarbons (CAHs). Trichloroethylene (TCE) is one of the most often detected contaminants in soil and groundwater. It is widely used as a degreaser, dry cleaning solvent, and extraction agent in industry and government facilities including military installations (Westrick et al., 1984). TCE was determined to be a suspected carcinogen (Infante and Tsongas, 1982), and the U.S. Environmental Protection Agency listed TCE as a priority pollutant in 1986, with a proposed Maximum Contaminant Level (MCL) in drinking water of 5 ppb (U.S. EPA.,1993). Remedial actions are often required to clean up TCE contamination to the MCL standard.

Long term land disposal of TCE through 1970s occurred at many military installations including McClellan Air Force Base, Sacramento, California. The McClellan AFB, which is the focus of this TCE bioremediation study, is a military installation that has been used to repair aircraft. TCE has been used at the site as a degreasing agent and extraction agent (CH2M HILL, ISCB report, 1994). The disposal of TCE in waste pits resulted in contamination of vadose zone and saturated zone. A large TCE contamination source was suspected to be located in the north portion of site 22, beneath the location of waste pits.

Based on the determination of TCE contamination at the pilot test area and area north of the site 22, on October, 1994. TCE hotspot concentrations, greater than 0.5 mg TCE/L, have been detected in groundwater at the McClellan AFB. The upper and lower A zone aquifer showed TCE concentrations significantly varied. TCE concentrations ranging from 3.2 to 8.0 mg TCE/L and 0.5 to 1.7 mg TCE/L have been detected in upper and lower A zone, respectively. High TCE concentrations ranging from 10 to 20 mg

TCE/L have been also observed in the down gradient of TCE source at north of the contaminated site 22.

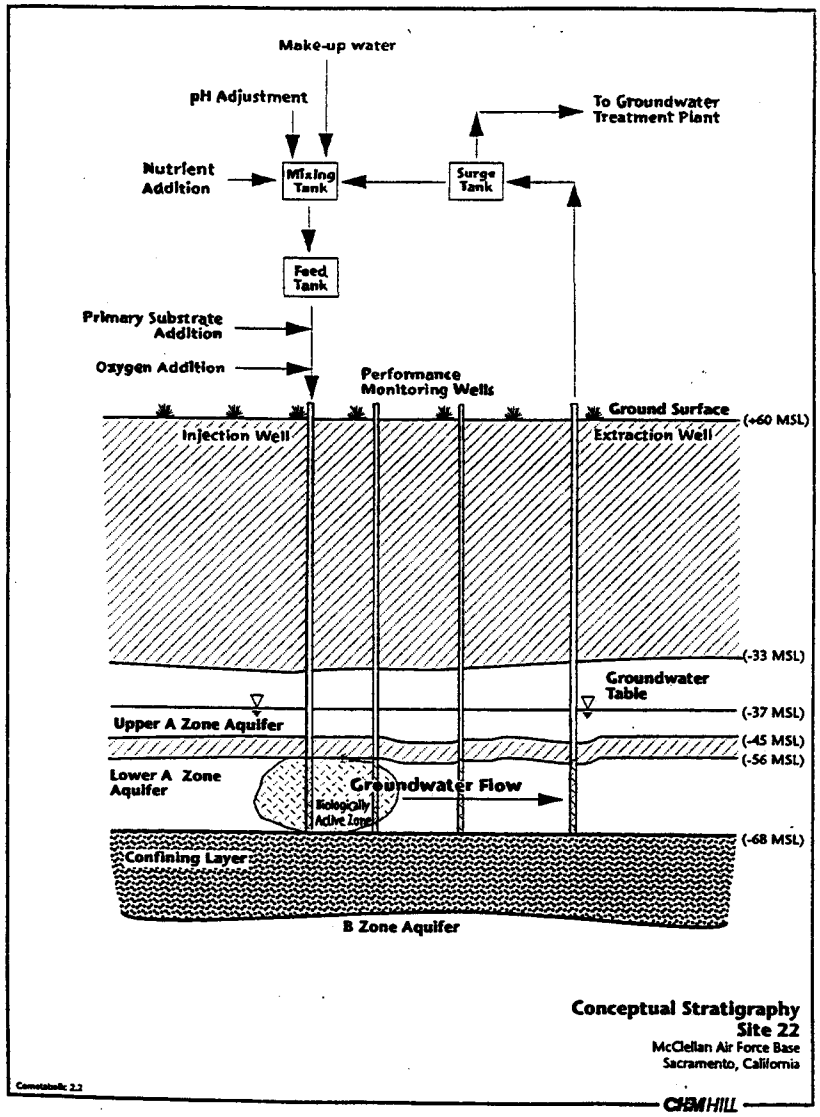

Figure 1.1 - The conceptual treatment design for in situ cometabolic bioremediation at Site 22 (McClellan Air Force Base, Sacramento, California.

In situ cometabolic bioremediation was eventually chosen to determine whether this process was effective for removal of TCE at McClellan AFB. A conceptual pilot study for contaminated soil and groundwater was designed on the ISCB (In-situ cometabolic bioremediation) pilot test area. Two well systems, injection well (EW 251) and extraction wells (EW313), were being constructed to evaluate in-situ cometabolic

biotreatment process (Figure 1.1). The lower A zone was selected for the pilot field demonstration because lower TCE concentrations were detected in this area. Performance monitoring wells were also installed between injection wells and extraction wells to measure TCE concentrations drowngradient. ISCB test was also designed for above ground processes in order to delivery nutrients and oxygen to the McClellan subsurface. The results of analysis of groundwater performed on July , 1994, indicated that groundwater needed to be amended with nutrients, since nitrogen and phosphorous as a minor nutrients were found to be limiting in the groundwater.

In addition to ISCB field demonstration, more research is needed to evaluate the feasibility of in situ bioremediation because many environmental factors may effect CAH cometabolism in the subsurface. Previous studies of in situ bioremediation indicated that cometabolic degradation of TCE and CAHs in soil and groundwater is complex. It is difficult to create a high efficiency treatment process in the subsurface environment (Semprini et al., 1990; Broholm et al., 1991). In order to evaluate in situ bioremediation of CAHs, preliminary bioremediation studies at the microcosms scale were performed to support ISCB field demonstration.

This study focused on determining the potential for aerobic cometabolism of TCE to non toxic end products using methane, propane and butane as a cometabolic growth substrates using batch incubated soil groundwater microcosms. The objectives of batch microcosm studies were :

1). to determine if indigenous microorganisms are present in the McClellan subsurface that utilize the specific cometabolic substrates of interest.

2). to evaluate how effective of the indigenous microorganisms are at transforming TCE and determine the ability of different substrates to promote TCE transformation.

3). to determine whether processes such as competitive inhibition and product toxicity are of concern.

4). to determine strategies for effective nutrient addition that might optimize TCE transformation.

5). to evaluate kinetic responses from the batch microcosm methods and to determine the transformation yields (T_y) (g CAH/ g substrate used) for a given specific substrate.

6). to study the affect of increasing TCE concentrations on the cometabolic processes for extended time periods.

7). to evaluate the transformation of CAH mixtures, including chloroform (CF), 1,1,1-trichloroethane (TCA), and trichloroethylene (TCE).

Microcosm Studies

A microcosm study is defined by Pritchard and Bourquin (1984) as "an attempt to bring an intact, minimally disturbed piece of an ecosystem into the laboratory for the study in its natural state." Microcosms have been widely used to determine the biodegradability of organic contaminants at the laboratory scale and under the impact of the site-specific physical, chemical and hydrogeologic conditions (Bedient et al., 1992). Several microcosms systems have been designed to identify biodegradable contaminants and study of chlorinated hydrocarbon degradation. Simple batch incubation systems and several systems with complex devices have been conducted to determine the metabolic pathways of biotic and abiotic transformations. Figure 1.2 shows three different types of microcosms design by Dunlap et al (1972), Bengtsson (1981), Wilson et al. (1981).

Microcosms appear to be a good screening method, as well as a method for determining process kinetic parameters. The results from microcosms studies are reproducible and allow appropriate controls to be employed. Microcosms also provide a more understandable determination of biodegradation rate and a time-efficient method for evaluating biodegradation potential at a field site. Furthermore, the input of the contaminant of interest into microcosms works well in evaluating the residual transformation yields (T_y), the ratio of contaminant degraded to growth substrate used, and also as a means of the studying the effects of contaminant concentration over a long

time period. Thus, microcosm studies are very useful for the application of in situ bioremediation.

Even though microcosms provides several experimental advantages, some limitations of microcosms should be considered when determining biodegradation processes. Higher mass transfer and higher surface to volume ratios in the microcosms can yield biodegradation rates that are not representative of in situ bioremediation. Incorrect extrapolations from microcosm results might be used to design the bioremediation treatment, impacting the design of field scale bioremediation.

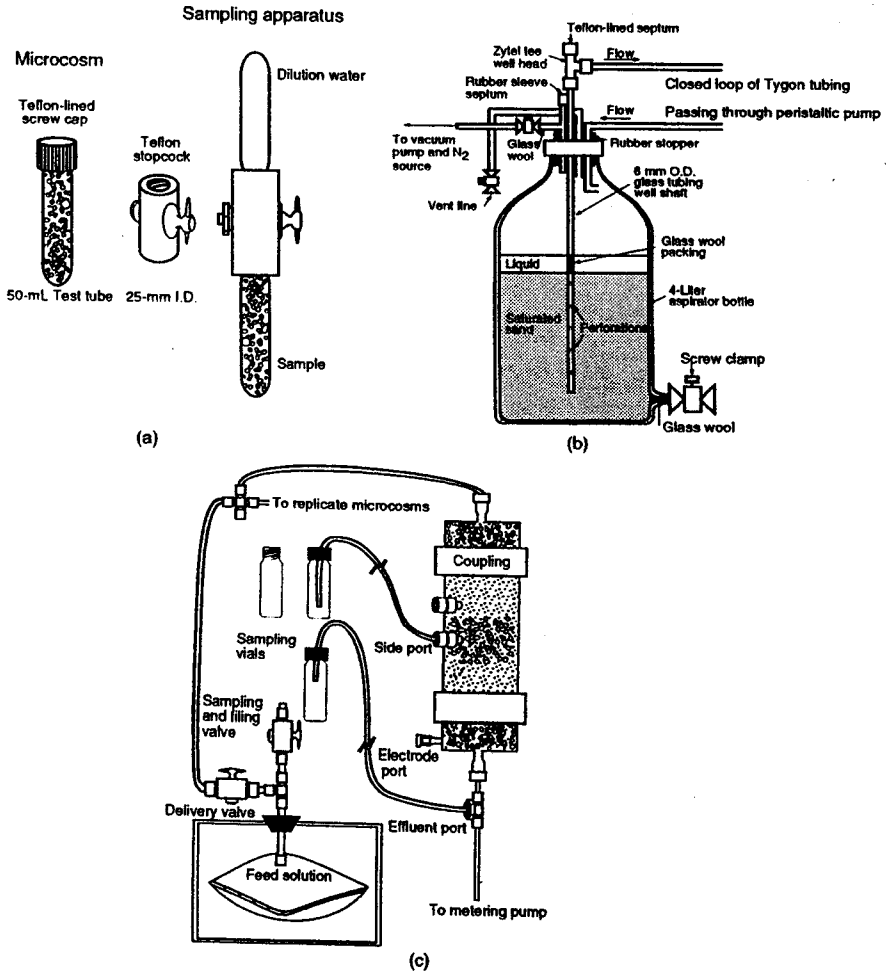

Figure 1.2 Example of several microcosm designs. (a) Wilson, 1981, (b) Dunlap, 1972, (c) Bengtsson, 1981. (Bedient et al., 1992).

Chemical Properties of the CAHs Studied

Trichloroethylene is a synthetic chlorinated organic compound that is highly volatile and colorless. It is also considered as nonflammable and nonexplosive compound at ambient temperature. TCE is commonly used in the industry as a excellent degreasing solvent and extraction agent because its boiling point provides low heat input and facilitates handling of work following degreasing operations. TCE is widely used in the metal-processing industry because it does not react with steel, copper, zinc, or other metal used in the industry. TCE is slightly soluble to water with a limited solubility of 1,100 ppm at 77 °F and considered to be a highly volatile compound that more favorably partitions in to the air than water (Mongomery,1991).

Table 1.1 Properties of selected groundwater contaminants.

Contaminant Properties	TCE	1,1,1 TCA	CF
Formular	$CHCl=CCl_2$	CCl_3CH_3	$CHCl_3$
Boiling point (°C)	87.2	74.1	61.7
Aqueous Solubility 20 °C (mg/L)	1100	480-1360	8000
Specific Density 20 °C	1.464	1.339	1.489
Henry's law constant (Hpc), 20 °C ($atm.m^3/mol$)	9.9×10^{-3}	1.5×10^{-2}	3.39×10^{-3}
Henry law constant (Hcc), 20 °C (dimentionless)	0.342	0.642	0.109
Log Octanol/Water Partition Coefficient.	2.29-3.30	2.18-2.49	1.90-1.97
U.S. Drinking Water MCL (μg/L)	5	200	100

The groundwater contaminants studied in this thesis are summarized in Table 1.1. 1,1,1 TCA and CF are also found at McClellan AFB and are often the contaminants that appear along with TCE. The properties in Table 1.1 were also major considerations for the analytical methods used in this research. The boiling point can be used to determine when compounds are expected to elude in gas chromatography analysis. The Henry's Law constants are used for the mass balance in the microcosm studies. With the Henry's Law constant and TCE headspace concentration, the liquid TCE concentration can be calculated, as well as the total mass of TCE in the microcosm.

The physical and chemical properties of contaminants also effect the migration and fate of contaminants in subsurface. Sorption is one of the main processes effecting transport in the groundwater and soil. Sorption of the contaminants can be predict by the octanol/water partition coefficient (K_{ow}). The moderate octanol/water coefficient for TCE indicates some affinity of TCE to sorb onto soil with high organic content. This will slow the movement (retardation) of TCE in an aquifer.

The solubility and specific density also affects the behavior and migration of the contaminants in groundwater. TCE has a greater specific density than water and can sink under gravity into the saturated zone. Thus, TCE is designated as a Dense Nonaqueous Phase Liquid (DNAPL).

CHAPTER 2

Literature Review

TCE Subsurface Contamination

Trichloroethylene (TCE) has been widely used as a degreasing agent, popular dry cleaning solvent, and extraction agent (Love and Eilers,1982; Westrick et al., 1984) in industries and government facilities including military installations since the 1940s. During 1940 to the 1970s, TCE was also used as an anesthetic by health professionals and extensively used in food production (Frank,1990). There was no federal, state, or local laws or regulations banning the use of TCE in early 1970s. In the mid 1970s, analytical methods became available to measure this compound at low concentrations. In the late 1970s, TCE was determined to be a suspected carcinogen (Infante and Tsongas, 1982), and the U.S. Environmental Protection Agency listed TCE as a priority pollutant in 1986, and a proposed maximum contaminant level in drinking water of 5 ppb (U.S. EPA,1993).

Long term land disposal of TCE through the 1970s occurred at many military installations. At the McClellan AFB, CA, which is the focus of this TCE bioremediation study, disposal in waste pits resulted in contamination of vadose and saturated zone. There are many sites with TCE contaminated groundwater (Westrick et al., 1984). The cost of TCE remediation has been estimated to be billions of dollars, and the clean up time required was estimated to be decades.

Remediation methods, such as pump-and treat, have been used for remediating groundwater contaminated with chlorinated compounds, including TCE (Symon, 1981). However, with a TCE drinking water standard of 5 ppb, pump-and treat remediation is an inefficient and expensive method for removing CAH pollutants from groundwater. The pumping of groundwater might also result in the transfer of the contaminants to the surface environment. A large volume of contaminated groundwater must also be extracted. The application and operation of pump-and treat is therefore expensive and

time consuming. It may require a time scale of decades to clean up a TCE contaminated aquifer to the drinking water standard (Mackay and Cherry, 1989).

Many previous researchers have indicated that in situ bioremediation has good potential to clean up contaminants without bringing groundwater to the surface. This technology may be capable to minimizing remediation costs and may reduce the time required for restoring contaminated aquifers. The contaminants are also completely degraded, and the subsurface can be used as bioreactor to eliminate above ground treatment (Semprini et al., 1991). The field experiments have demonstrated TCE cometabolism under aerobic (Semprini et al.,1990; Broholm et al., 1991) and under anaerobic conditions (Semprini et al., 1995). Aerobic cometabolism and anaerobic reduction now are considered important processes for the bioremediation of TCE and other chlorinated solvents.

Aerobic TCE Cometabolism

In situ bioremediation using anaerobic and aerobic processes are innovative technologies for cleaning up contaminated aquifers. Field demonstration studies have shown that under natural conditions (intrinsic), TCE can be anaerobically degraded to dichloroethylene, vinyl chloride, and ethylene (Major et al.,1991; Semprini et al., 1995; Beeman et al., 1994). However the anaerobic transformation of TCE requires long periods of time and a potential product is vinyl chloride, which is a known carcinogen. Complete degradation of vinyl chloride to ethylene and carbon dioxide under anaerobic conditions has been observed in only a few studies (Vogel and McCarty, 1985; Freeman and Gossett, 1989). However, under methanogenic (Freeman et al., 1989 and DiStefano et al., 1991) and hydrogen-utilizing conditions (DiStefano et al., 1992; Zinder et al., 1995), TCE has been completely transformed to ethylene in the laboratory studies.

Much research however has indicated that many CAHs, including TCE, can be aerobically degraded by a biological process known as cometabolism. Dalton and Stirling, 1982, defined cometabolism as "the transformation of a non-growth substrate in the

obligate presence of a growth substrate or another transformable compound." It was found that TCE can not support microbial growth. Therefore, cometabolic transformation of TCE requires other compounds (as a primary substrate) to be present to serve as the energy source for microbial growth.

Previous studies have demonstrated that many chlorinated organics, including TCE, can be cometabolically degraded into nontoxic end products by many types of aerobic microorganisms. Aerobic microorganisms expressing oxygenase enzymes required for utilizing growth substrates such as methane (Wilson et al., 1985), phenol (Nelson et al., 1987), toluene (Nelson et al., 1987; Wackett et al., 1988), ethylene (Henry et al., 1989), ammonia (Arciero et al., 1989), propane (Wackett et al., 1989), propylene (Ensign et al., 1992) have been shown to be responsible for the cometabolism. The oxidation of TCE does not provide the microorganisms with any benefit as a source of energy or nutrition. TCE is transformed by the microorganisms fortuitously. There are no microorganisms discovered to date that use TCE as a growth substrate.

Inhibitory effects of TCE cometabolism have been observed in previous studies. The transformation of TCE requires expression of an oxygenase enzymes and reducing energy source (NADH) to catalyze TCE oxidation. The depletion of enzymes and NADH significantly reduces TCE transformation ability (Chang and Alvarez-Cohen, 1995). In addition, the competition of TCE for the active sites with substrate, results in decreases in the TCE transformation rates. Direct toxicity of TCE at high concentrations and TCE transformation product toxicity also inhibits TCE transformation (Alvarez-Cohen and McCarty, 1991; Odenhuis et al., 1991).

TCE Cometabolism by Methane, Propane, and Butane-Utilizing Microorganisms

TCE oxidation by methanotroph bacteria (methane utilizing bacteria)

Methane-utilizing bacteria have been extensively studied for 25 years. Methane-utilizing microorganisms are widespread in transition zone between aerobic and anaerobic zones in subsurface where methane and oxygen are present (Hanson, 1980). These microorganisms are commonly called methanotrophs. The pathway for methane degradation shown in Figure 2.1 was first documented by Dalton and Stirling, 1982. In the first step, the enzyme methane monooxygenase (MMO) oxidizes methane to methanol. The methane oxidation reaction requires $NADH_2$ as an electron donor, which is generated

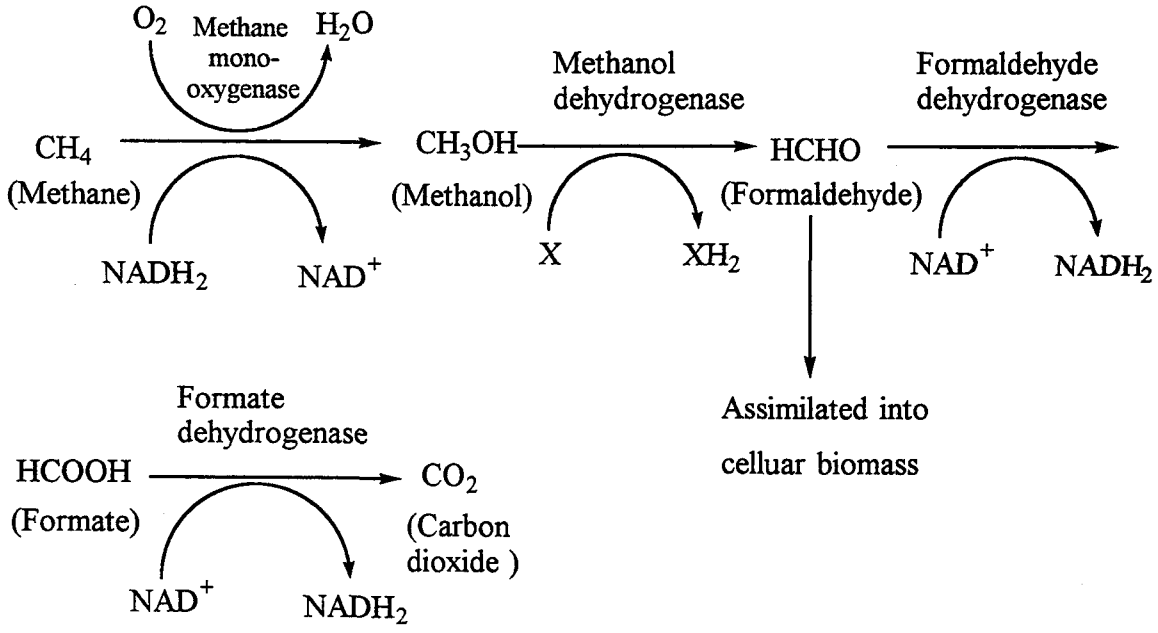

Figure 2.1 The pathway for methane oxidation by methanotrophic bacteria (Dalton and Stirling, 1982).

in the last two steps of methane reactions. Methanol is continuously transformed to formaldehyde, which is either metabolized into bacteria biomass or further oxidized to formate and carbon dioxide. The oxidation of formaldehyde to formate and the oxidation of formate to carbon dioxide also generates $NADH_2$, required for the initial oxidation of methane.

Methane-utilizing bacteria that cometabolize TCE were first discovered by Wilson and Wilson (1985). Their observations suggested that the enzymes that epoxidate ethylene transform TCE. In this study, TCE degradation was first observed in sandy soil column fed natural gas. After Wilson 's discovery, extensive research on TCE cometabolism by methanotrophic microorganisms was conducted (Fogel et al.,1986; Strand and Shippert, 1986; Hanson et al., 1988 and 1989). Fogel et al., 1986 showed that five chlorinated compounds including trichloroethylene (TCE), vinyl chloride, and cis- and trans-1,2-dichloroethylene, but not tetrachloroethylene, could be oxidized by methane-utilizing bacteria. The study also speculated that TCE epoxide may be a product of TCE oxidation by MMO. The study indicated that MMO is highly nonspecific enzyme, because most methanotrophs are capable of utilizing methane and other C_1 compounds as sole sources carbon and energy. Subsequent studies have demonstrated that faculative methanotrophs can also grown on more complex compounds, such as yeast extract, glucose, acetate, and methanol. These compounds also supported degradation of TCE (Filermans et al., 1988).

The pathways for aerobic cometabolic degradation of TCE were investigated by Little et al. (1988) (Figure 2.2). TCE was oxidized by MMO to TCE epoxide or trichloroacetaldehyde, which was hydrolyzed spontaneously to form dichloroacetic acid, glyoxylic acid, or one-carbon compounds such as carbon monoxide and formate (Little et al., 1988; Oldenhuis et al., 1990; Fox et al., 1990). The trichloroacetaldehyde is merely oxidized to trichloroacetate and partially transformed to trichloroethanol (Newman et al., 1991) Some carbon from TCE is incorporated into cells and converted to CO_2 (Fogel et al., 1986; Little et al., 1988).

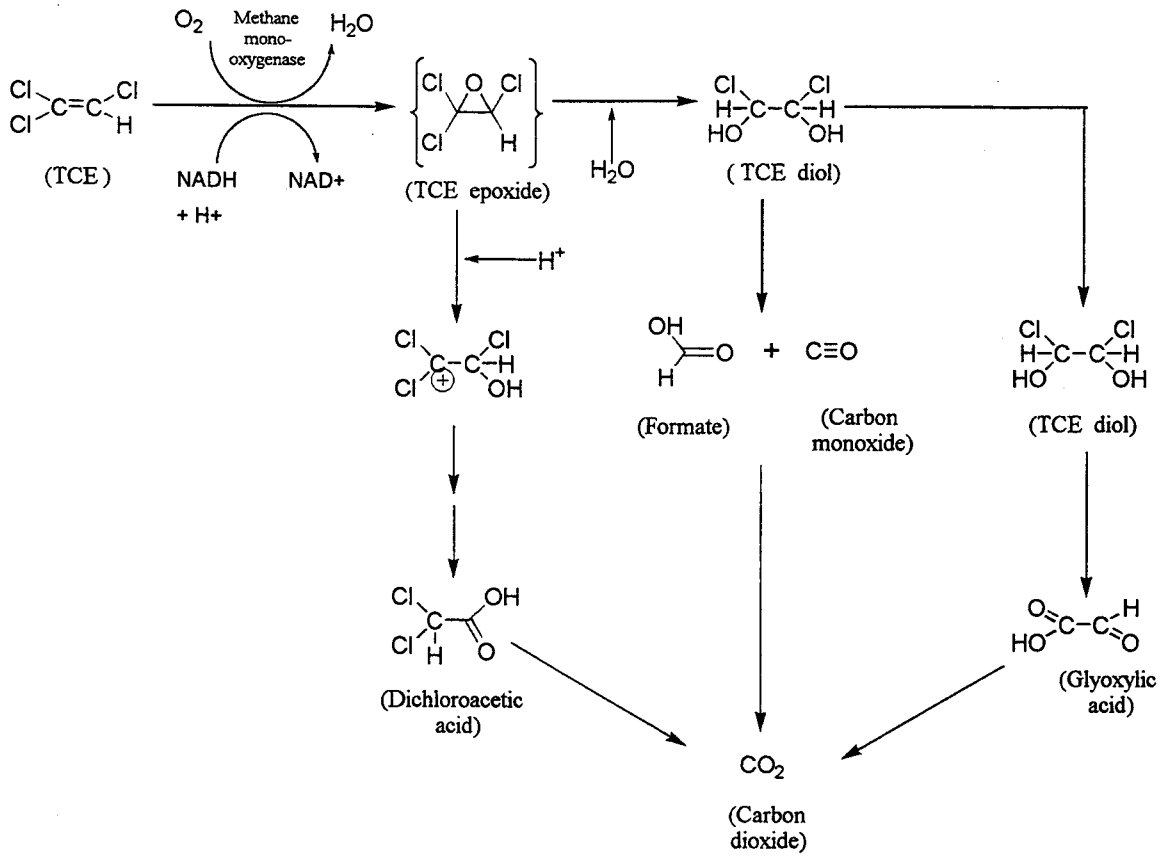

Figure 2.2 The mechanism of TCE transformation by methanotrophic bacteria (Little et al., 1988).

TCE transformation by-products have been found to inhibit TCE degradation (Alvarez-Cohen and McCarty, 1991; Oldenhuis et al., 1991; Henry and Grbic-Galic, 1991). However, hydrolysis products of the TCE epoxide and trichloroacetaldehyde did not inactivated MMO (Fox et al., 1990). Other resting cell studies showed that the depletion of electron donor for NADH regeneration and the product toxicity of TCE cometabolism are the factors affecting of the TCE transformation rate and transformation capacity. Methane, oxygen and TCE utilization were greatly decreased after TCE transformation occurred. Similar observations were documented in studies of chloroform (CF) and TCE degradation by methane-utilizing bacteria (Alvarez-Cohen et al., 1991). The study speculated that phosgene and TCE epoxide were responsible for the observed

product toxicity of CF and TCE, respectively. Both compounds have been speculated as an intermediate by-products which result in the decrease of transformation capacity. Studies on competitive inhibition also revealed that high methane concentrations have negative effect on TCE transformation (Broholm et al., 1992; Odenhuis et al.,1991; Semprini et al., 1991). High methane concentrations significantly compete with TCE for active site on MMO enzyme, resulting in a reduction of TCE transformation rates.

Methanotrophic biostimulation field experiments and laboratory column studies have demonstrated that the cometabolic biostransformation of chlorinated alkenes resulted from the biostimulation of indigenous methanotrophic bacteria (Semprini et al., 1990; Broholm et al., 1991). The studies of in situ bioremediation indicated that degradation of TCE and others CAHs in soil and groundwater is complex. Developing effective models for TCE and CAHs degradation is difficult. However, model simulations of in situ bioremediation of CAHs at Moffett Field site, including competitive inhibition between chlorinated ethylene and methane, successfully fitted the field experimental data (Semprini and McCarty,1992).

Mixed culture and pure cultures of methanotrophic bacteria have been studied to enhance cometabolic degradation of TCE (Broholm et al., 1993; Odenhuis et al., 1989 and 1991). Methanotrophs isolated from a TCE-contaminated aquifer, type II *Methylosinus Trichosporium* OB3b, expressing soluble methane monooxygenase, oxidized TCE at high rate under copper limited growth conditions. Long term TCE transformation activity was observed. The highest transfromation rate of TCE (200 nmole min^{-1} mg of $cell^{-1}$) by the OB3b culture was documented by Oldenhuis et al., 1991. The rate of TCE transformation was comparatively as high as the rate of methane degradation.

In addition to TCE, *M. Trichosporium* OB3b also degraded dichloromethane, chloroform, dichloroethane, cis- and trans-DCE, and 1,2 dichloropropane. This pure culture could not oxidized carbon tetrachloride and perchloroethylene (Odenhuis et al., 1989 and 1991). Similar observations was revealed by batch and chemostat reactor grown on mixed cultures of methanotrophs and pure *M. Trichlosporium* OB3b (Chang and Alvarez-Cohen, 1996). Resting cell studies indicated that CAH product toxicity to methane-oxidizing cells decreases in proprotion to the number of chlorine substitution on

the molecules. Cometabolic degradation by these cultures decreased with increasing chlorine substitution.

Kho et al., 1993 discovered a soluble methane monooxygenase produced in Type I methanotrophs, *Methylomonas Methanica* strain 68-1. TCE degradation by whole-cell sMMO activity of 68-1 was comparatively higher than sMMO activity in *Methylosinus Trichosporium* OB3b grown under the same conditions when copper was present. The research also showed that MMO gene probes from OB3b are almost genetic homology to those found on 68-1.

Broholm et al., 1992, and 1993 studied the different abilities of eight mixed culture of methane-oxidizing bacteria to degrade TCE. The experiment was conducted in batch reactors, at 10 °C, a common temperature for soils and groundwaters. TCE degradation was observed on three of the eight mixed culture, when the cultures were grown on methane. These three cultures were also able to transform TCE during the oxidation of methanol. These experiments demonstrated the ability of mixed cultures to degrade TCE varied significantly, even though all cultures were grown under the same conditions. The study also included model simulations for TCE degradation and methane oxidation. The model based on competitive inhibition kinetics was applied to laboratory batch experiments. The proposed mathematical model describing the growth of bacteria and the transformation of TCE, and uptake of methane successfully simulated the experimental results.

Chang and Alvarez-Cohen, 1994 compared methane, propane, toluene, and phenol-utilizers ability to transform TCE with microbes enriched from a contaminated site. All cultures were grown under chemostat conditions. The transformation capacity (Te), which represented as the difference between initial and final substrate mass divided by cell mass, was evaluated. The resting cell of methane culture exhibited highest transformation capacities for TCE, CF, and 1,2 DCE. The transformation capacities (Tc : mg TCE / mg cells) of TCE degradation by resting cells of four oxygenase expressing cultures were as follows: methane, 0.05; phenol, 0.031; toluene, 0.0073; and propane, 0.0065. The transformation yields (Ty : mg TCE / mg growth substrate), which represents the mass of CAH degraded per mass of growth substrate utilized, were also observed as : methane,

0.017; propane, 0.0056; toluene, 0.0021; and phenol, 0.017. The propane and methane cultures were able to transform both saturated and unsaturated chlorinated hydrocarbons. The culture of phenol and toluene degrade TCE but not transform CF, 1,2 DCA, or saturated organics. All of culture tested were unable to transform PCE and CCl_4.

Transfomation kinetics of chlorinated ethenes, including TCE, by *Methylosinus Trichosporium* OB3b were reported by Van Hylckama Vlieg et al., 1996. The detection of unstable epoxides of chlorinated ethenes was observed by using on-line gas chromatography analysis of headspace of well-mixed incubation mixtures. The method was used to evaluate the kinetics of transformation of all chlorinated alkenes and the kinetic of corresponding epoxides by *Methylosinus Trichosporium* OB3b expressing pMMO or sMMO. The study found significant amounts of all epoxides, except 1,1-DCE epoxide, leaving the cell. The results of the study also showed that methane and acetylene inhibited the degradation of cis-1,2-DCE epoxide, suggesting that cis-1,2-DCE epoxide is transformed by sMMO.

TCE oxidation by propane-utilizing microorganisms

Previous work has documented that microorganisms are able to use propane as a growth substrate under aerobic conditions. Propane-oxidizers have been enriched from soil and water samples (Perry, 1979; Hou et al., 1983). These microorganisms are able to degrade a broad range of aliphatic hydrocarbons. The propane oxygenase enzyme is also nonspecific enough to metabolize and oxidize short-chain alkenes (Hou et al., 1983) and other aliphatic hydrocarbons. The first oxidation step is to insert O from O_2 into propane molecule to form 2-propanol, which is further oxidized to acetone (Perry J.J, 1980). However, other studies by Stephen and Dalton (1986) concluded that the initial propane oxidation takes place on the terminal carbon atom in propane molecule.

In contrast with the significant study of methane-utilizing bacteria, little work have been done on study CAH cometabolism by propane-utilizing bacteria. To date, no work has studied the application of these microorganism for bioremediation. Wackett et al.,1989 first demonstrated that propane monooxygenase (PMO) could catalyze the

oxidation of TCE. The propane monoxygenase enzyme from five different bacteria could oxidize TCE when propane was used as growth substrate. Inhibition between propane and TCE was observed on this study, indicating that propane monooxygenase enzyme was involved in TCE degradation. In addition to TCE, propane monooxygenase in *Mycobacterium Vaccae* JOB5 transformed vinyl chloride and cis-and trans-DCE, but not tetrachloroethylene.

The degradation and inhibition of TCE and 1,1,1 TCA has been studied with a propane-oxidizing enrichment culture (Keenan et al., 1993). This is the first work demonstrated the degradation kinetics and inhibition of TCE and 1,1,1 TCA cometabolism by propane. The results of the study showed that propane inhibited TCE degradation and the data was best described by noncompetitive inhibition model. TCE degradation followed Michaelis-Manten kinetics with V_{max} = 0.0016 mg TCE / mg TSS .hr and a K_s = 0.6 mg TCE/L. The results also demonstrated that TCA was strongly inhibited by propane and a competitive inhibition model did not fit the experimental data.

TCE oxidation by butane-utilizing microorganisms

Among the gaseous alkanes, most research has focused on microorganisms that grown on methane as growth substrate. However, the production rates of biomass from methane are limited by the transfer rate of methane into culture, because the solubility of methane is relatively low. Instead of using methane, normal alkanes such as propane and, n-butane have been used for the production of higher biomass. The transfer rate and solubility limits of those alkanes are higher than that of methane. The yields of biomass expected on propane and butane are approximately 1.4 times as high as that expected on methane (McLee et al., 1972).

The potential for using butane as a substrate or biomass production has lead to the isolation of several strains of microorganisms. One study demonstrated that the pure culture, *Pseudomonas Butanovora*, was able to grow on butane as sole of energy and carbon source (McLee et al., 1972; Takahashi et al.,1980). The strain was isolated from activated sludge and sampled from an oil refining plant. In addition to butane, these

microorganisms utilized C1-C8 alkanes, and C2-C4 alcohols and carboxylic acid, but did not oxidized C10 and more, C1 compounds, alkenes and sugars.

Toccalino et al., (1993) studied the effects of nitrogen on propane and butane biodegradation in an unsaturated sandy soil. The results of the study indicated that butane-utilizing bacteria overcame N limitations. The biological N_2 fixation was not observed on propane-amended soil. Propane-oxidizing microbes became N limited after about three months in propane-amended soil.

In a survey of chlorinated hydrocarbons (CAHs) degradation by butane-utilizing microorganisms, there are one report of butane-utilizing bacteria that are capable of chloroform degradation (Kim, 1996). Batch microcosms studies were performed to study the cometabolism of chloroform by butane-utilizing bacteria from the Hanford subsurface. The studies indicated that effective CF transformation was induced by butane-utilizers. Complete transformation of 1200 ug/L of CF in aqueous solution was observed. The study also concluded that oxygenase enzymes of butane utilizers are involved in CF transformation. This is the first demonstration of butane as a cometabolic substrate for CF transformation.

The Effect of Nutrients on Aerobic Cometabolism

Meeting nutrient requirements to maintain effective microbial growth in the subsurface environment is one of the major factors that influence TCE cometabolism potential for in situ bioremediation. Nitrogen, particularly nitrate, is one of the most essential nutrients that is often found to be limiting in subsurface aquifers. The addition of nitrogen sources such as nitrate or ammonia to the nitrogen-deficient subsurface may enhance TCE and CAHs degradation.

Methane-utilizers are categorized into two groups (Type I and II) based on their internal membranes. Both types can express the form of enzyme called particulate MMO (pMMO). Only Specific Type II methanotroph can express sMMO (soluble forms) that are responsible to transform a broad range of substrates, and are most active toward

transforms TCE and many other chlorinated hydrocarbons. sMMO can be produced under the copper limited conditions. In contrast, Type I organisms that express pMMO require copper for growth (Brusseau et al., 1990; Odenhuis et al., 1989; Tsien et al., 1989).

Prior studies revealed that Type II methanotrophs appear to be selected during nitrogen-limited conditions. Type I strains appear to be present in almost all methane-enrichment locations when other nutrient such as nitrogen are available (Graham et al.,1993). They showed that *M. Trichosporium* OB3b, Type II strains, can be selected under nitrogen limitations. Type I organism are unable to fix molecular nitrogen, while Type II methanotroph typically are a nitrogen fixers and prefer nitrate limited conditions and low oxygen tensions. Nitrogen-fixing methane-oxidizer (sMMO), grown at low oxygen tension, were also found to degrade TCE rapidly and exhibited high TCE transformation capacity (Chu and Alvarez-Cohen, 1996). These results suggest that reactor systems can be used to manipulate the species selection of methane utilizing bacteria for removing specific CAHs. On the other hand, most selection under in-situ conditions is less promising due to copper availability in the subsurface (Grahalm et al., 1993).

Methane-utilizers can produce poly-β-hydroxybutyrate (PHB) as an endogenous energy source under nitrate limited conditions for regeneration of NADH during TCE transformation (Asenjo, J. A. et al.,1986; Henrysson and McCarty, 1993). PHB is an intracellular reserve polyester polymer whose synthesis serves as an electron sink for microorganisms under growth limited condition such N, P, S, Mg , and/or O_2 limitations (Dawes and Senior, 1973). The intracellular reducing equivalents to improve and extend TCE transformation might be due to the catabolism of stored PHB content in methane utilizers (Henrysson and McCarty, 1993; Henry and Grbic-Galic, 1991). High accumulation of PHB content was also observed upon depletion of nitrate on the study of Type II, *Methylosinus Trichosporium* OB3b (Shan et al., 1996).

The effect of the nitrogen source on propane and butane-utilizers have also been observed in an unsaturated sandy soil (Toccalino et al., 1993). The results from this study showed that the microorganisms in soil amended with nitrate degraded butane and

propane more rapidly than nitrogen limited controls. With butane and propane amended soil became N-limited reducing the rate of propane and butane utilization. However, the butane-amended soil overcame their nitrogen limitation by fixing nitrogen.

Compared with methanotrophic bacteria cellular-lipids studies, no work has documented the influence of endogenous storage lipids (PHB) during cometabolism of TCE by propane and butane-utilizers. In addition, no work have been indicated the effect of nutrient addition on TCE transformation by butane and propane microorganisms. The synthesis of cellular lipids on these organisms and the effect of nutrient have also not yet clearly identified. Only one study has documented the large accumulation of cellular lipids of a Nocardia strain grown on propane and butane (Davis,1964). There are at least three lipid products which accumulate to the Nocardia cells such as glyceride, aliphatic waxes, or close structure to poly-β-hydroxybutyrate. All of these materials may be considered as carbon and energy reserve materials.

Reactor Systems for the Bioremediation of TCE and Other CAHs

Experimental bioreactors have been designed to study the cometabolic degradation of TCE and other CAHs. Many types of bioreactors using methanotrophic bacteria have been studied including a biofilm reactor with continuous purging of methane and oxygen (Strand et al., 1990), a two-state bioreactor (a dispersed-growth reactor followed by a plug flow reactor) (Alvarez-Cohen et al., 1991; McFarland et al., 1991), a sequential anaerobic-aerobic reactor system for mixed chlorinated solvents treatment (Long et al 1991), and a multi-state bioreactor with pure methane-utilizing bacteria (Tschantz et al., 1995).

Since competitive inhibition greatly affects the transformation of TCE and the utilization of the growth substrate (as methane), several researchers have constructed the reactors which avoid the competitive inhibition to increases TCE transformation efficiency. The dual or multiple reactors configurations described above have some

advantages over a single reactor. Here, the cells are grown in the absense of the CAHs. The cell are then mixed with the CAHs in the absence of the growth substrate, and the CAHs are transformed. This system works because the cells have a finite capacity to transform CAHs in the absence of the growth substrate. Competitive inhibition between the growth substrate and TCE are avoided because the growth and transformation process are separated.

There have been several reports of the effect of TCE loading on methanotrophic cultures in the reactors (Strand et al., 1990; Strand et al., 1991). A mixed methanotrophic culture was maintained with continuous supply of methane and nutrients with TCE loading increasing from 4 to 10 μg TCE/ (mg protein-d). The maximum sustainable ratio of TCE transformed to methane consumed was 6 μg TCE/mg methane. The study concluded that aerobic cometabolism of TCE by methanotrophs in a continuous TCE-fed system is an unstable process. The degradation of TCE can not be maintained due to product toxicity. Population shifts and changes in enzyme activity occurred with long-term exposure of a mixed culture to high levels of TCE.

The kinetics of methane utilization and the cometabolic degradation of TCE and 1,1,1 TCA by mixed methanotrophic culture was studied in close-system reactor (Strand et al., 1990). Continuous increases of TCE into the reactor showed that the activity of methanotrophic culture ceased at aqueous TCE concentrations of 7,770 μg/L. However, dissolved TCA concentrations less than 4,470 μg/L had no inhibiting effects on the mixed methane culture oxidation rates. The results also showed that for TCA, but not TCE, biodegradation rates were inhibited by the presence of dissolved methane at concentrations in excess of 0.25 mg/L. Lower TCE and TCA biodegradation rates were observed for mixtures of TCE and TCA.

Besides a bioreactor fed with single methane as growth substrate, there is one report using methane and propane as mixed substrates for a continuous-recycle packed and expanded bed bioreactor (Phelps et al., 1990). This study have shown substantial TCE degradation in a reactor fed both methane and propane. When methane alone was added to the reactors as an sole of energy source, TCE transformation decreased by about 60%, compared with the reactor in which both methane (5% by volume) and propane (3%

by volume) were fed. When propane alone was added to the reactor, the extent and rate of TCE degradation were similar to that observed when methane and propane were fed into the reactor. The increased efficiency of propane mixed with methane, or with propane alone indicated that the consortia use propane more efficiently as a growth substrate, or that propane does not complete as effectively as methane with TCE-transforming enzymes. The results of the study also indicated that propane-fed reactor more effectively transformed TCE than the methane-fed reactor.

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CHAPTER 3

Comparison of TCE Transformation in Long-term Batch Microcosms by Methane and Propane-Utilizing Microorganisms Stimulated from the McClellan Subsurface

Abstract

Subsurface microorganisms from McClellan Air Force Base were grown in batch aquifer microcosms on methane, propane, and butane as gaseous cometabolic substrates. The potential for aerobic TCE cometabolic transformation was determined under batch microcosms consisted of aquifer solids, site groundwater, and a air filled headspace. Stimulation of microbes on all of the substrates tested indicate a diverse microbial community exists in the McClellan subsurface. The lag periods before active substrate consumption was observed was about 2 weeks for methane, and three weeks for propane and butane. Methane and propane utilizers enriched from the site were active toward TCE cometabolism, while butane-utilizers showed no ability to transform TCE. TCE and methane or propane were successively transformed in the soil microcosms for up to 1 year. The mass of TCE added was gradually increased while the mass of growth substrate added was held essentially constant. TCE was transformed most rapid during the period of active methane consumption, and continued at a slower rate for about 1 week after methane was consumed. The propane culture remained active for up to four weeks after propane was consumed, and the rate followed first order kinetics. All of the microcosms remained active toward primary substrate utilization, while gradually increasing TCE concentrations. Different TCE transformation yields developed in replicate microcosms with time. Changes in TCE transformation ability resulted from changes in TCE concentration or TCE product toxicity. Both methane and propane-utilizers shows linear correlation between initial TCE transformation rates and primary substrate utilization rates. The correlation between the ratio of zero order TCE transformation rates to

primary substrate utilization rates are directly proportional to transformation yields. The ratio of the rate was about 50 % of the ultimate transformation yield for methane-utilizers, and 20 % of the transformation yield for propane-utilizers. The maximum observed TCE transformation yields were 0.068 g TCE/g methane and 0.048 g TCE/g propane.

Introduction

Trichloroethylene (TCE) is one of the most widespread contaminants in soil and groundwater, due to its use as a degreaser, dry cleaning solvent, and extraction agent in industry and government facilities including military installations (Imfante and Tsongas,1982; Westrick et al., 1984). Long term land disposal of TCE through 1970s occurred at many military installations including McClellan AFB. TCE concentrations greater than 0.5 mg TCE/L have been detected in groundwater at the McClellan AFB. Higher TCE concentrations, ranging from 10 to 20 mg TCE/L, have been observed in the down gradient of TCE source at north end of the contaminated site (ISCB Field Demonstration Report). Previous studies have demonstrated that in situ bioremediation has good potential to clean up contaminants without bringing groundwater to the surface. Here, the subsurface can be used as the bioreactor to eliminate above ground treatment. This technology may be capable of minimizing remediation costs and may reduce the time required for restoring contaminated aquifers (Semprini et al, 1990,1991).

Chlorinated aliphatic hydrocarbons (CAHs), including TCE, can be cometabolically degraded into nontoxic end products by many types of aerobic microorganisms (McCarty, 1992). Aerobic microorganisms expressing oxygenase enzymes required for utilizing the growth substrate, such as methane (Wilson et al., 1985), ammonia (Arciero et al.,1989), toluene (Nelson et al., 1987; Wackett et al., 1988), and phenol (Nelson et al., 1987) have been observed to be responsible for the CAHs cometabolism. Much research has focused on TCE cometabolism by methane-utilizing mixed and pure cultures (Alvarez-Cohen and McCarty, 1991; Fogel et al., 1986; Little et al 1988; Henson et al., 1988; Odenhuis et al., 1989; Tsien et al 1989). In contrast, there

are only a few investigations of CAH transformation by propane (Wackett et al., 1989; Keenan et al., 1993; Wilcox et al., 1995) and butane-utilizing bacteria (Kim, 1996). Transformation capacity (T_c) and transformation yields (T_y) of TCE transformation by resting cells of mixed methane, propane, toluene, and phenol utilizing culture were evaluated by Chang and Alvarez-Cohen, 1995, and 1996.

Aerobic TCE cometabolism by methane-utilizers has revealed inhibitory effects have limited TCE transformation. TCE oxidation requires the expression of an oxygenase enzyme and a source of reductant (e.g. NADH). The loss of enzyme activity and/or reductant supply significantly reduces the capacity to transform TCE (Chang and Alvarez-Cohen, 1995, and 1996). In addition, competitive inhibition between TCE and the substrate sites decreases the TCE transformation rate (Broholm et al., 1990; Odenhuis et al., 1991; Semprini et al 1991). Direct toxicity of TCE at high concentrations and TCE product toxicity also inhibits TCE transformation (Alvarez-Cohen and McCarty, 1991; Odenhuis et al., 1991).

Methane-utilizers can maintain TCE transformation ability for a limited time after methane was consumed by regenerating a source of reducing energy (NADH) using methanol and formate. Alternative energy sources such as formate and methanol, methane catabolic intermediates, temporally enhance TCE transformation (Odenhuis et al., 1989; Alvarez-Cohen and McCarty, 1991; Semprini et al.,1991; Janssen et al., 1988). Methane-utilizers can also use poly-β-hydroxybutyrate (PHB) as an endogenous energy source for regeneration of NADH during TCE transformation (Asenjo, J. A. et al.,1986; Henrysson and McCarty, 1993). PHB is an intracellular reserve polymer whose synthesis serves as an electron sink for microorganisms under growth-limited condition. The intracellular reducing equivalents improves the extended TCE transformation due to the catabolism of stored PHB (Henrysson and McCarty, 1993; Henry and Grbic-Galic, 1991).

Among the gaseous alkanes, most research has focused on microorganisms that grown on methane. However, biomass production rates from methane are limited by methane mass transfer, because the solubility of methane is relatively low. Therefore, normal alkanes with higher transfer rate and solubility limits such as propane and, n-butane have been used for higher biomass production. Biomass yields with propane and butane

are approximately 1.4 times as higher than with methane (McLee et al., 1972). Butane and propane are also cheap, readily available substrates. They are also non toxic, and are not regulated chemicals. Thus, regulator approval to add these compounds for enhanced in situ bioremediation is possibly.

Previous studies have indicated that long term in situ bioremediation might prove difficult due to microorganisms inability to retain their TCE degradation for extended periods. Transformation product toxicity is one of the potential reasons for loss in TCE transformation ability. Fields studies have evaluated TCE transformation potential for a periods of months (Robert et al., 1989; Semprini et al., 1990,1991,1995; McCarty, P.L, 1993; Hopkin et al., 1993). The column microcosm studies with indigenous microorganisms grown on phenol showed loss in TCE transformation ability after an extended time period of 280 days (Munakata et al., 1997). Reported here is TCE transformation in long term batch incubated microcosms by indigenous methane and propane-utilizing microorganisms stimulated from subsurface aquifer solids and groundwater from McClellan AFB. The ability to maintain long term TCE transformation was determined as TCE concentrations were gradually increased over a period of one year.

Material and Methods

Long term batch microcosm studies with aquifer solids

The studies were performed in batch microcosms constructed with aquifer material and groundwater from McClellan Air Force Base. Methane, propane and butane were used as growth substrates for each of the microcosm studies. The microcosms method was adapted from Broholm et. al., (1990) and Yi Mu and Scow, (1994). Duplicate microcosms were prepared for each of substrates tested. The microcosms were constructed using 125 ml amber serum bottles (Wheaton Class Co., Millville, NJ.). Aquifer material from the McClellan Air Force Base, Sacramento, CA, was wet sieved

with site groundwater under a laminar flow hood using a No. 8 sieve (2.38 mm opening) to remove large particles. The site groundwater was filtered (0.45 μm sterilized filter) before use. 15 ml of wet solids and 50 ml of filtered ground water were added to each batch microcosm, leaving a 60 ml air-filled headspace as a source of oxygen. The headspace permitted sampling of the gaseous substrate, oxygen, and TCE. The microcosms were crimp sealed with a Teflon™ butyl rubber cap (Kimble Co., IL), then inverted and incubated at room temperature on a shaker table at 100 rpm. The microcosms were maintained for a one year period, with periodic groundwater exchanges and readdition of growth substrates and TCE.

Control microcosms

Control microcosms included: 1) TCE control microcosms containing aquifer solids, groundwater, and TCE, but lacking the growth substrate; 2) sterilized microcosms prepared in the above manner, but exposed for 11 hours to a Cobolt 60 gramma irradiation source. After irradiation, filtered (0.45 μm) ground water was added under a laminar flow hood. The addition of 0.45 μm filtered groundwater potentially resulted in a source of microorganisms to these controls.

Groundwater amendment

The microcosms were maintained for a one year period, with periodic groundwater exchange and substrate readdition. The exchange of 25 ml of groundwater was performed in the batch microcosm prior to readditions of the growth substrate and TCE. The groundwater was amended with nitrate to 30 mg/L, since nitrogen was found to be limiting in the groundwater. The microcosms were centrifuged for 20 min at 1000 rpm to keep the microorganisms in the microcosms. The serum caps were then removed under a laminar flow hood. 25 ml of groundwater was replaced with new ground water and the

microcosms were then resealed. During each exchange, the mass of TCE added was increased while maintaining a constant mass addition of growth substrate.

Chemicals

Trichloroethylene (TCE; >99 %) were purchased from Aldrich Chemical Co. (Milwaukee, WIS.). Methane (>99.9 %) was purchased from Airco (Vancouver, WA.). Propane (10 % in nitrogen) and butane (10 % in nitrogen) were obtained from Aldrich Chemical Co. (Milwaukee, WIS.). A saturated TCE stock solution was prepared by adding 4 ml of pure TCE in a 125 ml capped serum bottle. The bottle was shaken and allowed to settle for at least 24 hours before use. The aqueous TCE concentrations were measured before use, using procedures described below. Methane, propane, and butane were transferred from gas containers to batch microcosms by direct volume additions with gas-tight syringes (Hamilton Co., Reno, NEV.).

Analytical methods

Methane, propane, butane, TCE, and oxygen were determined by headspace sampling of the microcosm. TCE concentrations were measured with a Hewlett Packard (Wilmington, DE) 5890 gas chromatograph equipped with a ^{63}Ni electron capture detector. Separation was obtained by a stainless steel packed column (1/8" x 8' ; 15 % squalene; CPAW-DMCS; 80/100; 5327PC, Alltech, Deerfield, IL) operated isothermally at 80 °C. An argon/methane (95/5) mixture at head pressure of 60 psi was used as the carrier gas. A 100 µl headspace sample was analyzed. The method was calibrated using external standards.

TCE aqueous concentrations were quantified by purge and trap, using a modified version of standard EPA Method 8010. A Hewlett Packard Purge and Trap model 7695 was used in conjunction with a Hewlett Packard 5890 gas chromatography equipped with

an Hall conductivity detector. A 100 µl sample was diluted in 5 ml of glass distilled water and then transferred into the trap of the purge and trap unit. Separation were obtained by a capillary column (HP-624; 19091v-433; 1.4 µm; 30 m length; Hewlett Packard, Wilmington, DE) operated with temperature gradient.

Headspace oxygen concentrations were determined on a Fisher Model 25V gas partitioner using nitrogen as a carrier gas. A 100 µl headspace sample were obtained with a Pressure-Lok gas tight syringe (Hamilton Co., Reno, NEV.). Separation were obtained by a stainless steel packed column (Supelco, INC., Bellefonte, PA). Oxygen gas standard was used to calibrate the method.

Methane, propane and butane concentrations were quantified by headspace analysis using a Hewlett Packard 5890 gas chromatography equipped with a flame ionization detector coupled with a 1.0 m - Hayesep Q stainless steel micropacked column (Restek Corporation, Bellefonte, PA). A 100 µl sample was used. The method was calibrated using external standards.

Nitrate concentrations were determined on a Dionex 4000I ion chromatograph. A Dionex Ionpac AS4A column, which utilizes a regenerant containing H_2SO_4 and Na_2CO_3 and $NaHCO_3$ eluent composition, was used for the chromatography separation. A 50 µl aqueous sample was analyzed. The method was calibrated using external standards.

Results

The evaluation of indigenous microbial activity in the McClellan subsurface cores

The batch microcosm studies were performed to determine indigenous microbial activity in the McClellan subsurface. Methane, propane, and butane microcosms were successively fed with growth substrate prior to TCE addition. Table 3.1 presents the mass histories of growth substrate addition and the lag time for substrate utilization during the initial incubation of the microcosms. The results demonstrate a diverse microbial community exists in the McClellan subsurface. Microbes could be stimulated on all of the

substrates tested. Stimulation of methane-utilizers in active microcosms were most rapid. The lag time in propane and butane utilizers were similar and about twice that observed for methane-utilizers. Complete removal of the substrate was observed within 2 to 3 days in active microcosm after substrate consumption was observed. Oxygen uptake in each batch microcosms correlated well with substrate utilization.

Table 3.1 The lag time for growth substrate utilization during the initial microcosm incubation.

Growth Substrate	Microcosms	Mass of Substrate Added (mg)	Lag time before Substrate Utilization (days)	Average Time Required for Substrate Utilization (days)
Methane	M#1	4.0	10	2
	M#2	4.5	10	2
	M#3 (Control)	4.5	55	5
Propane	P#1	4.0	24	2
	P#2	5.0	25	2
	P#3 (Control)	5.5	50	2
Butane	B#1	4.5	20	3
	B#2	5.5	20	3
	B#3 (Control)	5.7	45	15

The uptake of growth substrate was also eventually observed in the sterilized control microcosms. The lag time in the control microcosms was about 45 to 55 days for methane, propane, and butane. The longer lag times indicate indigenous microorganisms were stimulated in microcosms with much shorter lag periods than the sterilized control

microcosms. The presence of microorganisms in all sterilized control microcosms may have resulted from 0.45 μm filtered ground water used in the microcosms. Each of sterilized control microcosm (M#3, P#3, and B#3), was continuously fed with substrate prior to addition of TCE in order to compare with the active methane (M#1 and M#2), propane (P#1 and P#2), and butane (B#1 and B#2) microcosms. TCE addition was started after five additions of growth substrate. The transformation yields for TCE reported in Table 3.2 is the maximum observed upon increases in TCE concentration with successive readditions of TCE and the growth substrate. Methane and propane-utilizing microorganisms enriched from the site were active toward TCE cometabolism, while the butane-utilizers exhibited no ability for TCE transformation. The maximum transformation yields of 0.068 g TCE/ g methane and 0.048 g TCE/ g propane were observed on the methane-utilizers (M#3) and propane-utilizers (P#3), respectively. The stimulated controls showed the highest transformation yields.

Table 3.2 TCE transformations yields achieved with the different substrates.

Growth Substrate	Microcosms	Maximum Transformation Yields for TCE (g TCE/g substrate)
Methane	M#1	0.060
	M#2	0.048
	M#3 (Control)	0.068
Propane	P#1	0.028
	P#2	0.023
	P#3 (Control)	0.048
Butane	B#1	0
	B#2	0
	B#3 (Control)	0

Long-term batch microcosm studies and the effect of TCE concentration

Long term batch microcosm studies were performed to study the effect of increasing of TCE concentrations, the rates and extents of TCE transformations, and culture activities. The aqueous TCE concentration was gradually increased from 0 to 7000 μg TCE /L in methane and propane microcosms over a 1 year period, while maintaining a constant mass of substrate addition. The maximum sustainable ratio of TCE transformed to substrate consumed was determined. The long term studies also determined microorganisms ability to cope with increasing TCE concentrations.

Figures 3.1 and 3.2 shows the mass histories of methane, propane, and TCE over the period of 150 to 200 days after stimulation started. Methane, propane, TCE and nitrate were added while increasing the TCE concentration. The results showed increasing the aqueous TCE concentrations from 0 to 900 μg/L, did not greatly effect the rate of methane and propane utilization and extent of TCE transformation. The zero-order rates (μg TCE/day) of TCE transformation increased in all methane and propane microcosms as TCE concentration was increased over this range. Higher rates of substrate consumption were associated with higher rates of TCE transformation (Figure 3.1 and 3.2). In both the methane and propane microcosms, the rate and the extent of TCE transformations varied among microcosms and was correlate well with the rate of primary substrate utilization.

The maximum TCE transformation extent (greater than 95% TCE removal) was observed in methane-utilizing microcosm M#2. Microcosms M#1 and M#3 had a continued history of less TCE removal than microcosm M#2. Maximum TCE transformation was observed in propane microcosm P#3, with greater than 98 % TCE removal achieved. Microcosms, P#2 and P#1, had a continued history of less removal than microcosm P#3.

The methane-utilizers and propane-utilizers showed different ability to remain active toward TCE cometabolism after the primary substrate was consumed. During the period of methane consumption, the rate of TCE transformation was rapid. As methane was depleted, TCE transformation slowed significantly. However, TCE transformation

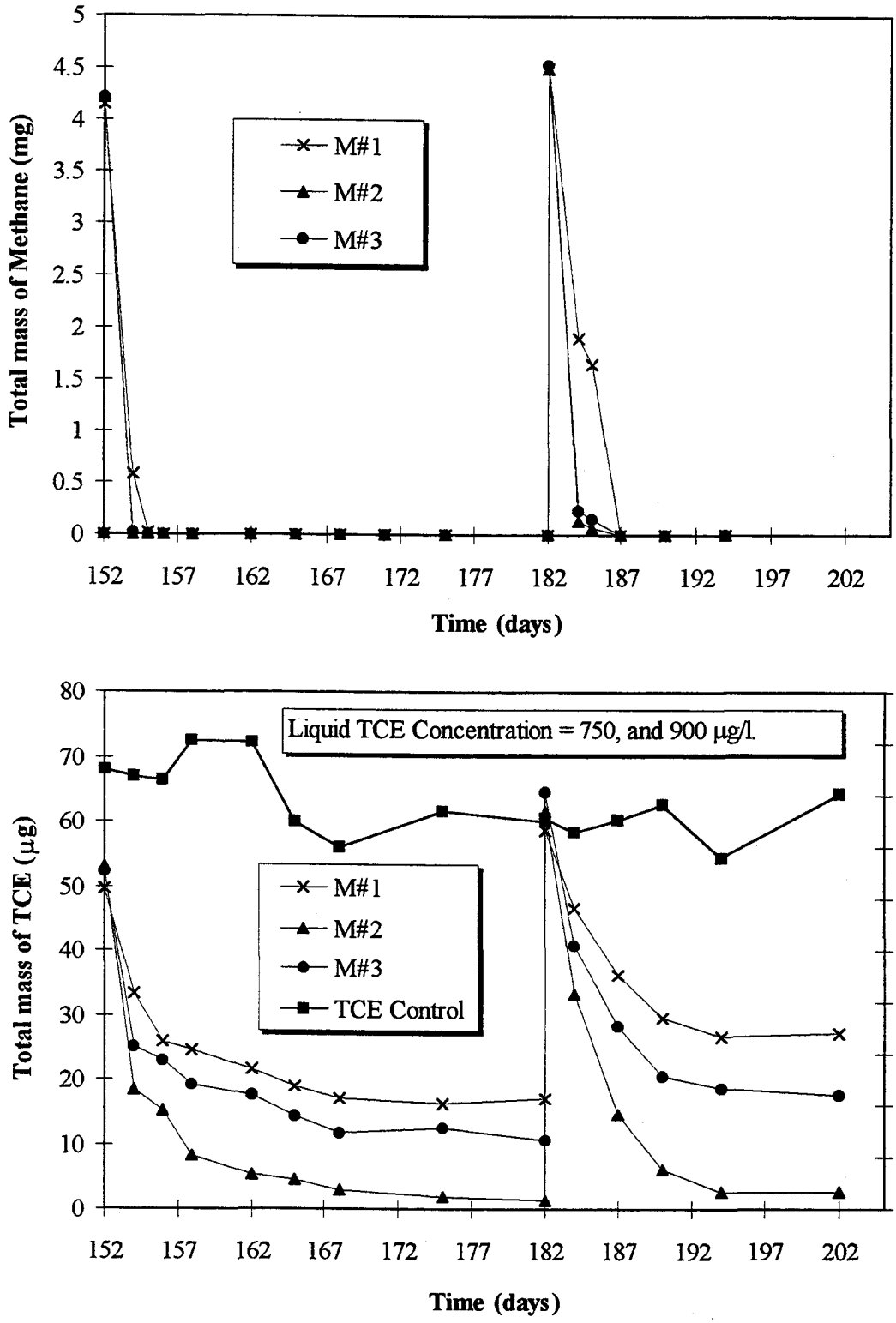


Figure 3.1 Mass histories of methane and TCE with increasing TCE concentrations.

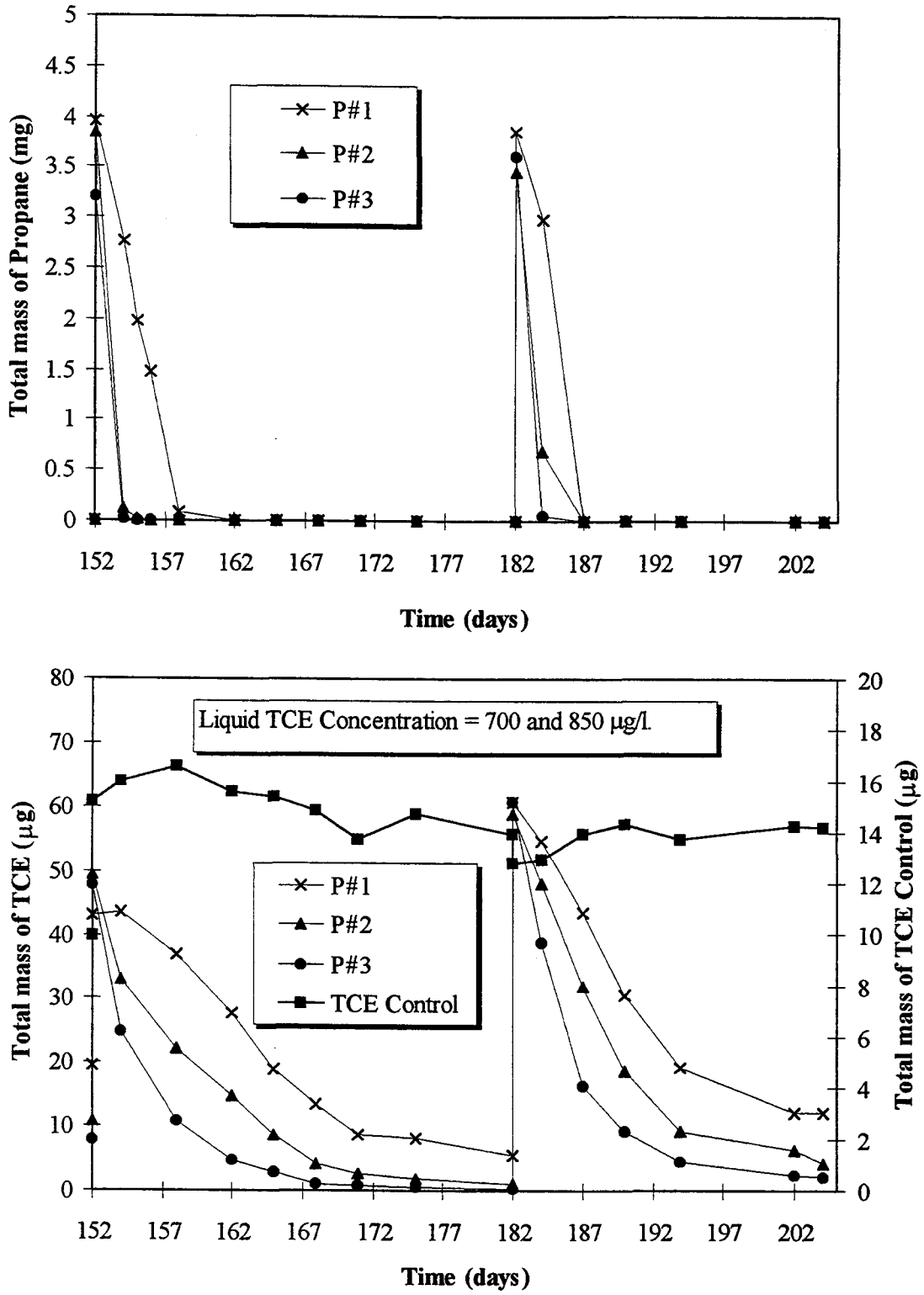


Figure 3.2 Mass histories of propane and TCE with increasing TCE concentrations.

continued for a period of about 10 days after methane was consumed. The propane culture remained active up to 30 days after propane was consumed. This prolonged activity was very reproducible in the successive readditions of propane and TCE.

Methane and propane microcosms (#1, 2, and 3) were maintained with constant supply of substrate as TCE loading was gradually increased from 1000 to 7000 μg TCE/L, until the maximum sustainable ratio of TCE transformed to substrate consumed were achieved. Figure 3.3 and 3.4 show the mass histories of methane, propane, and TCE, with gradually increases in TCE concentration. The increase in TCE concentration resulted in different TCE transformation activities. All the microcosm remained active toward primary substrate utilization over one year period. The microcosms showed differences in transformation yield developing with time. Again, the rate and the extent of TCE transformation varied among microcosms and was well correlated with the rate of primary substrate consumption.

The results presented in Figures 3.3 and 3.4 show that at elevated TCE concentrations, the rate of methane and propane utilization and TCE transformation was affected. When TCE concentration exceeded 5000 μg TCE / L, the rate of methane utilization and rate of TCE transformation declined in the methane microcosms. Upon exposure to elevated TCE concentration, TCE transformation abilities were loss in propane microcosms (P#1 and 2). Propane microcosms (P#1 and 2) showed less TCE transformation and lower rate of propane utilization when TCE concentration exceeded 2000 μg TCE / L. However, over the course of this study, the propane microcosm (P#3) remained active with prolonged activity for up to four weeks after propane was consumed.

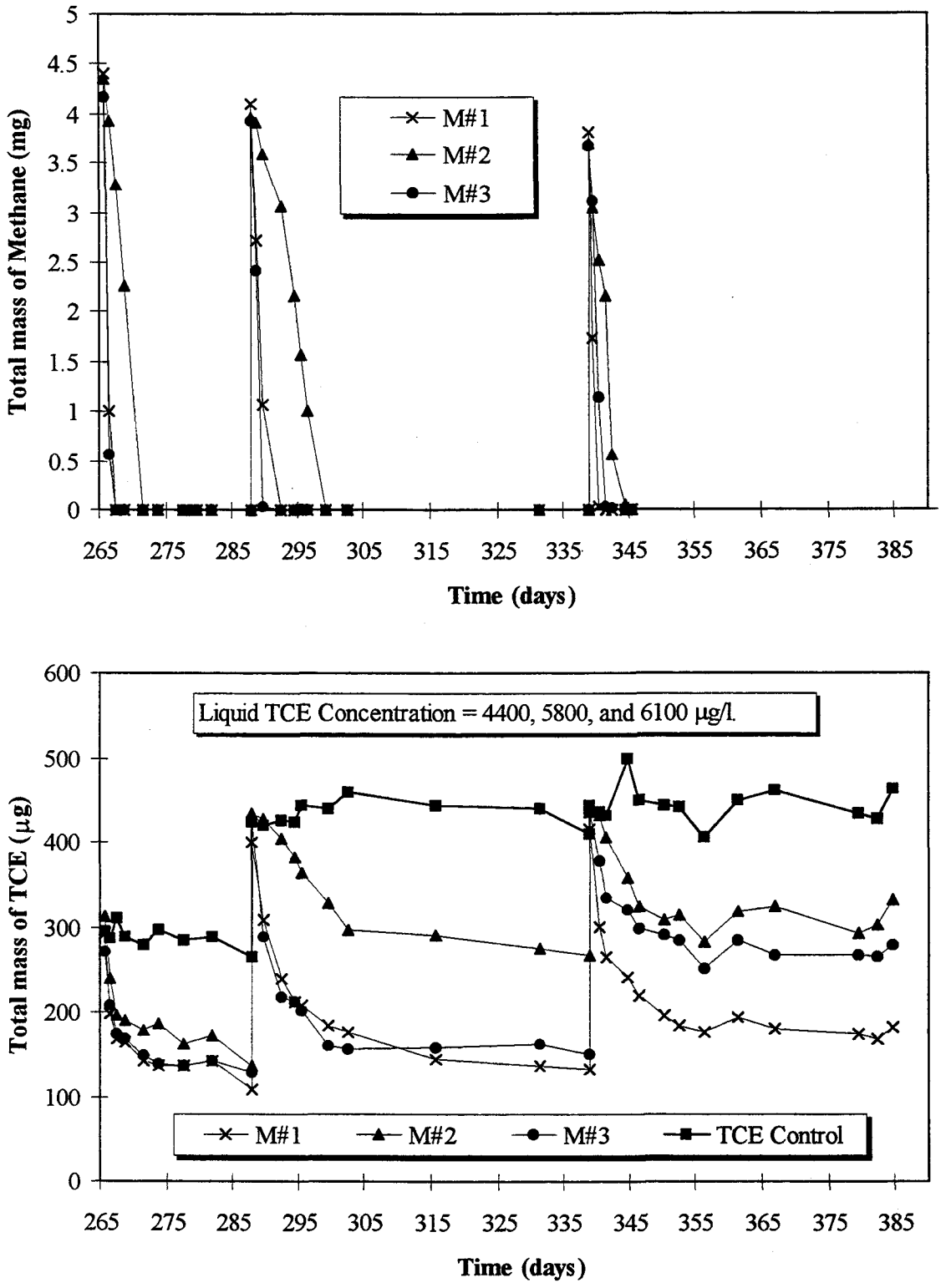


Figure 3.3 Mass histories of methane and TCE with increasing TCE concentrations.

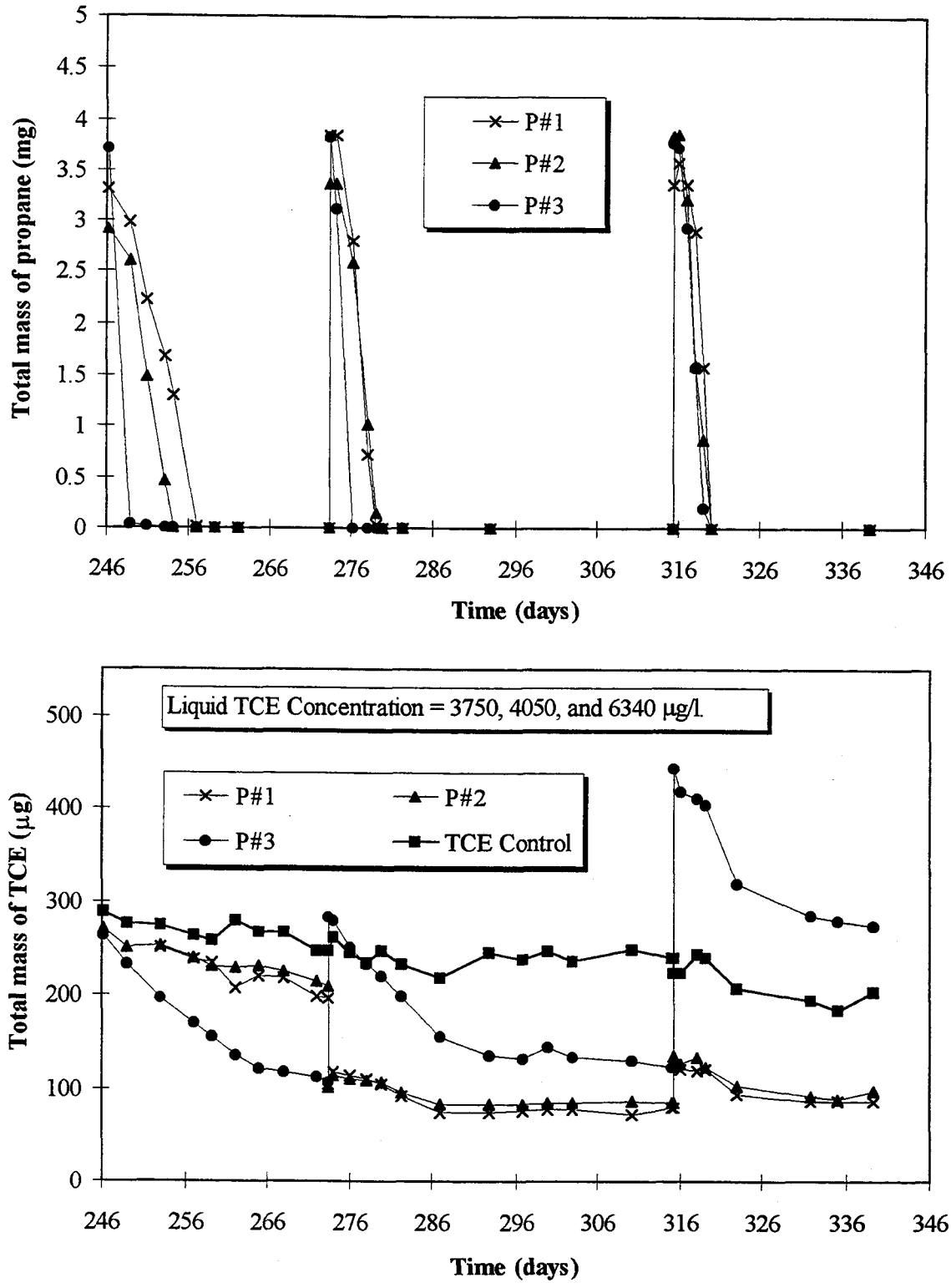


Figure 3.4 Mass histories of propane and TCE with increasing TCE concentrations.

The prolonged TCE activity of methane and propane utilizers after consumption of substrate

The methane and propane utilizers showed different TCE transformation abilities after primary substrate was consumed. Figure 3.5 and 3.6 illustrate TCE transformation abilities on propane utilizers compared to the methane-utilizers. The logarithm of TCE mass versus time is presented. The rates of TCE transformation are correlated with the rates of methane utilization. Higher rates of TCE transformation were observed when methane was being consumed. The rate of TCE transformation decreased soon after methane was depleted and followed a first-order rate of removal until transformation ceased.

The propane microcosms (Figure 3.6) follow first-order transformation kinetics for up to 20 days after propane was consumed. The rates of TCE transformation were correlated with the rates of propane utilization. The first-order TCE transformation and correlation with rates was very reproducible upon successive readditions to the microcosms.

Table 3.3 shows the average period of measurable TCE activity and the associated first order rate coefficient for the three methane and three propane utilizers after primary substrate was consumed. The values were estimated from individual estimates from successive additions of TCE and the growth substrate over the one year period of the study. All methane cultures show ability to transform TCE for a period of about 9 days with the averaged first order kinetic ranging from 0.04 day^{-1} to 0.08 day^{-1}.

The propane cultures remained active for longer periods (up to 20 days) and had higher first-order rate coefficient. Propane microcosms (P#3) remained active for the longest period of TCE transformation (about 23 days) after propane was consumed. It is interesting that the average first-order rate is similar for propane microcosm P#3 compared to P#1 and P#2, despite the longer activity. Microcosm P#3 was stimulated from the radiated control and this culture may have resulted from microbes in groundwater that passed through the 0.45 μm filter.

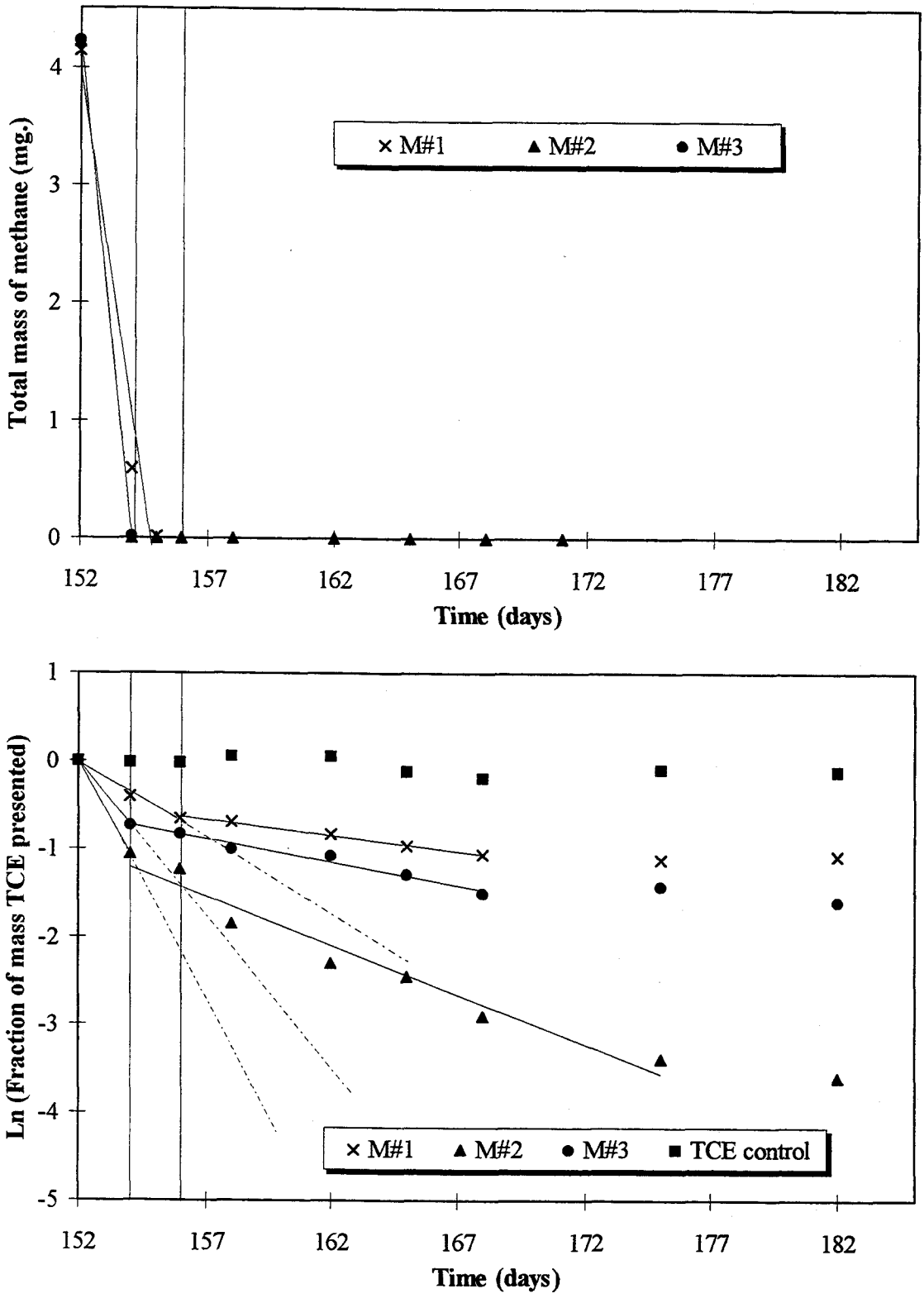


Figure 3.5 First order rate of TCE transformation and mass histories of methane consumption versus time.

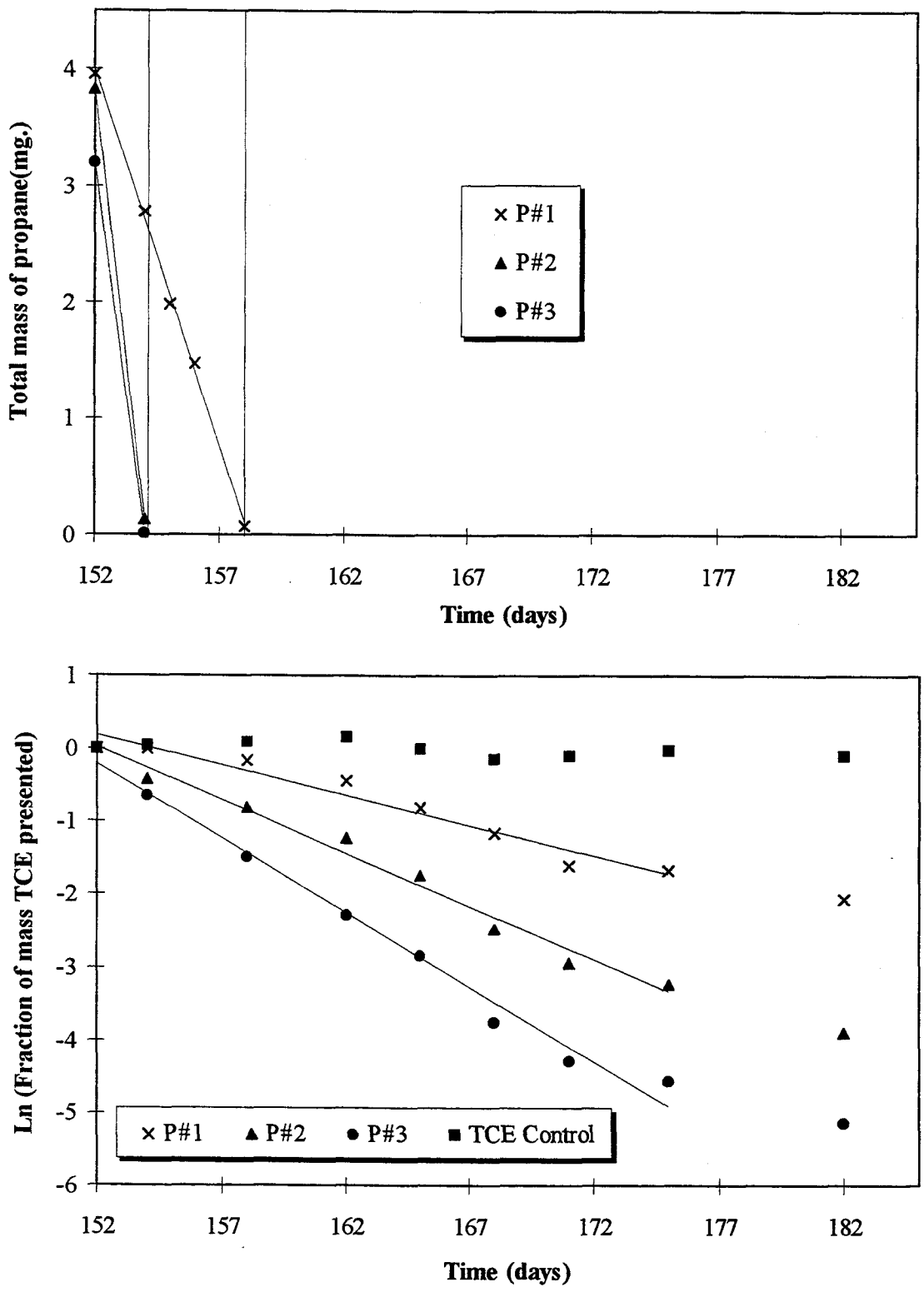


Figure 3.6 First order rate of TCE transformation and mass histories of propane consumption versus time.

Table 3.3 The prolonged transformation of TCE after the primary substrate was consumed.

Growth substrate	Mixed enrichment cultures	n*	Averaged period of TCE transformation after the primary substrates was consumed (days)	Averaged first order rate of TCE transformation (day $^{-1}$)
Methane	M#1	10	9.3 ± 4.0	0.036 ± 0.01*
	M#2	9	10.8 ± 5.8	0.083 ± 0.06
	M#3	11	9.7 ± 4.8	0.043 ± 0.02
Propane	P#1	6	15.5 ± 7.6	0.096 ± 0.07
	P#2	6	16.0 ± 9.8	0.102 ± 0.06
	P#3	10	23.5 ± 4.7	0.101 ± 0.07

n* = number of estimates

* = methane value is for after methane was consumed

Effect of TCE concentration on the zero order rate of TCE transformation and methane or propane utilization

Methane and propane utilization rates and initial TCE transformation rates were compared over a range of TCE concentrations studies. The zero order rates are presented for comparison purposes. Figure 3.7 shows the effect of aqueous TCE concentration on the rates of the methane and TCE transformation. In general at low TCE concentration, TCE transformation rates were associated with enhanced methane rates. TCE transformation and methane utilization rates decreased in all the microcosms when aqueous TCE concentration were increased above 5000 μg TCE/L. TCE concentration, TCE product toxicity, and competitive inhibition are potential processes causing the decreased rates.

Figure 3.8 shows the effect of aqueous TCE concentration on the initial rate of propane utilization and TCE transformation over the one year study period. Two of the

propane cultures (P#1 and P#2) did not maintain TCE transformation abilities. TCE transformation and propane utilization rates significantly decreased when aqueous TCE concentration exceeded 2000 μg TCE/L. Microcosm (P#3), however, remained active toward TCE transformation despite the lower uptake rates of propane at the high TCE concentrations. TCE rates remained constant after the TCE concentration reached to 2000 μg TCE / L.

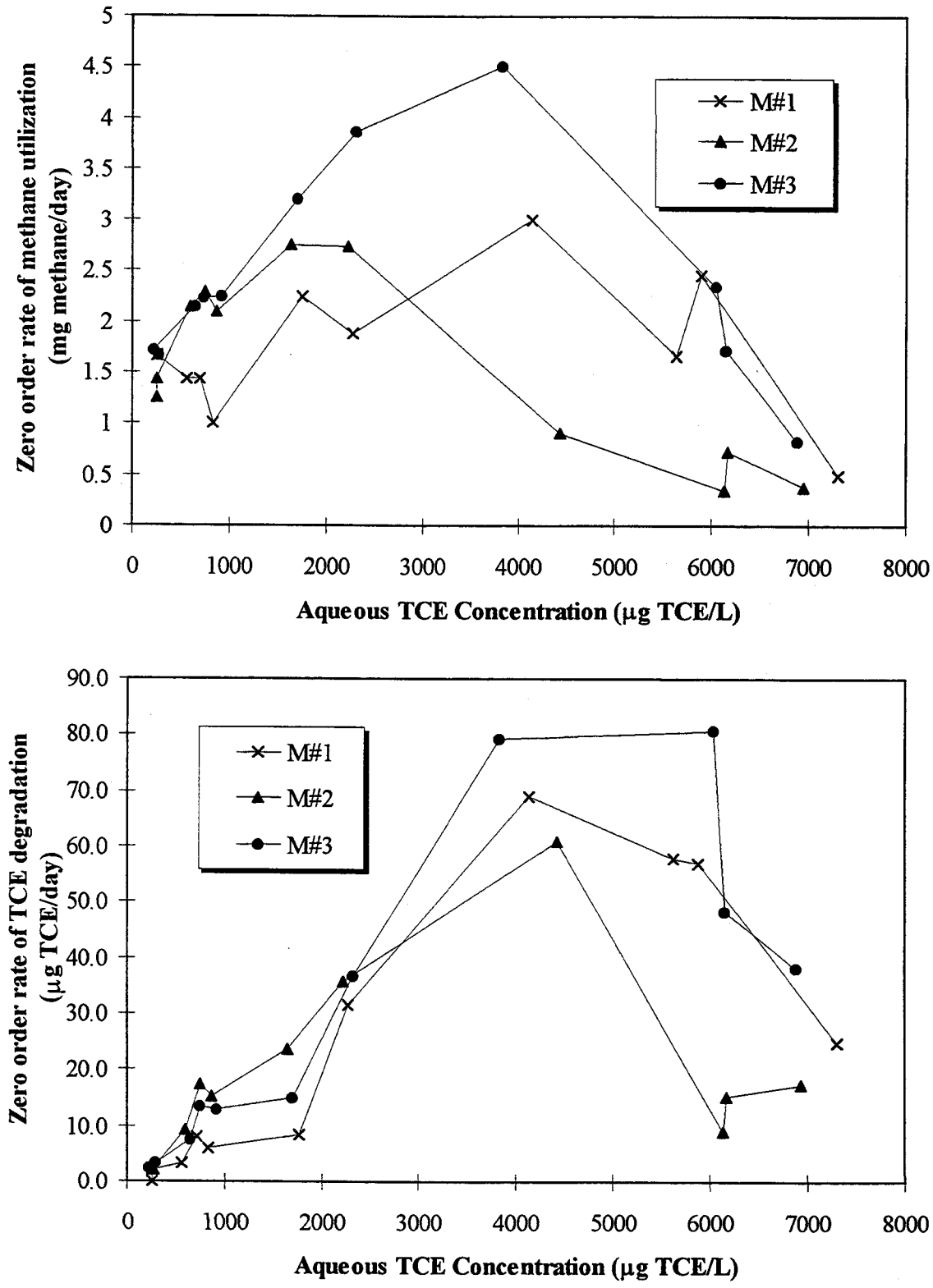


Figure 3.7 The initial rates of TCE transformation and methane degradation over a range of TCE concentrations during a one year period.

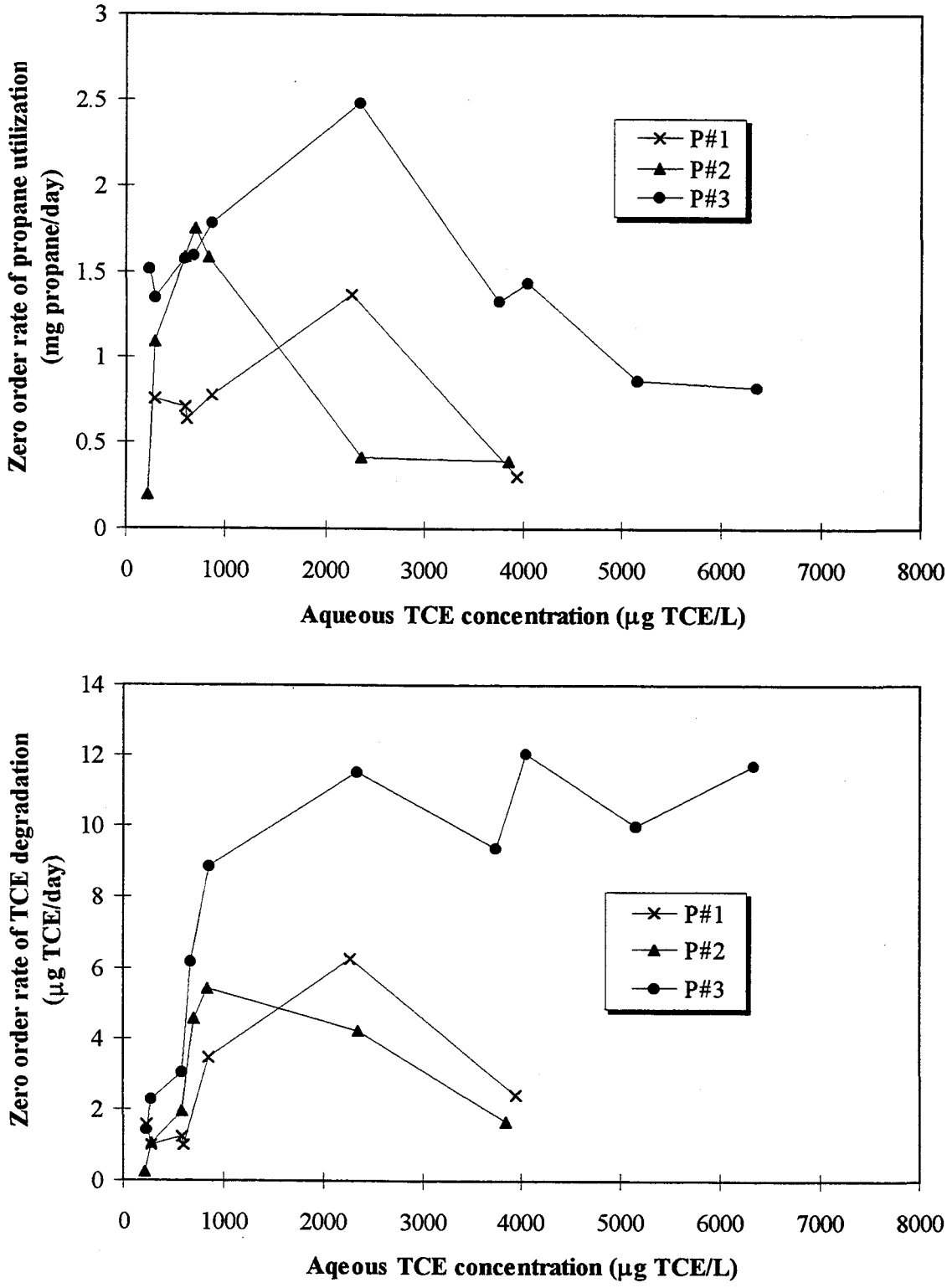


Figure 3.8 The initial rates of TCE transformation and propane degradation over a range of TCE concentrations during a one year period.

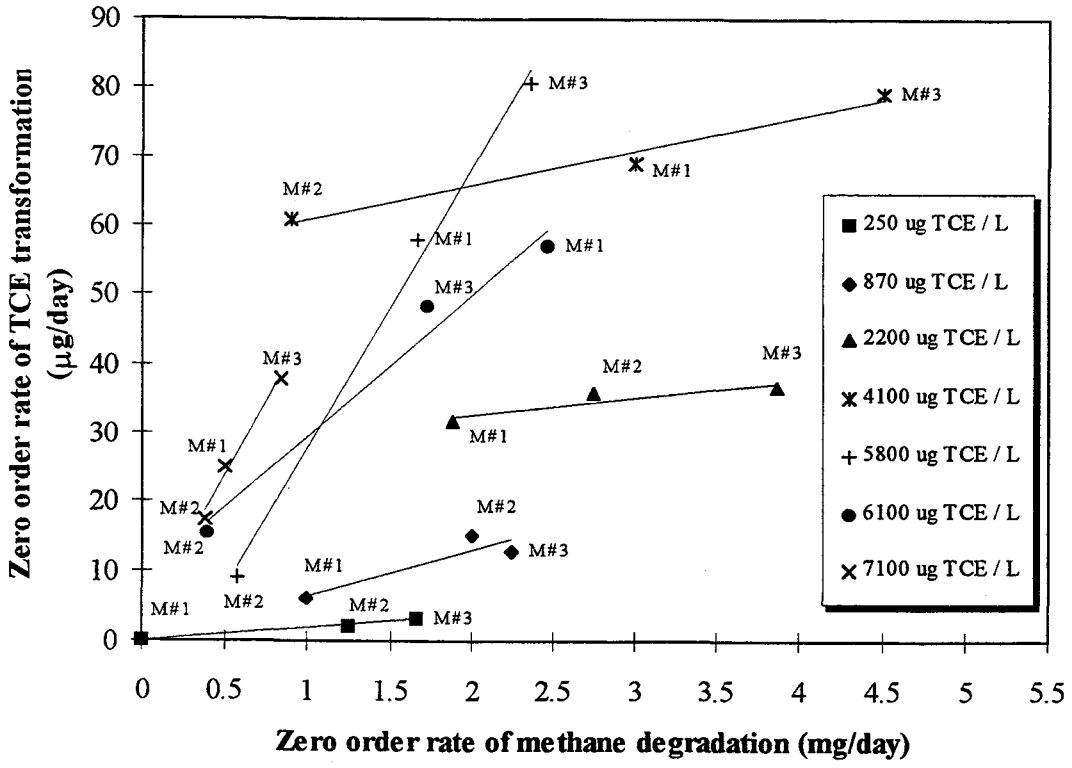


Figure 3.9 The effect of aqueous TCE concentration on the initial TCE transformation and methane utilization rates in three methane-utilizing microcosms. Solid lines are the linear regression best-fits at different aqueous TCE concentrations.

Figure 3.9 shows the correlation of initial zero order rates methane utilization and TCE transformation at different TCE concentration over a one year period. The rate and extent of TCE transformation varied among methane microcosms and was correlate well with the rate of methane consumption. A linear relationship between TCE transformation and methane utilization rates is shown. The slope of all linear regression fits, at different TCE concentration, are positive. All linear regression coefficients (r^2) were about 0.98. The results indicate that three methane microcosms show a strong correlation between initial TCE transformation rate and methane utilization rate at constant TCE concentration. The slope of linear line also increases when aqueous TCE concentrations increase.

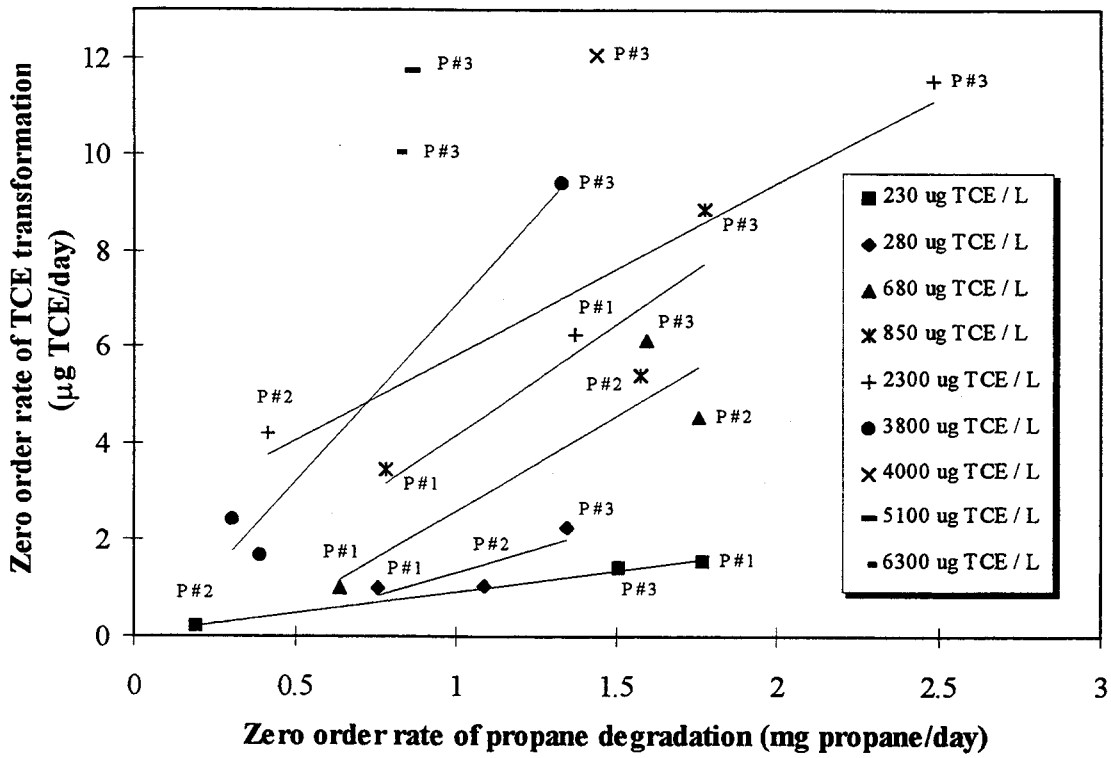


Figure 3.10 The effect of aqueous TCE concentration on the initial TCE transformation and propane utilization rates in three propane-utilizing microcosms. Solid lines are the linear regression best-fits at different aqueous TCE concentrations.

Figure 3.10 shows the correlation of initial zero order rates of TCE transformation and propane utilization in the three propane microcosms at different TCE concentrations. Similar results as methane were observed, but with lower linear regression coefficients (r^2) values of about 0.8 were achieved. All the slopes were positive, indicating that the initial rate of TCE transformation also dependents on the initial rate of propane utilization. The initial rate of TCE transformation by the propane-utilizers was much lower than the methane-utilizers, even though the initial rates of propane and methane utilization were similar.

An interesting observation is that even though propane has prolonged TCE activity, the initial rates of TCE transformation and propane utilization are correlated.

The results also showed loss of TCE transformation ability of two propane-utilizers (P#1 and P#2) at high TCE concentration, despite continued propane utilization. Only one propane-utilizers (P#3) remained active with a high rate of TCE transformation and propane utilization.

The ratio of initial TCE transformation rates to substrate consumption rates versus transformation yields by methane and propane utilizers

To determine what is the major cause of TCE transformation ability of methane and propane-utilizers, the ratio of TCE transformation rates to primary substrate utilization rates are plotted with the transformation yields. The correlation between the ratio of TCE transformation rate to methane utilization rate versus transformation yields are shown in Figure 3.11. A linear relationship ($r^2 = 0.89$) was observed when the competitive inhibition data at high TCE concentrations are omitted. The linear relationship indicates the ratio of TCE rates to methane rates are directly proportional to transformation yields. Since the slope is approximately 0.50, the ultimate transformation yields for the methane-utilizers is about a factor of two greater than that based on the ratio of the rates. The different may be attributed to slow TCE transformation activity after methane was consumed (Figure 3.5).

Figure 3.12 shows that the relationship between the ratio of initial zero-order TCE transformation rates to propane degradation rates versus transformation yields for the three propane microcosms. The linear correlation (r^2 was about 0.8) was achieved with competitive inhibition data omitted. An r^2 of 0.66 was observed when all data are included. The slope of the correlation ranged from 0.23 to 0.24. The transformation yields represented by the ratio of initial zero-order rates corresponded to only 20 to 30 % of the transformation yields. This difference results from the long term TCE activity after propane is consumed (Figure 3.6). It is interesting that the ratio of zero order rate between TCE and propane degradation are correlated with the transformation yields of propane-utilizers, despite the fact that most of the transformation occurs after propane is

utilized. This results from the correlation of rate of long term activity to the initial rate of propane utilization previously discussed.

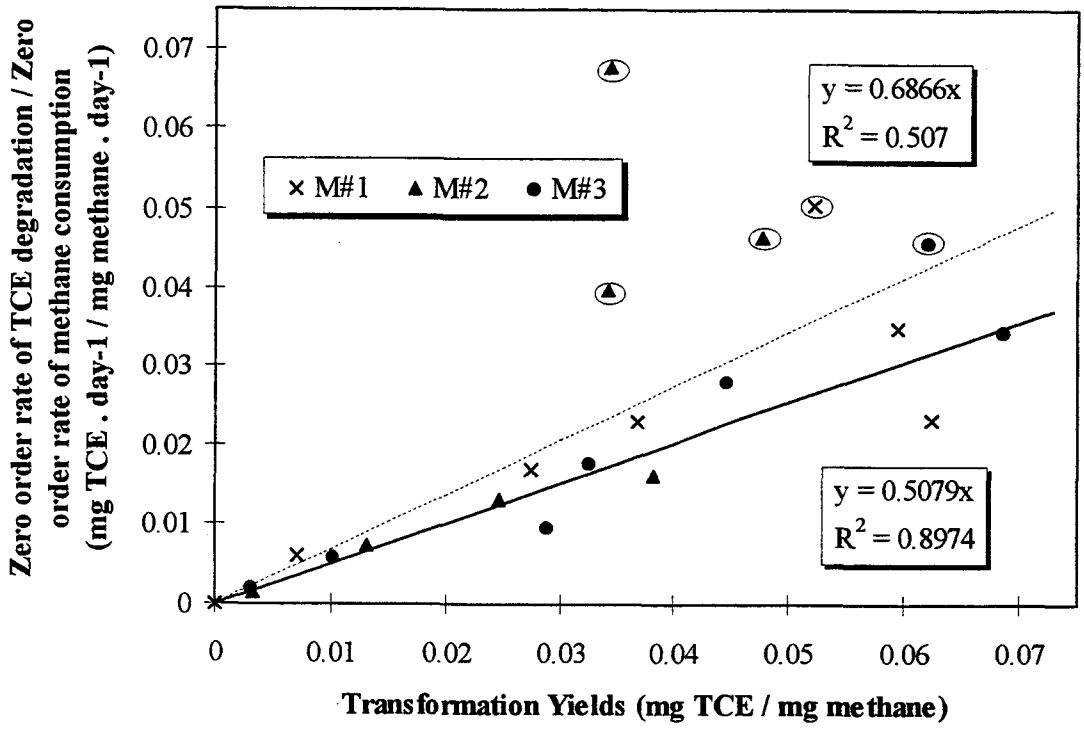


Figure 3.11 The ratio of initial TCE transformation rates to initial methane utilization rates versus transformation yields for methane microcosms {× M#1, Δ M#2, • M#3 }. {⊗ M#1, ▲ M#2, ⊙ M#3} are the results at high TCE concentrations when competitive inhibition between methane and TCE was observed. The dashed line represents the linear regression fit using all the data. Solid line is the linear line with competitive inhibition data included.

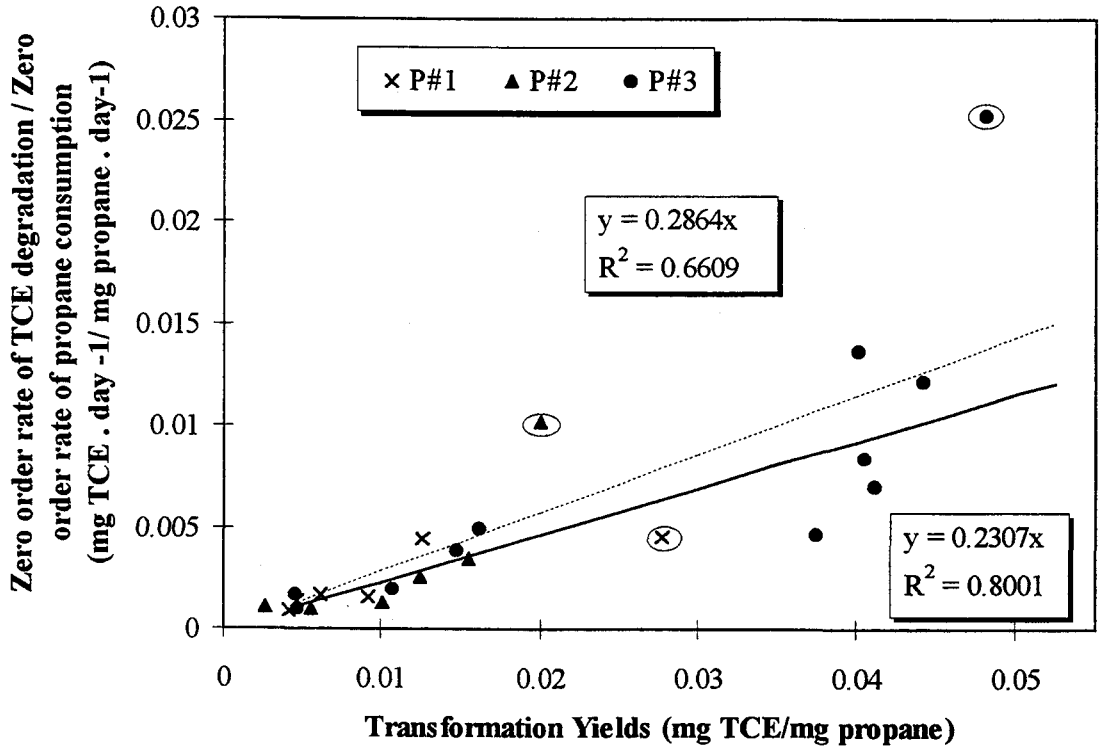


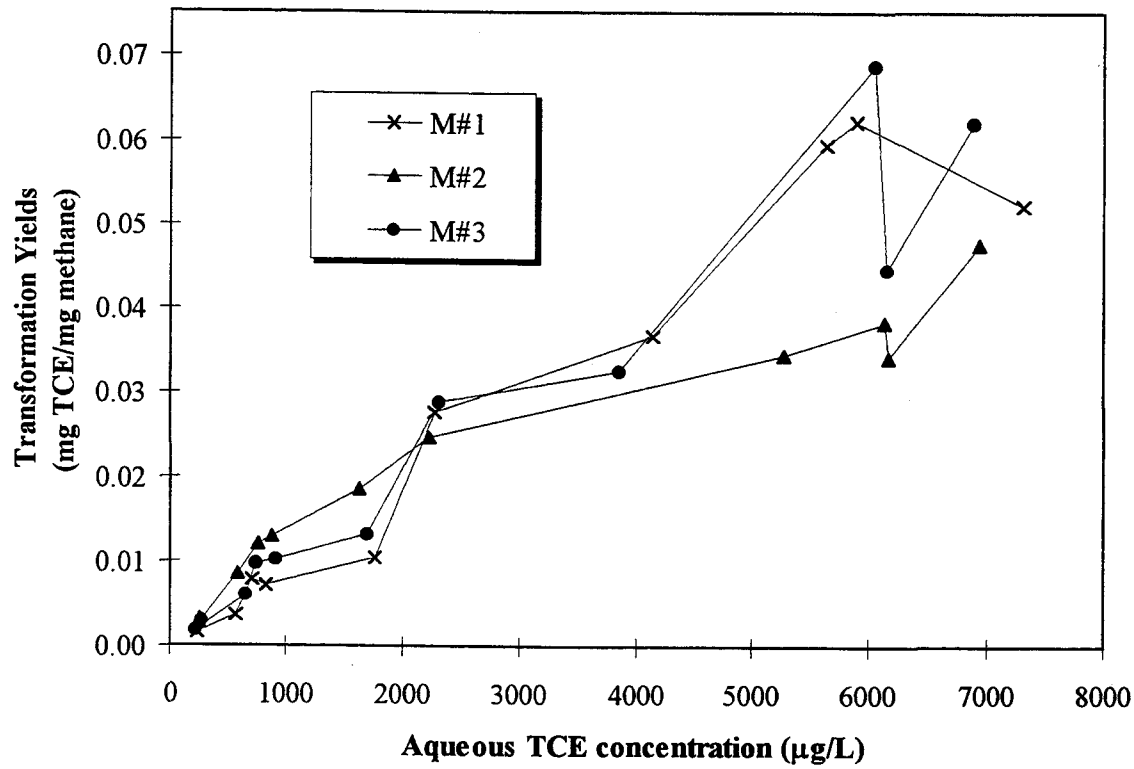
Figure 3.12 The ratio of initial TCE transformation rates to initial propane utilization rates versus transformation yields for propane microcosms {× P#1, Δ P#2, • P#3}. {⊗ P#1, ▲ P#2, ⊙ P#3} are the results at high TCE concentrations when competitive inhibition between propane and TCE was observed. The dashed line represents the linear regression fit when all data is included. The solid line omits data that clearly showed competitive inhibition.

Transformation yields over a range of TCE concentrations by methane and propane utilizers

Figure 3.13 shows the transformation yields for the three methane and propane microcosms with increasing TCE concentrations over a one year period. Initially, two of the methane microcosms (M#1 and 3) exhibited a lower TCE transformation yields than microcosm (M#2), but after exposure to high TCE concentrations, the situation was reversed. The results also show that transformation yields of all methane cultures

increased with time. The increase in transformation yield is likely due to an increasing of biomass with successive readditions of growth substrate, and the yields being limited by the TCE mass present at low TCE concentrations. The maximum transformation yield was 0.068 mg TCE/ mg methane observed on methane microcosm M#3. Methane-utilizers were able to maintain high sustainable yields at high TCE concentrations.

Two propane microcosms (P#1 and P#2) showed a decreased ability to cometabolize TCE with increasing TCE concentrations above 2000 μg/L. The results suggests that long term exposure to high TCE levels caused a loss in the cometabolic capabilities of the propane-utilizers. However, propane microcosms (P#3) remained active with increasing TCE concentration, yielding a maximum transformation yields of 0.048 mg TCE/mg propane. The gradual increase in TCE concentrations over a one year period resulted in different TCE transformation activities. The change in TCE transformation ability in propane and methane microcosms resulted from changes in TCE concentration, possible caused by TCE product toxicity. Population shifts in the microcosm may have also occurred, resulting in changes in TCE transformation ability. The results also indicate that methane-utilizers showed a better ability to cometabolize and cope with higher TCE concentrations than the propane-utilizers.



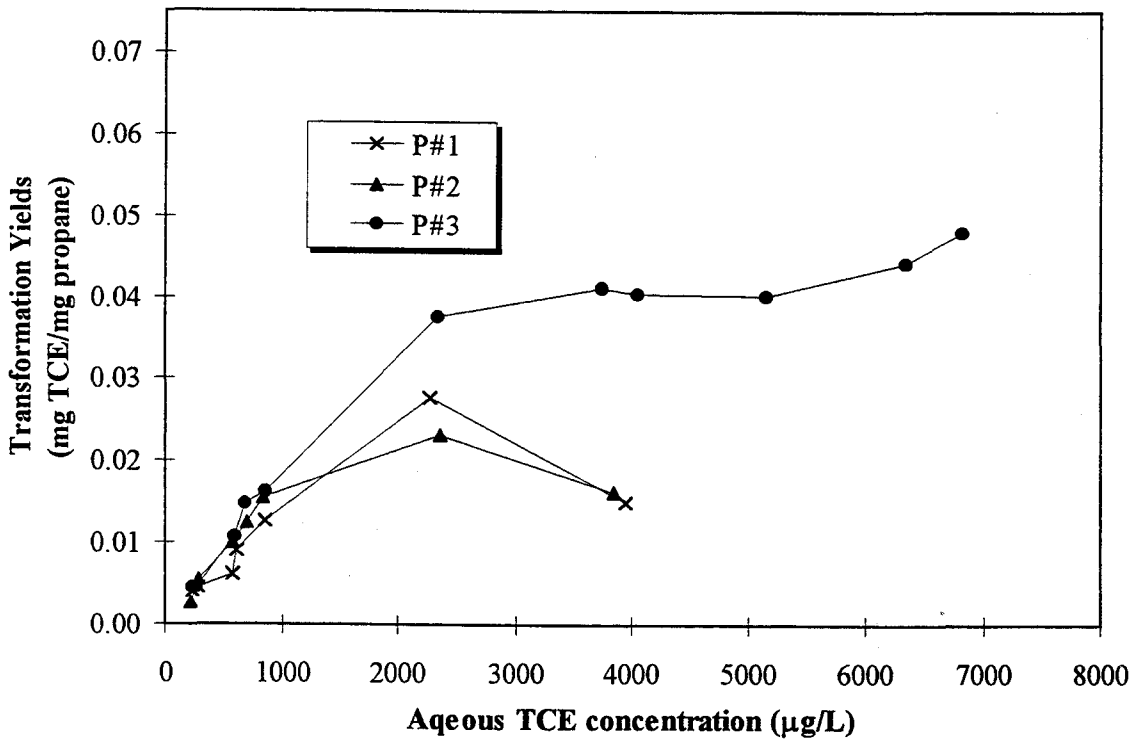


Figure 3.13 The ratio of mass of TCE transformed to mass of methane or propane consumed (T_y) as TCE concentrations were gradually increased over a one year period.

Discussion

The ability to stimulate microbes on methane, propane and butane was demonstrated in the microcosms constructed with McClellan subsurface solids and groundwater. The study showed that McClellan subsurface appears to have a diverse microbial community. Stimulation of propane and butane had twice the lag period of methane utilizers. The longer lag times indicate either lower numbers of these microorganisms in the McClellan's subsurface or slower growth rates. The results indicated that propane and butane are readily stimulated in the McClellan's subsurface and might be useful for bioremediation of CAHs.

Methane and propane-utilizers were able to transform TCE, while butane had no ability to TCE cometabolism. The reason why butane-utilizing microorganisms are unable to degrade TCE is not known. Previous studies indicated that butane-utilizing microorganisms, *Pseudomonas butanovora*, isolated from an oil refining plant, were unable to utilize normal alkenes compounds (McLee et al., 1972; Takahashi et al., 1980). However, in another study, butane-utilizing bacteria were able to cometabolize TCE and CF (Kim, Arp, and Semprini, 1997).

The results of the long term batch microcosms showed that the three methane and propane fed microcosms exhibited a different ability to transform TCE. The observed differences in TCE transformation may be due to different inherent abilities among the cultures to degrade TCE. However, the rate of substrate consumption among cultures correlated well with the rate and extent of TCE transformation. Higher rates of substrate consumption corresponded to higher rates of TCE transformation. Similar results were observed on study of eight mixed methane-utilizers (Broholm et al., 1993). They revealed that the ability of mixed cultures of methane-utilizing bacteria to degrade TCE varied significantly, even though the cultures were grown under the same conditions.

This study provides the observations of the prolonged TCE transformation activity by propane-utilizing microorganisms after propane was consumed. TCE transformation followed first-order kinetics for about 3 to 4 weeks after propane was consumed. In contrast, methane shows TCE transformation activity for about 1 weeks after methane

was consumed. The reasons for prolonged TCE activity of propane-utilizers is unknown. It may be that by-products of propane oxidation possibly serve as an alternative energy source to further transform TCE after metabolic degradation of propane is utilized. The propane-utilizers might also effectively storing energy reserves that are later used to drive the TCE transformation.

Previous studies showed that formate and methanol, a methane catabolic intermediates, could be use as an alternative energy source to increase and extend TCE transformation in pure and mixed methane utilizing cultures (Alvarez- Cohen and McCarty,1991; Brusseau, G.A. et al., 1990; Henry and Grbic-Galic,1991; Odenhuis, et al.,1991; Semprini et al., 1991). Energy reserves stored during growth limited conditions of methane-utilizers possibly are another possible reason for the prolonged TCE activity. Previous observations has been suggested that the energy reserves (as Poly-β-Hydroxybutyrate (PHB)) of methane-utilizers (Dawes and Senior, 1973) can provide intracellular reducing equivalents to extend and improve the TCE transformation (Henrysson and McCarty, 1993; Henry and Grbic-Galic,1991).

A similar explanation on long term TCE activity of the propane-utilizers can be postulated. Intermediate by-products associated with catabolism of propane may provide alternative energy source to drive TCE transformation. Previous studies have shown that propane grown microorganisms are able to degrade broad range of aliphatic hydrocarbons including short chain alkenes (Hou et al.,1983; McLee et al., 1972; Takahashi et al., 1980). Intermediate by products from metabolic degradation of propane have been proposed (Perry, 1979; Stephen and Dalton, 1986). Acetone is formed which is further oxidized from 2-propanol (Perry J.J 1980). Acetone was shown to be an excellent carbon and energy source for propane-microorganisms. The organisms isolated by enrichments acetone as substrate were normally able to oxidize propane (Lukins and Foster, 1963). However, it may also be that propane-utilizing microorganisms can also store more energy reserves than methane-utilizers. The synthesis of a copolymer of PHB by propane-utilizing microorganisms has been reported (Davis, 1964). These propane species also can synthesize aliphatic waxes during growth on propane as substrate. More research is needed to determine what is causing of the prolonged TCE activity of propane-utilizers.

It is also interesting that long term TCE activity was achieved with aquifer solids and the background groundwater chemistry. To our knowledge, this is the first observation of such activity under groundwater conditions.

Changes in the ability to transform TCE was observed with both methane and propane-utilizers as the TCE concentration was increased over a one year period. High TCE concentrations cause inhibition of methane and propane utilization. Lower rates of substrate utilization and TCE transformation were observed when TCE concentrations reached to 5 mg TCE/L and 2 mg TCE/L, respectively, in methane and propane microcosms. The propane cultures in microcosm (P#3) exhibited increased TCE transformation rates above 2 mg TCE/L, even though lower propane utilization rates was observed. Except of microcosm (P#3), all methane-utilizers showed a better ability to transform and cope with higher TCE concentration than the propane utilizers. The sterilized control microcosms (M#3 and P#3) may have been inoculated by the filtered ground water replacing into the control microcosms. Small size of microorganisms, that possibly passed through 0.45 μm filter, may have been stimulated. If so, microbes that better tolerate TCE product toxicity may have been selected. More experiments are required to confirm this possibility.

The ratio of zero order TCE transformation rates to substrate utilization rates were correlate with transformation yields for TCE on methane-utilizers and propane-utilizers. The ratio yielded about half of the observed transformation yields for methane-utilizers and 20 to 30 % of the transformation yields for propane-utilizers. The long-term TCE activity with propane-utilizers results in the lower percent. Even though the zero order TCE transformation rate for methane-utilizers are much higher than the propane-utilizers, the maximum transformation yields on methane and propane-utilizers are similar. This results from the long-term activity of the propane-utilizers.

Resting cell, mixed and pure cultures, studies have yielded the different transformation yields of TCE for given specific cometabolic substrates (Change and Alvarez-Cohen, 1995; Dolan and McCarty, 1993; Wilcox et al., 1995). Table 3.4 compares our methane and propane results to previous studies. The transformation yields for TCE observed on our study with both methane and propane-utilizers are in the range

of those observed by prior studies. However, resting cell transformation yields and transformation yields in the presence of growth substrate, can not be directly compared.

Table 3.4 Comparison of transformation yields for TCE (mg TCE/ mg primary substrate).

CONDITION	SOURCE	TRANSFORMATION YIELDS
Methane-utilizers (resting cells)	Dolan and McCarty, 1993	0.0400
Methane-utilizers (resting cells)	Change and Alvarez-Cohen, 1995	0.0170
Methane-utilizers (resting cells)	Change and Alvarez-Cohen, 1996	0.180
Methane-utilizers	reported here	0.0680
Propane-utilizers (resting cell)	Change and Alvarez-Cohen, 1995	0.0056
Propane-utilizers	Wilcox et al., 1995	0.0050
Propane-utilizers	reported here	0.0480

The microcosms exhibited TCE transformation activity with gradual increases in TCE concentration over a one year period. Two of propane microcosms (P#1 and 2) eventually recovered TCE transformation abilities after reducing the TCE concentration. Indigenous microbes therefore remained active toward TCE transformation and substrate utilization over one year of continuous TCE transformations.

The effectiveness of TCE cometabolism by an indigenous phenol fed microorganisms declined significantly during a 280 day experiment (Munakata et al., 1997). However, the results from our study and the results from phenol column microcosm study can not be directly compared. Much more TCE was transformed in

column microcosm studies which may have generated higher TCE transformation product toxicity, causing more significant inactivation.

The batch microcosms method used here appears to be a good screening method for testing long-term activity. More research is needed on determining kinetic parameters from this batch incubation method. The readdition of TCE into microcosms works well in evaluating transformation yields and to studying the effect of increasing the TCE concentration.

Acknowledgments

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CHAPTER 4

Long-term Batch Microcosms Studies of CAH Cometabolism by Methane, Propane, and Butane-Utilizing Microorganisms Stimulated from McClellan Air Force Base

Introduction

Chlorinated aliphatic hydrocarbons (CAHs) such as 1,1,1 TCA and CF are among the most widespread contaminants in groundwater and soil. Like TCE, both compounds are widely used as industrial solvents and military extraction agents. 1,1,1 TCA and CF have also been detected along with TCE in the groundwater and subsurface of McClellan AFB. CF is known recalcitrant compound exhibiting slow transformation rates in the subsurface. 1,1,1 TCA can be abiotically converted to 1,1-DCE in the subsurface (Vogel and McCarty, 1987). CF and 1,1,1- TCA are of particular concern due to their toxicity and carcinogenicity. They are regulated by EPA to a maximum contaminant level of 0.1 mg CF/L and 0.2 mg 1,1,1-TCA/L (Cook,1987; McCarty and Semprini, 1994).

Under aerobic conditions, many chlorinated hydrocarbons can be cometabolically degraded by microorganisms grown on methane (Wilson et al., 1985), propane (Wackett et al., 1989), phenol (Nelson et al., 1987), and toluene (Nelson et al., 1987; Wackett et al., 1988). The selection of suitable microorganisms to transform specific CAHs in soil and groundwater are of interest for in-situ bioremediation. Microorganisms which catalyze the transformation of a significantly broad range of contaminant substrates are desirable for enhancing in situ bioremediation, since groundwater and soil are commonly contaminated with multiple chlorinated hydrocarbons.

Methane-utilizing bacteria have been reported to degrade broad range of chlorinated hydrocarbons including TCE, CF and 1,1,1 TCA (Fogel et al., 1986; Chang and Alvarez-Cohen, 1994 and 1996; van Hylckama Vlieg et al., 1996; Little et al., 1988; Oldenhuis et al., 1989 and 1991). Among TCE, CF and 1,1,1-TCA, methane-utilizing cultures in resting cell studies exhibited highest transformation capacity for TCE, follows

by CF and 1,1,1- TCA (Change and Alvarez-Cohen, 1996). However, significantly lower transformation capacities for CF and 1,1,1 TCA were obtained in the methane resting cell studies.

Heterotrophic bacteria grown on phenol and toluene were able to degrade TCE, but not saturated compounds such as CF and TCA (Wackett et al., 1988; Nelson et al., 1986; Fliermans et al., 1988; Chang and Alvarez-Cohen, 1994). Previous resting cell studies also shown that organism grown on propane were able to degrade all three compounds, but with transformation capacity values less than those of methane-utilizers (Chang and Alvarez-Cohen, 1994). The degradation and inhibition of TCE (Wackett et al., 1989) and 1,1,1 TCA (Keenan et al., 1993) by propane has been observed by propane-oxidizing cultures. They demonstrated the propane-monooxygenase enzyme was responsible for the degradation kinetics and inhibition of TCE and 1,1,1 TCA cometabolism. Moreover, the propane oxygenase enzyme which is responsible for initial oxidation of propane, is nonspecific enough to metabolize and oxidize short-chain alkenes and other aliphatic hydrocarbons (Hou et al., 1983; Perry, 1980).

Previous research has shown that CAHs such as TCE and CF exert transformation product toxicity to methane-utilizers, decreasing the ability of the organisms to transform CAHs (Broholm et al., 1990; Alvarez-Cohen and McCarty, 1991; Speitel et al., 1993; Oldenhuis et al., 1989 and 1991). Previous studies proposed that the presence of CF and 1,1,1 TCA in the groundwater are of concern, since they impact TCE transformation. Competitive inhibition among growth substrates and the CAHs can also decrease the rate of CAHs transformation (Odenhuis et al., 1991). Toxicity resulting from CAHs transformation also causes inactivation and limited transformation capacity (Alvarez-Cohen and McCarty, 1991).

CF and 1,1,1 TCA were selected as the chlorinated hydrocarbons of interest in this study because these compounds, like TCE, are substituted with three chlorines. These compounds are also observed in the subsurface of McClellan AFB. The study also wanted to evaluate a chlorinated methane, ethane, and ethene to determine how the changes in molecular structure affected on cometabolic transformation potential. In this study, we examined the transformation of individual compounds and mixtures of CF, 1,1,1-TCA and

TCE in the presence of growth substrates (methane, propane, and butane). Moreover, the study was conducted to evaluate the effect of CF and 1,1,1 TCA on TCE transformation on methane and propane-utilizers. The maximum transformation yields (g CAH/g substrate used) for individual and multiple of CAHs for methane, propane, and butane were determined. The affect of long term batch incubation in the presence of these compound was also determined.

Objectives

The objectives of the batch microcosm studies were to :

1). Determine whether indigenous microorganisms utilizing methane, propane and butane from the McClellan subsurface are able to transform TCE, CF and 1,1,1-TCA.

2). Determine the maximum transformation yields for these compounds using methane, propane and butane as growth substrates.

3). Evaluate if competitive inhibition among compounds exists by evaluating transformation of CAH mixtures and product toxicity from CF or 1,1,1-TCA.

4). Examine the effect of CF and 1,1,1 TCA on TCE transformation by methane and propane-utilizers.

6). Study the affect of long term transformations on the ability to maintain TCE cometabolism.

Material and Methods

Batch microcosm with construction and operation

Batch microcosms were prepared and operated as previously described (in Chapter 3). Active methane, propane, and butane microcosms, that had previously transformed TCE for over 1 year were used to study of transformation of individual compound and

mixtures of chloroform (CF), 1,1,1-trichloroethane (1,1,1-TCA), and trichloroethylene (TCE). The batch microcosms were continuously operated with periodic groundwater exchanges and additions of growth substrates. The groundwater exchanges and addition of growth substrates were performed as described in the previous studies. Control microcosms included aquifer solids, groundwater and the CAH of interest, but lacking growth substrates were used.

Transformation studies of individual CAHs were conducted in the presence of growth substrate. 1,1,1 TCA transformation studies proceeded sequential incubations with CF. The mass of 1,1,1 TCA or CF was increased in batch microcosms with a constant amount of growth substrate added. Maximum transformation yields for TCA and CF were determined. Incubations and groundwater exchanges were performed as described in Chapter 3.

In order to study the transformation of the CAHs mixtures, equivalent aqueous concentration of TCE, CF, and TCA (1 mg CAH/L) were added to the microcosms along with the growth substrate. After the study of transformation of mixture of the three CAHs was completed, the transformation studies were continued with TCE and TCA, without CF present, and followed by final incubation with TCE alone. All batch microcosms were incubated and exchanged with groundwater as described previously.

After the study with mixed CAHs transformation, methane and propane microcosms were used to retest their TCE transformation ability. The experiments also determined the effect of CF and 1,1,1-TCA transformation on TCE transformation ability. Methane and propane microcosms were purged with nitrogen to remove residual of CAHs from prior studies before perform TCE experiments.

Chemical sources

Chloroform (CF; >99.9 % gas chromatography {GC} grade), 1,1,1-trichloroethane (1,1,1-TCA; 99.9 % {GC} grade), and trichloroethylene (TCE; >99 %) were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). Methane (>99.9 %) was

purchased from Airco. (Vancouver, Wa.). Propane (10 % in introgen) and butane (10 % in introgen) were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). The stock saturated CAH solution was prepared by adding 4 ml of pure CAH in a 125 ml capped serum bottle. The bottle was shaken and allowed to settle for at least 24 hours before use. The aqueous CAH concentrations were measured before use, using the procedure described below. The Henry law's constant at 24 ^{0}C of 0.392, 0.703, and 0.15 for TCE, 1,1,1-TCA, and CF, respectively, were used to determine partitioning between the gaseous and aqueous phase (Gossett, 1987). Methane, propane and butane were transferred from gas containers to batch microcosms with gas-tight syringes (Hamilton Co., Reno, Nev.). Direct volume addition achieved the desired mass concentration in the microcosms.

Analytical methods

TCE, 1,1,1-TCA, and CF concentrations were determined from analysis of the microcosm headspace. A Hewlett Packard (Wilmington DE) 5890 gas chromatography equipped with a 3393 A integrator and a ^{63}Ni electron capture detector was used to quantified CAH concentrations. Separation was obtained by a capillary (HP-624; 19091v-433; 1,4 μm length; Hewlett Packard, Wilmington, DE.) operated isothermally at 80 °C. An argon/methane (95/5) mixture at head pressure of 60 psi was used as the carrier gas. Injections of 100 μl were used for CAH headspace analysis. The method was calibrated with external standards. The Henry law's constant at 24 ^{0}C of 0.392, 0.703, and 0.15 for TCE, 1,1,1-TCA, and CF, respectively, were used to determine total mass of CAHs in microcosm (Gossett, 1987).

The analysis of TCE, 1,1,1-TCA, and CF concentrations in liquid phase were quantified by the purge and trap method using a modified version of standard EPA Method 8010. A Hewlett Packard Purge and Trap model 7695 was used in conjunction with a Hewlett Packard 5890 gas chromatography equipped with an OI Hall conductivity detector. A 100 μl sample was diluted in 5 ml of glass distilled water and then transferred

into the trap of the purge and trap unit. Separation were obtained by a capillary column (HP-624; 19091v-433; 1.4 μm length; Hewlett Packard, Wilmington, DE.).

Oxygen was measured by gas partitioner as previously described (Chapter 3). Methane, propane and butane headspace concentrations were also measured by gas chromatography as previously described (Chapter 3). A 100 μl sample from the microcosm headspace was injected into GC to obtain the substrate concentration. External gas standards were used to calibrate the method.

Results and Discussion

The transformation of 1,1,1 TCA by three propane-utilizers is shown in Figure 4.1. All the propane-utilizers effectively transformed TCA. Propane utilizers, P#1 and #3, show higher TCA transformation than that observed in P#2 on the first incubation with TCA. Higher TCA transformation was observed on propane microcosm (P#2) with the second addition of TCA. Propane degradation may have been inhibited by TCA. The propane uptake rates decreased with the increase of 1,1,1 TCA concentration in the second addition. Higher inhibition between propane and TCA were observed and the time periods to achieve total degradation of propane and TCA was longer than when the concentration of TCA was low. The transformation of TCA coincided with propane utilization in all three microcosms, providing evidence of competitive inhibition among propane and TCA. Prolonged activity toward TCA transformation in the absence of propane was not observed, except microcosm P#1.

Figure 4.2 shows the transformation of CF in three propane microcosms at an initial CF concentration of 4.0 mg CF/L (225 μg of CF). Complete CF transformation was achieved in microcosms (P#1 and P#3). CF transformation was correlated with the rates of propane utilization. Maximum transformation yields for CF of 0.07 g CF/ g propane were observed in microcosms (P#1 and #3), and 0.02 g CF/g propane in P#2. Slower propane uptake was also observed compared to the incubation with 1,1,1 TCA.

This potentially resulted from CF transformation product toxicity and/or competitive inhibition.

Figure 4.3 shows the transformation of TCA in methane microcosms. All methane microcosms showed no ability to transform TCA, despite of methane utilization. Similar results were observed in butane microcosms. All butane microcosms, shown in Appendix E, were not able to transform TCA even after readditions of butane was performed in the microcosms.

Figure 4.4 shows the transformation of CF in methane microcosms. The methane uptake rate was slower than when the culture exposed to TCE. Slower uptake of CF and methane was also observed in the microcosms. The rates of CF transformation were correlated with the rates of methane utilization. All methane utilizers exhibited the transformation yields of about 0.02 g CF/ g methane. The slower rate of methane utilization likely resulted from transformation product toxicity. The results indicated that methane-utilizers are less effective at transforming CF than propane-utilizers. In addition, unlike methane and propane, butane-utilizers exhibited no CF transformation (data shown in Appendix E).

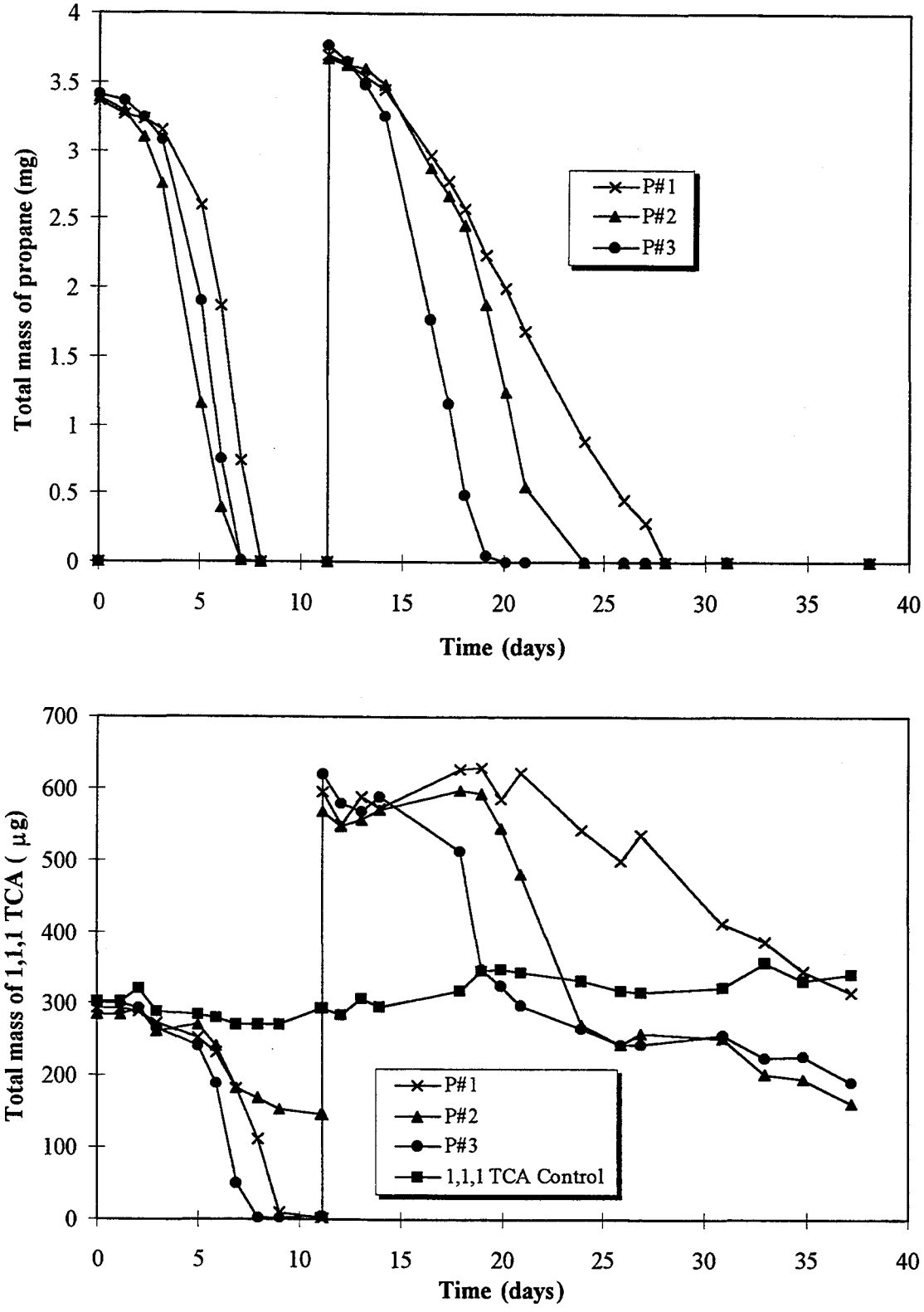

Figure 4.1 The transformation of 1,1,1 TCA in propane fed microcosms P#1, P#2, P#3, with increasing 1,1,1 TCA concentrations.

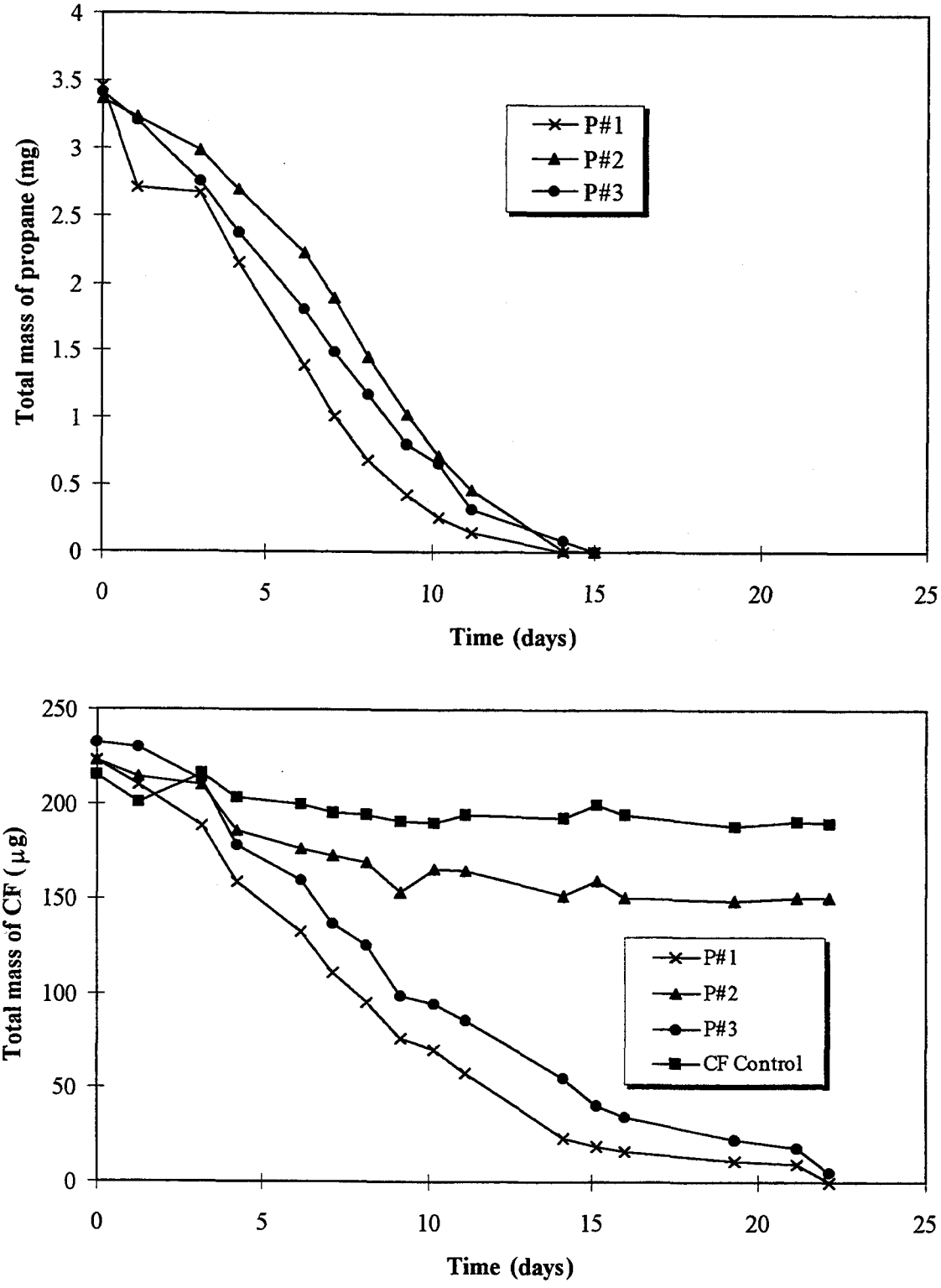

Figure 4.2 The transformation of CF in propane fed microcosms P#1, P#2, P#3.

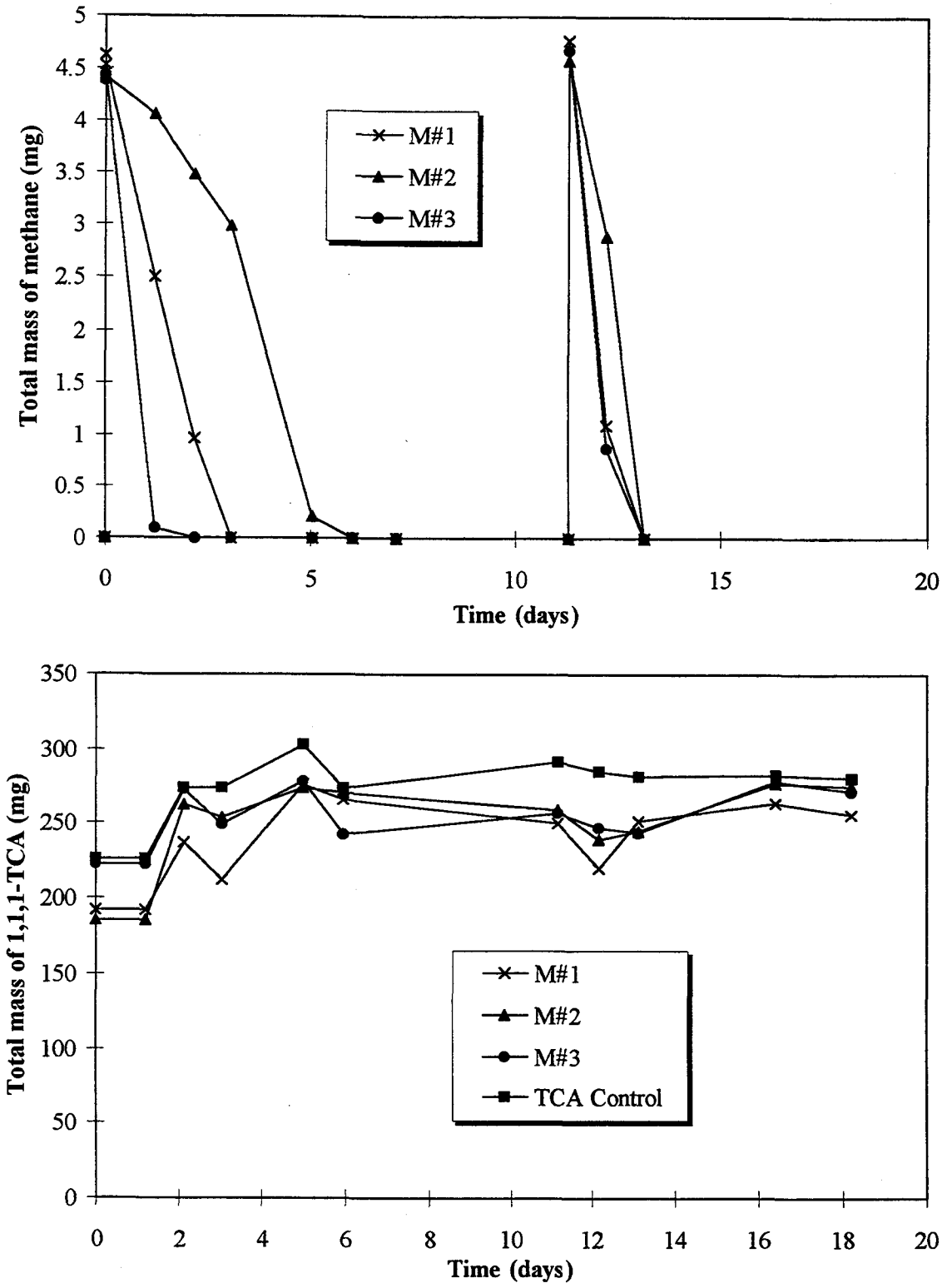

Figure 4.3 The transformation of 1,1,1-TCA in methane fed microcosms M#1, M#2, M#3.

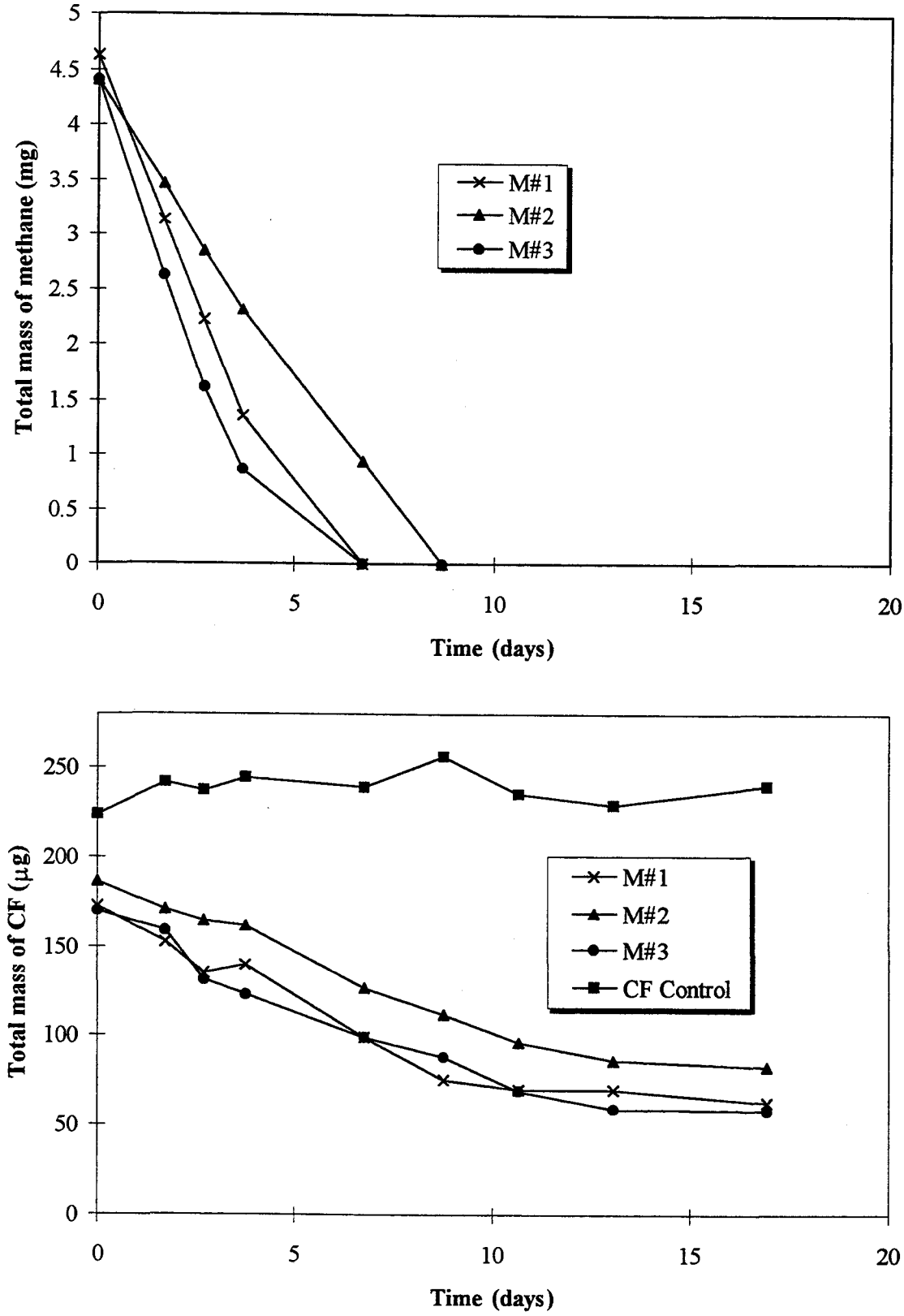

Figure 4.4 The transformation of CF in methane fed microcosms M#1, M#2, M#3.

Table 4.1 and Figure 4.5 presented the maximum transformation yields for individual incubations of TCE, TCA, and CF by methane and propane-utilizers. Propane-utilizers (P#1 and P#3) were more effective to transforming TCA and CF than methane-utilizers. The methane-utilizers more effectively transformed TCE, but have no ability to transform TCA. Butane-utilizers showed no ability to transform any of the CAHs tested. The transformation of CF appeared to inhibit both methane and propane-utilizers. Furthermore, CF transformation appeared to inhibit propane-utilizers more than methane-utilizers. Studies after chloroform transformation showed that propane-utilizers had a more difficult time recovering from their exposure to chloroform. This may have resulted from propane-utilizers degrading more CF, so more product toxicity could have resulted. Interestingly, unlike TCE, when propane cultures were exposed to TCA, long term of TCA transformation was not observed after propane was consumed, except propane microcosm P#1.

Table 4.1 Maximum transformation yields (g CAH/g substrate) for TCE, 1,1,1 TCA and CF achieved by methane and propane fed microcosms.

Growth substrate	Mixed enrichment culture	TCE	1,1,1 TCA	CF
		T_y [a] (g TCE/ g substrate)	T_y [a] (g 1,1,1 TCA/ g substrate)	T_y [a] (g CF/ g substrate)
Methane	M#1	0.060	0	0.024
	M#2	0.048	0	0.022
	M#3	0.069	0	0.024
Propane	P#1	0.028	0.088	>0.065 [b]
	P#2	0.020	0.098	0.020
	P#3	0.048	0.106	>0.068 [b]

[a] The maximum transformation yields of CAH (g CAH/g growth substrate).

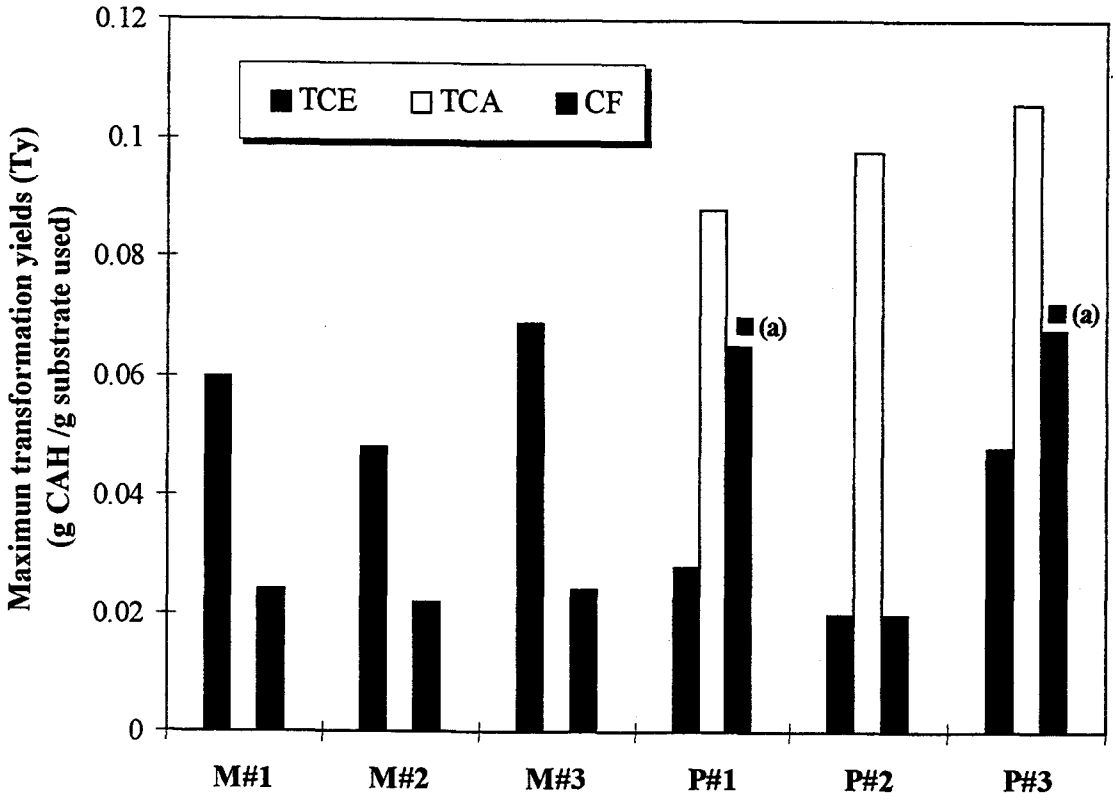

Figure 4.5 - Maximum transformation yields (g CAH/g substrate) for TCE, 1,1,1 TCA, and CF achieved by methane and propane fed microcosms.
(a). Transformation yields may have been limited by the mass of CAH present.

Transformation of CAHs mixtures (TCE, 1,1,1-TCA, and CF) by methane and propane-utilizers

Studies of transformations of mixtures of TCE, 1,1,1-TCA, and CF were then performed at CAH concentrations of 1 mg /L for each compound. The results from the methane microcosm M#3 are presented in Figure 4.6. TCE was the most effectively transformed followed by CF. No transformation of TCA was observed. These observations are consistent with the results from the individual compound tests (Figure 4.5). Methane utilization appeared to be inhibited by TCE and CF transformation, since

slower methane uptake rates were observed in all the microcosms. However, transformation of TCE and CF was more rapid during methane degradation. In the absence of methane, the culture continued to degrade TCE and CF at slower rates several days after methane was consumed.

The transformation of mixtures of CF,1,1,1-TCA, and TCE in the propane microcosm P#3 is shown in Figure 4.7. Effective transformation of CF, TCA, and TCE was observed. CF was most rapidly transformed and followed by TCA and TCE. Complete transformation of 1.0 mg/L of CF (68 μg total mass of CF) and TCA (82 μg total mass of TCA), and nearly complete transformation of TCE (70 μg total mass of TCE) in aqueous solution were observed. Transformation of CF, TCA, and TCE was observed during propane utilization, but transformation rates were most rapid after the propane was reduced to low concentration, suggesting competitive inhibition of propane on CAH transformations. The long lag time may have been caused by the previous transformation of CF. The results also suggested that CF is the most competitive CAHs tested followed by TCA and TCE.

Similar results were observed in microcosm P#1, but not for propane microcosm P#2 (Appendix E). Propane utilizers in P#2 showed no transformation of mixed CAHs tested, even though this culture previously transformed the individual CAHs (Figure 4.5). It was unclear that why the transformation of CAH mixtures was not observed. A possible explanation is that the transformation on CF in the prior study of individual CAHs diminished the cometabolic potential of the P#2 cultures.

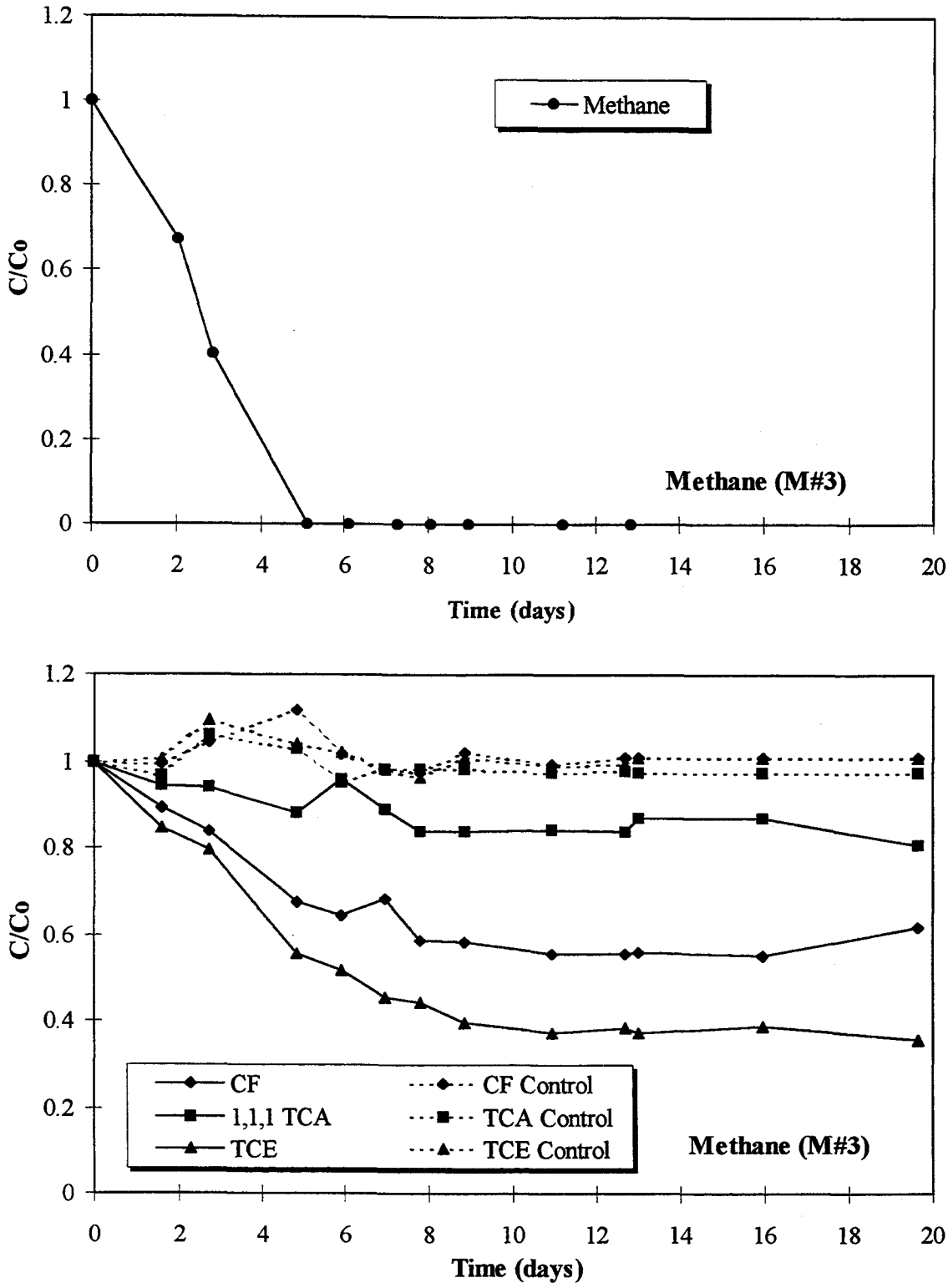

Figure 4.6 Methane utilization and CAH transformation in the methane microcosm M#3.

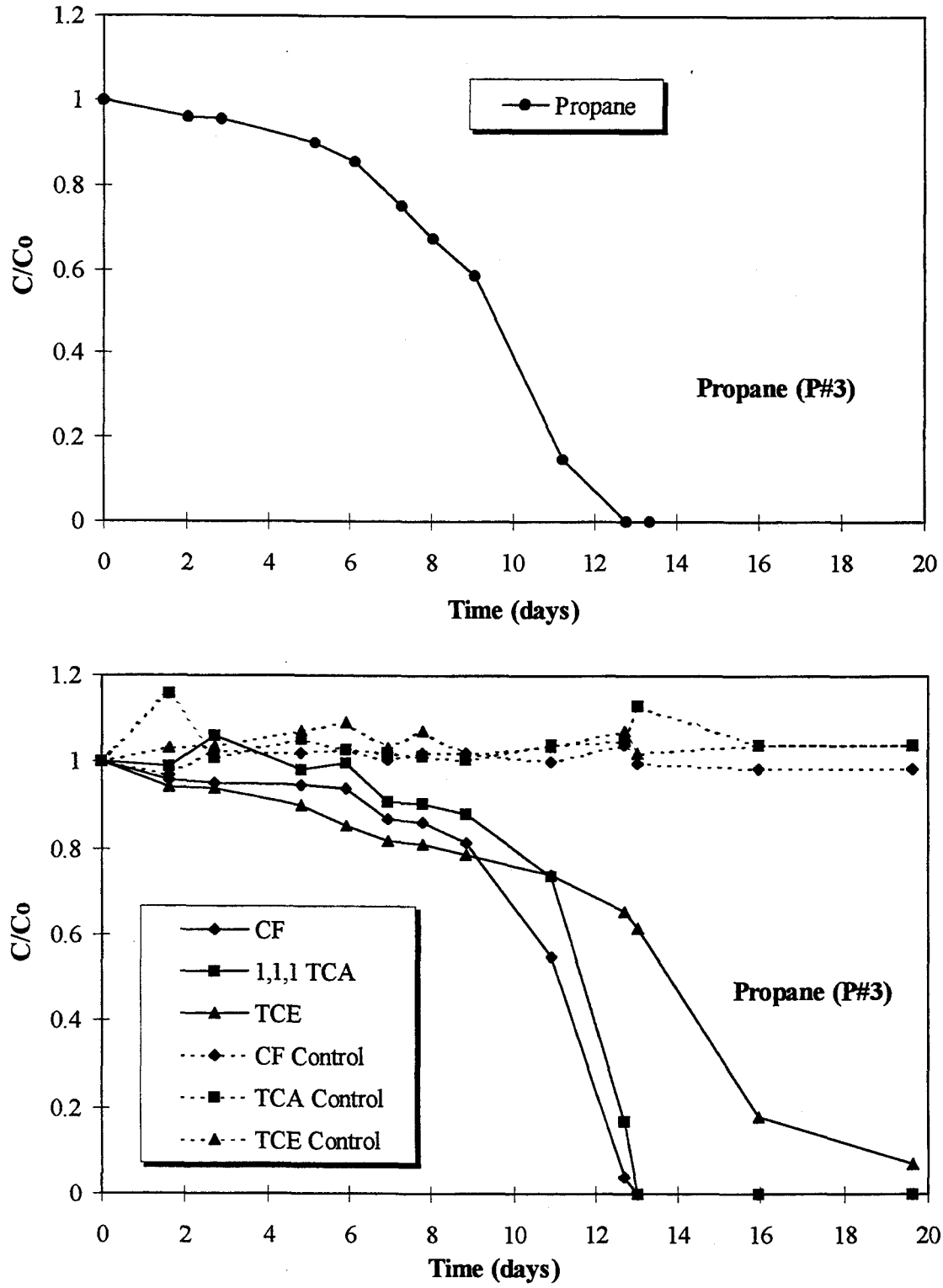

Figure 4.7 Propane degradation and CAH transformation in the propane microcosm P#3.

The comparison of transformation yields in the presence of growth substrate for the CAH mixtures for methane and propane-utilizers are shown in Figure 4.8. The methane-utilizers were able to transform CF and TCE, but not 1,1,1-TCA. Higher transformation yields for TCE compared to CF were observed and consistent with the results for single compounds (Figure 4.5). Propane-utilizers effectively transformed TCA , CF, and TCE. The T_y results represented conservative estimates, since complete transformation of CAH mixtures was observed. No transformation of CAHs was demonstrated by propane-utilizers (P#2). For the two effective cultures P#1 and P#3, the observed transformation yields of the propane-utilizers with mixed CAHs transformation were much higher than those of methane-utilizers. It is of interest that TCE was more effectively transformed, compared to when the single CAH were tested (Figure 4.5). Here the simultaneous transformation of CF and TCE appears to have a greater impact on methane-utilizers than propane-utilizers.

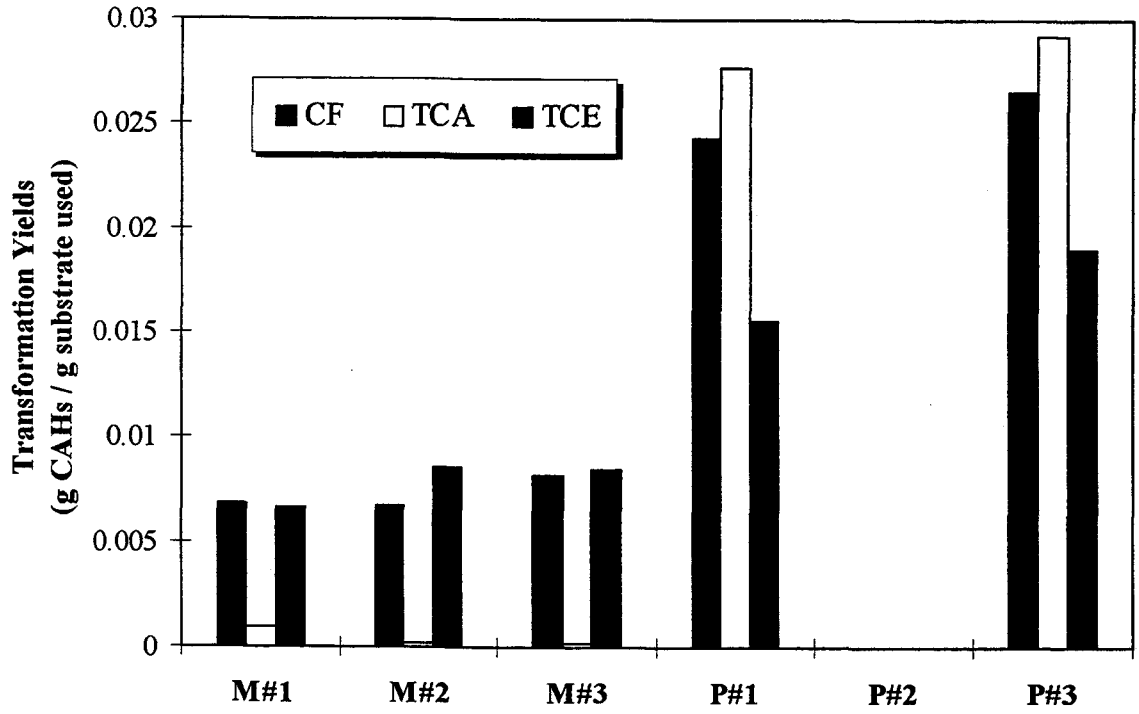

Figure 4.8 Comparison of transformation yields for mixed CAHs by methane and propane-utilizers.

Transformation of 1,1,1-TCA and TCE without exposure to CF

In order to study the effect of CF on the transformation of TCA and TCE, the transformation of TCA and TCE in each microcosm was measured in the absence of CF. The results for the methane microcosm M#3 are presented in Figure 4.9. The rates of TCE transformation and the rates of methane utilization were more rapid in the absence of CF. Higher TCE transformation yields were also obtained by all the methane-utilizers when CF was not present. The results indicate that CF transformation significantly inhibited methane utilization and TCE transformation.

Propane utilization and 1,1,1 TCA and TCE transformation in the absence of CF are shown in Figure 4.10. The rate of propane degradation significantly increased and TCA was removed more rapidly in the absence of CF. Complete TCA transformation was observed and higher rate of TCA transformation was observed after propane was utilized. The results also shows that TCE transformation occurred after propane was removed to low TCE concentration, and continued for 15 days after propane was consumed. The culture had more prolonged TCE activity than when CF was present.

Similar results were obtained in propane microcosm P#1, but not in P#2 (Appendix E). Propane microcosm P#2 did not transform either TCA or TCE, even when CF was removed. The results indicate CF transformation reduced the rates of propane utilization and TCA and TCE transformation. CF transformation from the previous incubation appears to have a detrimental effect on microcosm P#2. However, transformation yields for TCE by propane-utilizers in the absence of CF, are not different from those observed in the presence of CF. This results is surprising, since CF is likely draining energy reserves needed for TCE transformation if the same microorganisms degrade both CF and TCE. It may be that the effect of CF transformation was actually observed in the subsequent test when CF was removed. In both tests, most of the TCE was transformed after propane was utilized and CF was transformed. It is also possible that different propane-utilizers in the mixed cultures degrade different CAHs

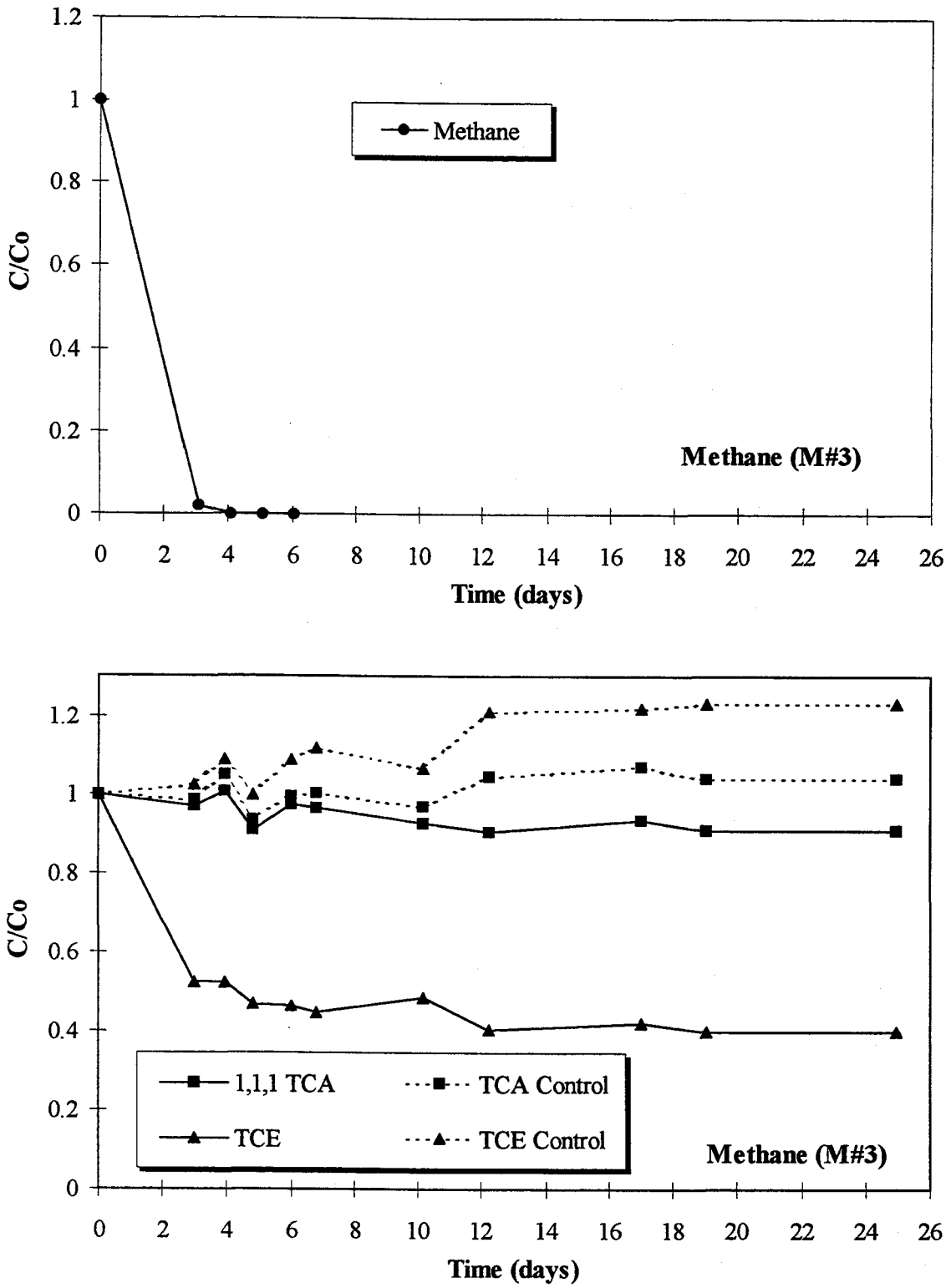

Figure 4.9 Methane degradation and transformation of 1,1,1 TCA and TCE without exposure to CF in microcosm M#3.

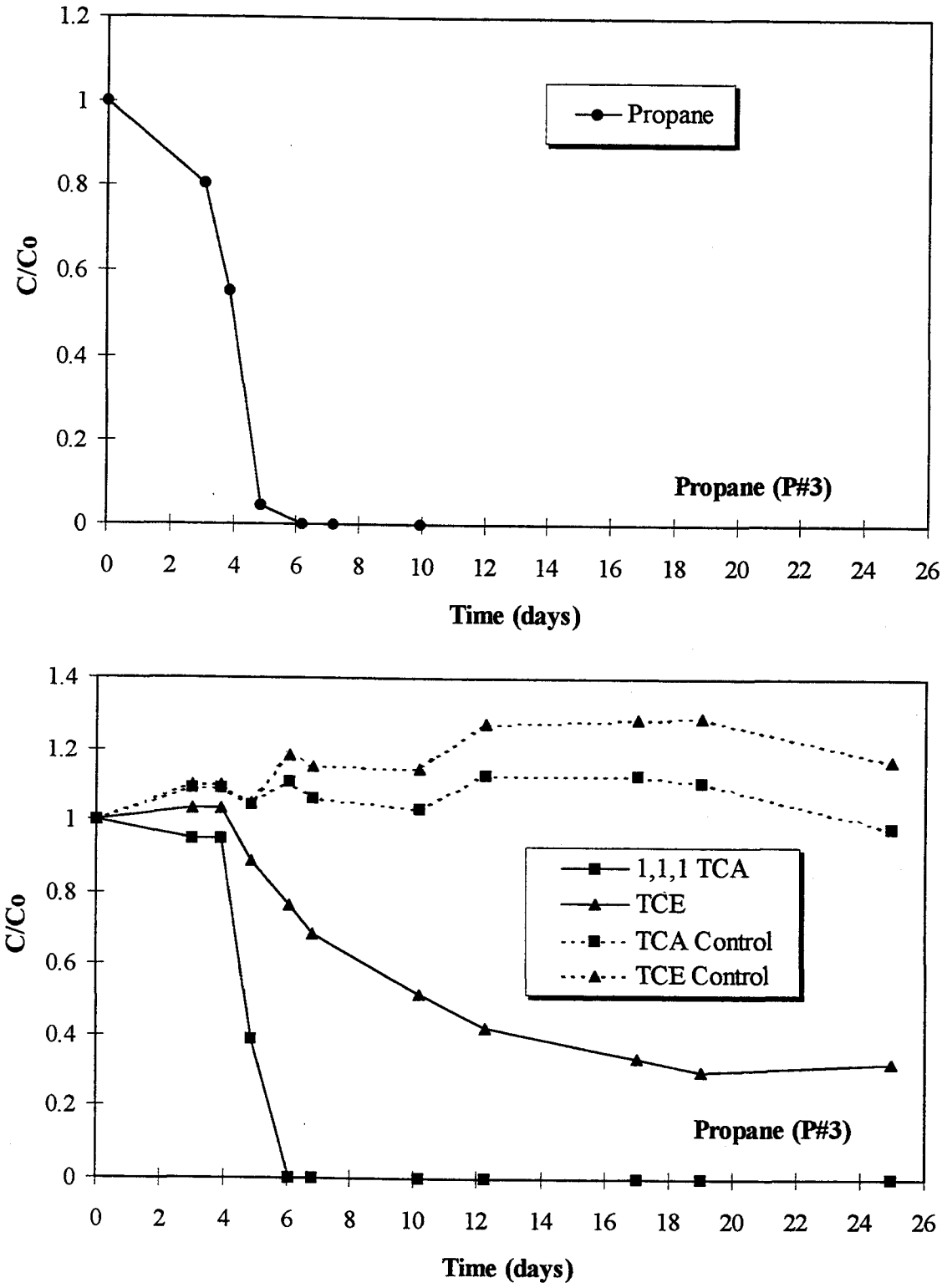

Figure 4.10 Propane degradation and transformation of 1,1,1 TCA and TCE without exposure to CF in microcosms P#3.

Transformation of TCE by methane and propane-utilizers without exposure to 1,1,1-TCA and CF

One year after the initial TCE transformation test and after the study with individual and CAH mixtures, all methane and propane microcosms were retested for their ability to transform TCE. These results permit a comparison with the results from the original TCE studies. Studies of transformation of mixtures of TCE and 1,1,1-TCA were performed at CAH concentrations of 1 mg /L for each compound. The effect of 1,1,1-TCA on TCE transformation was also evaluated. The transformation of TCE in the absence of TCA and CF in methane microcosms are presented in Figure 4.11. The rates and extents of methane utilization and TCE transformation was similar to when this culture was exposed to 1,1,1 TCA. Unlike CF, the presence of 1,1,1 TCA did not have an impact on methane degradation and TCE transformation. Observed transformation yields for TCE by all methane-utilizers in the absence of 1,1,1 TCA equivalent to those observed when the cultures were exposed to 1,1,1 TCA.

Figure 4.12 shows the propane utilization and TCE transformation in the propane microcosms in the absence of 1,1,1 TCA and CF. In comparison with the previous incubation, the presence of 1,1,1 TCA appeared to inhibit propane degradation. The rates of propane utilization increased and shorter lag time was observed in the absence of 1,1,1 TCA. 1,1,1 TCA, however, appeared to have less impact on TCE transformation because the observed TCE transformation yields in the absence and presence of TCA were similar. Unlike 1,1,1 TCA, TCE transformation normally occurred after propane degradation was completed. The results also indicated long term TCE transformation of propane utilizers , P#1 and P#3, after propane was consumed, similar to the results when this culture was exposed to 1,1,1 TCA. TCE transformation continued for about 15 days with and without 1,1,1 TCA present. Data for propane microcosms P#1 and #2 are also shown in Figure 4.12. Propane-utilizer (P#2) recovered their ability to transform TCE in this experiment. If CF transformation caused the earlier loss of TCE transformation ability, the microcosm was able to recover TCE transformation ability, TCA inhibition of TCE transformation in this microcosm also can not be ruled out.

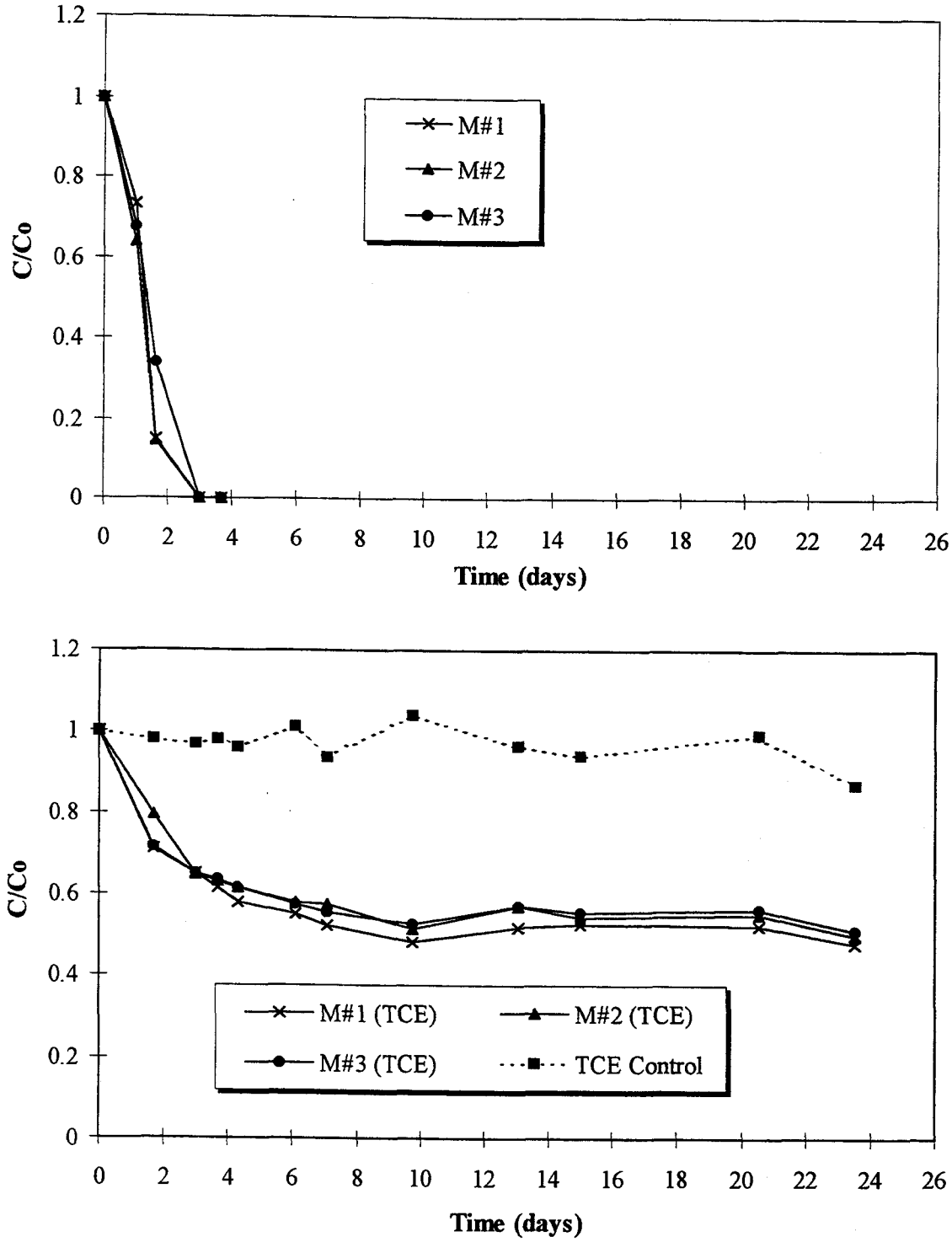

Figure 4.11 Methane degradation and transformation of TCE without exposure to CF and 1,1,1 TCA in microcosm M#1, M#2, M#3.

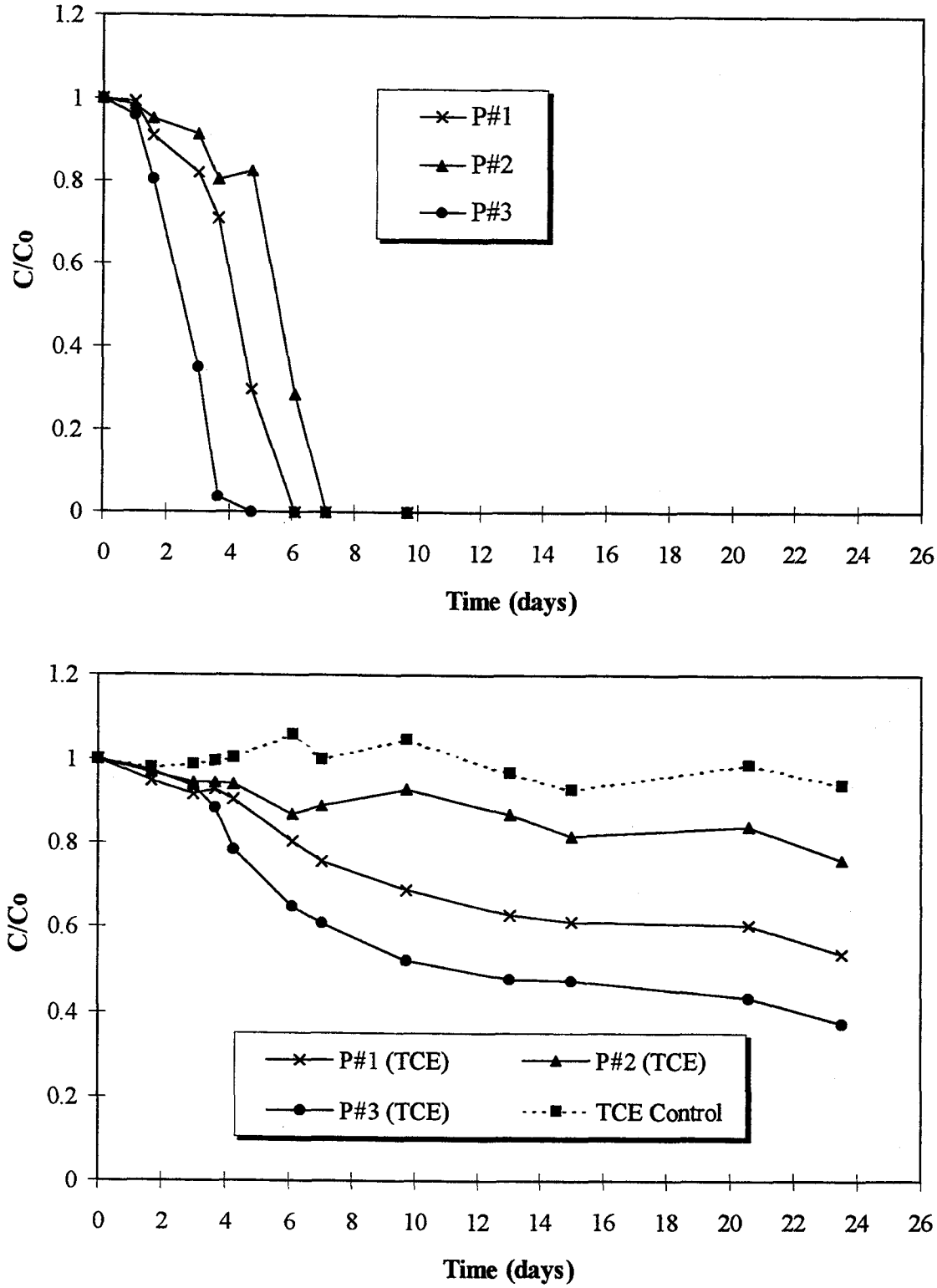

Figure 4.12 Propane degradation and transformation of TCE without exposure to CF and 1,1,1 TCA in microcosm P#1, P#2, P#3.

Figure 4.13 presents the TCE transformation yields achieved by methane and propane-utilizers for different CAH mixture conditions. For the methane-utilizers, the transformation yields for TCE in the presence of CF was lower than when the cultures were exposed to TCE alone or TCE with TCA. CF transformation had a greater impact on TCE transformation on methane-utilizers than propane-utilizers (except P#2). The presence of CF strongly hindered the methane-utilizers ability to transform TCE. However, TCA exerted no observable effect on methane-utilizers ability to transform TCE. No TCA transformation by methane-utilizers occurred, thus toxicity effects of TCA on methane-utilizers or consumption of energy for cometabolism was not observed, therefore TCE transformation ability was not effected. Competitive inhibition of TCA on TCE transformation was not apparent.

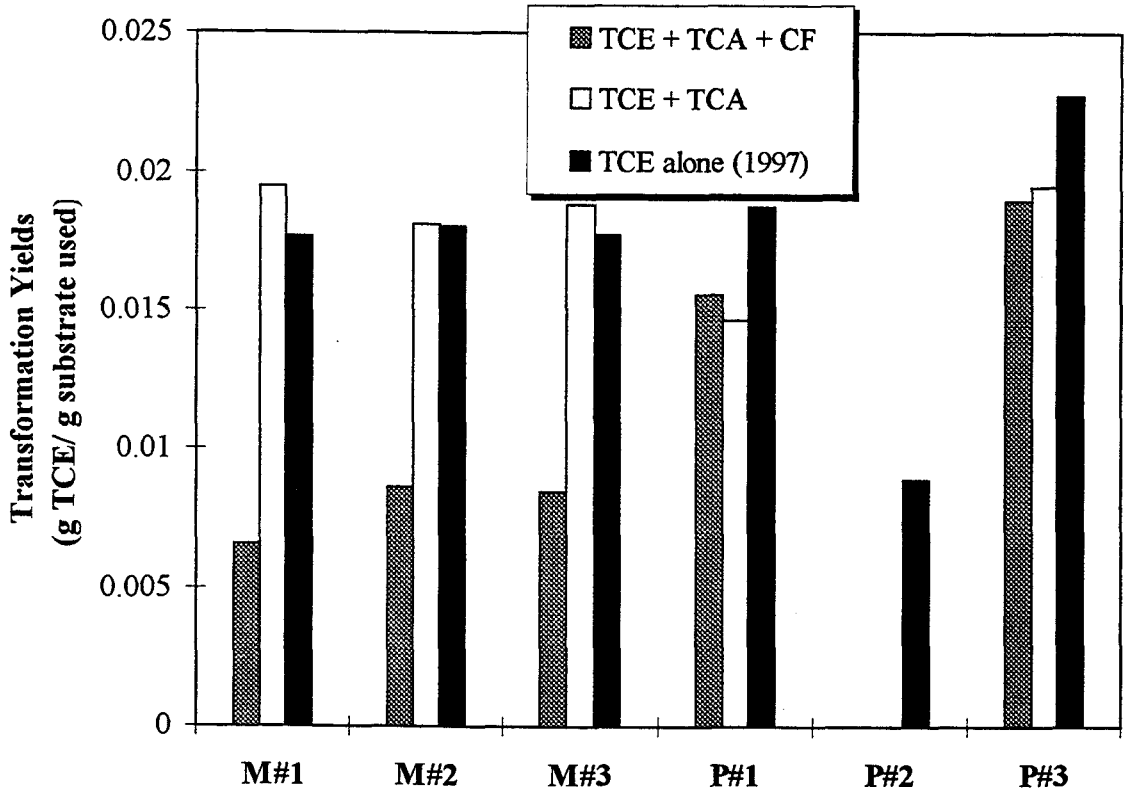

Figure 4.13 Comparison of TCE transformation yields for different CAHs mixture conditions by methane and propane-utilizers. The aqueous TCE, 1,1,1-TCA, and CF concentrations were 1.0 mg/L.

The propane-utilizers had higher ability to transform TCE than methane-utilizers in the presence of CF and TCA. The transformation yields for TCE in presence of all CAHs mixtures is not very different from that observed in the presence of CF and TCA. This indicates that CF and TCA exerted less impact on TCE transformation by propane-utilizers. The results in Figure 4.12 also showed that methane and propane-utilizers remained activity toward TCE cometabolism for over one year. The different TCE transformation yields can result from many factors, including CF exposure. Effective TCE transformation was maintained in all the microcosms. In addition, propane-utilizers (P#2) can recovered their ability to transform TCE after the exposure to high TCE and CF. Longer term cometabolic activity can be maintained under microcosm conditions. The batch microcosms appears to be a reliable method for evaluating the in situ cometabolic bioremediation potential of TCE and CAH mixtures.

Summary and Conclusions

Summary

The results presented in this study have demonstrated that indigenous microorganisms grown on propane are capable of transforming TCE, CF and 1,1,1 TCA. Microorganisms grown on methane transformed TCE and CF, but not TCA. Butane-utilizers did not transform any of the CAHs tested. Methane-utilizers exhibited highest transformation yields for TCE. The propane-utilizers effectively transformed CF, TCE, and 1,1,1-TCA.

TCE transformation rate of methane-utilizers was most rapid during methane utilization and the rate of TCE transformation slowed significantly after methane was consumed. Propane-utilizers showed lower rates of TCE transformation than methane-utilizers, but transformation continued for extended periods after propane was consumed.

TCA transformation studies indicated that propane utilization are strongly inhibited by TCA transformation. CF transformation product toxicity likely occurred for both methane and propane-utilizers. The decrease of methane and propane utilization rates occurred after exposure to CF. CF transformation product toxicity or high competitive inhibition between CF and growth substrate are possible reasons for the decreased rate. Previous research has shown that CF transformation product toxicity to methane-utilizers, decreasing their ability to transform CF (Broholm et al., 1990; Alvarez-Cohen and McCarty, 1991; Oldenhuis et al., 1989 and 1991). However, propane-utilizer exhibited much higher CF transformation yields than methane-utilizers, indicating that the propane-utilizers stimulated here have higher ability to cometabolize CF.

The transformation of CAH mixtures (TCE, CF and 1,1,1-TCA) resulted in higher transformation yields for CAHs by propane-utilizers than the methane-utilizers. Propane-utilizers were much more effective to transforming CAH mixtures. Thus, indigenous propane-utilizers from the McClellan subsurface appear to have a better potential for in situ bioremediation of groundwater contaminated with CAH mixtures. Since CF and 1,1,1TCA are the contaminants that have been detected along with TCE in the McClellan AFB, stimulation of propane-utilizers would be desirable for in-situ bioremediation at this site.

The results indicated that the presence of CF and 1,1,1 TCA in the groundwater are of greater concern when methane-utilizers are stimulated for TCE transformation. CF lowers the TCE transformation ability of methane-utilizers. This would be a major concern when cometabolic TCE degradation by methane cultures are used in clean up processes. Lower observed transformation yields for TCE are likely when CF is present. However, the propane-utilizers had higher ability to transform TCE than methane-utilizers in the presence of CF and TCA. Higher TCE transformation ability of propane-utilizers in the presence of mixed CAHs possibly results from faster removal of CF than TCE, with TCE transformation occurred after CF is removed. The processes causing this behavior need to be investigated in much more detail.

Conclusions

The following conclusions can be drawn from this study.

1. McClellan's indigenous microorganisms grown on methane and propane were capable of transforming TCE and CF. Indigenous methane-utilizers could not transform 1,1,1-TCA, while propane-utilizers very effectively transformed TCA. The butane-utilizers were not able to degrade any of the CAH tested.
2. Based on individual transformation yields, methane was the most effective substrate for TCE removal. The propane-utilizers exhibited the highest transformation yields for both CF and 1,1,1 TCA. When CF was present, propane-utilizers had higher TCE transformation yields than methane-utilizers.
3. TCA transformation was inhibited propane-utilization, suggesting propane-oxygenase enzyme was involved in TCA transformation.
4. CF was observed to inhibit both methane and propane utilization. This likely resulted from transformation product toxicity.
5. The observed transformation yields of CAH mixtures by propane-utilizers were much higher than those of methane-utilizers. Propane-utilizers were much more effective at transforming CAHs mixtures than methane-utilizers.
6. The presence of CF and 1,1,1 TCA in the groundwater will likely have a greater negative effect on methane-utilizers ability to TCE transformation. The presence of CF lowers TCE transformation ability.
7. Methane and propane-utilizers remained activity toward TCE transformation with repeated transformation of TCE, CF, and TCA over a one year period. The results indicate the transformation in the presence of growth substrate help to maintain transformation ability.
8. The batch microcosms appear are reliable for evaluating in situ cometabolic bioremediation potential for TCE and CAH mixtures.

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CHAPTER 5

The Effect of Nutrient Addition on TCE Cometabolism by Methane, Propane, and Butane Utilizing Microorganisms Stimulated from McClellan Air Force's Aquifer Solids

Introduction

Nutrient requirements are one of the major factors that influence the potential for chlorinated aliphatic hydrocarbon (CAH) transformation in situ. Nutrients are needed for maintaining the growth of subsurface microorganisms that cometabolize TCE and other CAHs. Nitrogen is one of the most essential nutrients that can be limiting in groundwater, with nitrate usually being the available nitrogen source. The addition of a nitrogen source such as nitrate or ammonia to the nitrogen-deficient subsurface may be required to enhance TCE and CAH cometabolism.

Methane-utilizing bacteria have been extensively studied for 25 years. Methane-utilizing microorganisms are widespread in transition zone between aerobic and anaerobic zone in the subsurface where methane and oxygen are present (Hanson, 1980). Methane-utilizers are categorized into two groups (Type I and II) based on their internal membranes. Both types can express a particulate enzyme form called particulate methane monooxygenase, (pMMO). Only Type II methanotrophs can express soluble methane monooxygenase, (sMMO) that can cometabolize a broad range of substrates, including TCE and many other CAHs. Previous studies have shown that soluble forms of MMO (Type II) can be produced under the copper limited growth conditions, while Type I organisms that express pMMO require copper for growth (Brusseau et al., 1990; Odenhuis et al., 1989; Tsien et al., 1989). Recent studies have also found that methanotroph Type II that efficiently cometabolize TCE can also fix nitrogen under conditions of low oxygen tension (de Bont, 1976; Murrell and Dalton, 1983; Chu and Alvarez-Cohen, 1996; Graham et al.,1993).

Type II methanotrophs appear to be selected under nitrogen-limiting conditions, while Type I strains appear to be present under all methane-enrichment conditions when nitrate or ammonia are available (Dugan et al., 1978; Graham et al.,1993). Graham et al., (1993) found that *M. Trichosporium* OB3b, Type II strains, can be selected under nitrogen limited conditions and the condition of low oxygen tension. Type II methanotrophs typically are nitrogen fixers, while Type I organisms are unable to fix molecular nitrogen and require dissolved nitrogen such as nitrate, ammonia or organic nitrogen for growth.

Nitrogen-fixing methane utilizers expressing sMMO, grown at low oxygen tensions, were able to degrade TCE rapidly with a high transformation capacity (Chu and Alvarez-Cohen, 1996). These results indicate that reactors can be used to manipulate methane-utilizing bacteria species selection to optimize TCE and other CAHs removal. Nitrate is one of the primary factors influencing methanotrophic species selection. On the other hand, most research claims that expression of sMMO under in situ conditions may prove difficult due to copper availability in the subsurface. Thus, it might be difficult to control conditions to select of Type II dominant species on the subsurface environment (Graham et al., 1993).

In the absence of growth substrate or external electron source, methane-utilizers can also produce poly-β-hydroxybutyrate (PHB) as an endogenous electron donor or source of required reducing power. PHB is an intracellular reserve polyester polymer whose synthesis serves as an electron sink in microorganisms grown under limited conditions (such as limitations of N, P, S, Mg , and/or O_2) (Dawes and Senior, 1973). The PHB may be used for the regeneration of NADH during TCE transformation (Asenjo, J. A. et al.,1986; Henrysson and McCarty, 1993). Intracellular reducing equivalents to improve and extend TCE transformation might be due to the catabolism of stored PHB contents in methane-utilizers. A positive correlation was observed between PHB contents and the naphthalene oxidation rate (a measure of soluble MMO activity), as well as between PHB and the TCE transformation rates and capacity (Henrysson and McCarty, 1993; Henry and Grbic-Galic, 1991). High accumulation of PHB was also

observed upon depletion of nitrate in *Methylosinus trichosporium* OB3b cultures, Type II strains (Shah et al., 1996).

The goal of this study was to determine the effect of nutrients on methane and propane-utilizers stimulated on McClellan's aquifer solids. The effect of nitrate on TCE cometabolism was determined in batch microcosms with groundwater and aquifer solids. A comparison of the effect of nutrients on methane and propane enrichments cultures was also investigated in the batch microcosms with groundwater or media, without aquifer solids present.

Materials and Methods

Indigenous microcosm studies with aquifer solids

The studies were performed in batch microcosms constructed with aquifer material and groundwater from McClellan Air Force Base. Methane, propane and butane were used as growth substrates for each of microcosm studies. Microcosm method was adapted from Broholm et. al., (1990) and Mu and Scow, (1994). The microcosms were prepared for each of substrates tested. The microcosms were constructed using 125 ml amber serum bottles (Wheaton Class Co., Millville, NJ.). Aquifer material from the McClellan Air Force Base, Sacramento, CA, was wet sieved with site groundwater under a laminar flow hood using a No. 8 sieve (2.38 mm opening) to remove large particles. The site groundwater was filtered (0.45 μm sterilized filter) before use. 15 ml of wet solids and 50 ml of filtered ground water were added to each batch microcosm, leaving a 60 ml air-filled headspace as a source of oxygen. The headspace permitted sampling of the gaseous substrate, oxygen, and TCE. The microcosms were crimp sealed with a Teflon™ butyl rubber cap (Kimble Co., IL), then inverted and incubated at room temperature on a shaker table at 100 rpm.

Batch microcosms were prepared and operated as described previously. Active methane (M#1, 2, and 3), propane (P#1, 2, and 3), and butane microcosms (B#1, 2, and 3)

were used to study the effect of nitrate addition on substrate utilization and TCE cometabolism. The exchange of 50/50 groundwater was performed in the methane (M#2 and 3), propane (P#1 and 3), and butane (B#2 and 3) microcosms prior to the readdition of the growth substrate and TCE. The groundwater was amended with nitrate (30 mg/L), since nitrogen (as nitrate) was found to be limiting in the groundwater. Groundwater exchanges lacking nitrate were performed in microcosms, M#1, P#2, and B#1. Nitrate-limited microcosms were used to compare TCE transformation and substrate utilization with nitrate-rich microcosms. Groundwater exchange procedure were performed as described in the previous study (Appendix A).

Enrichment microcosms with media and groundwater

Mixed methane and propane enrichment cultures used in this study were obtained from batch microcosm studies on McClellan AFB. Methane and propane enrichment cultures were obtained by transferring 1 ml of groundwater and aquifer solids from the previous stimulate microcosm M#1 and P#3. The enrichment cultures were grown in the media containing with ; 15 mM $K_2HPO_4 + NaH_2PO_4$ (a buffer 7.5 solution), 0.5 mM $MgSO_4$, 0.1 mM $CaCl_2$, 23.5 mM $NaNO_3$, 0.796 mM $(NH_4)_2SO_4$, 0.1 g of yeast extract, and trace element solution containing ; 22.6 μM $FeSO_4.7H_2O$, 1.52 μM $MnCl_2.4H_2O$, 0.51 μM $ZnSO_4.7H_2O$, 1.0 μM H_3BO_3, 0.45 μM $Na_2MoO_4.7H_2O$, 0.1 μM $NiCl_2.6H_2O$, 0.1 μM $CuCl_2.2H_2O$, and 0.1 μM $CoCl_2.6H_2O$. Each of suspended cultures in 750 ml serum bottles were then shaken and incubated at 30 °C on a shaker table at 190 rpm. Each of 750 ml serum bottles was supplied by methane or propane (10 % in the headspace) to sustain the microbial growth before use in the experiments.

Enrichment cultures were used to study the effect of nutrient addition on TCE cometabolism. Three different media compositions were tested included ; 100 % media, 100 % groundwater, and 50/50 % media /groundwater. Three serum bottles were constructed for each of substrate tested. The 125 ml serum bottles contained 60 ml of medium were inoculated with 1 ml of the enrichment cultures, growth substrate and TCE. The serum bottles were then crimp sealed with a Teflon™ butyl rubber (Kimble Co., IL)

then inverted and incubated at room temperature on a shaker table at 100 rpm. The serum bottles were maintained with repeated additions of growth substrate and TCE for a period of 150 days without exchanging groundwater or adding nutrients.

Analytical methods

Methane, propane, butane, TCE, and oxygen were determined by headspace sampling of the microcosms as previously described (Chapter 3). The nitrate concentration was determined using 50 µl aqueous sample as previously described (Chapter 3).

Results

The effect of nitrate addition on indigenous microcosm studies with aquifer solids

The batch microcosm studies with aquifer solids were conducted to determine the effect of nitrate addition on indigenous microbial activities and TCE cometabolism. Methane, propane and butane were used as growth substrates for each of microcosm studies. Active methane (M#1, 2, and 3), propane (P#1, 2, and 3), and butane microcosms (B#1, 2, and 3) were used to study the effect of nitrate addition on substrate utilization and TCE cometabolism.

The uptake of methane and propane, TCE transformation, and the effect of nitrate addition is shown in Figure 5.1 and 5.2. In active microcosms, complete methane and propane utilization required about 2 to 3 days of incubation. TCE removal was observed in nitrate amended microcosms, M#2, M#3, P#1, and P#3. Methane and propane microcosms (M#1 and P#2) showed limited microbial activity under nitrate limited conditions during the first incubation with substrate and TCE. No uptake of methane or TCE transformation was observed in microcosm M#1, while microcosm P#2 showed slow

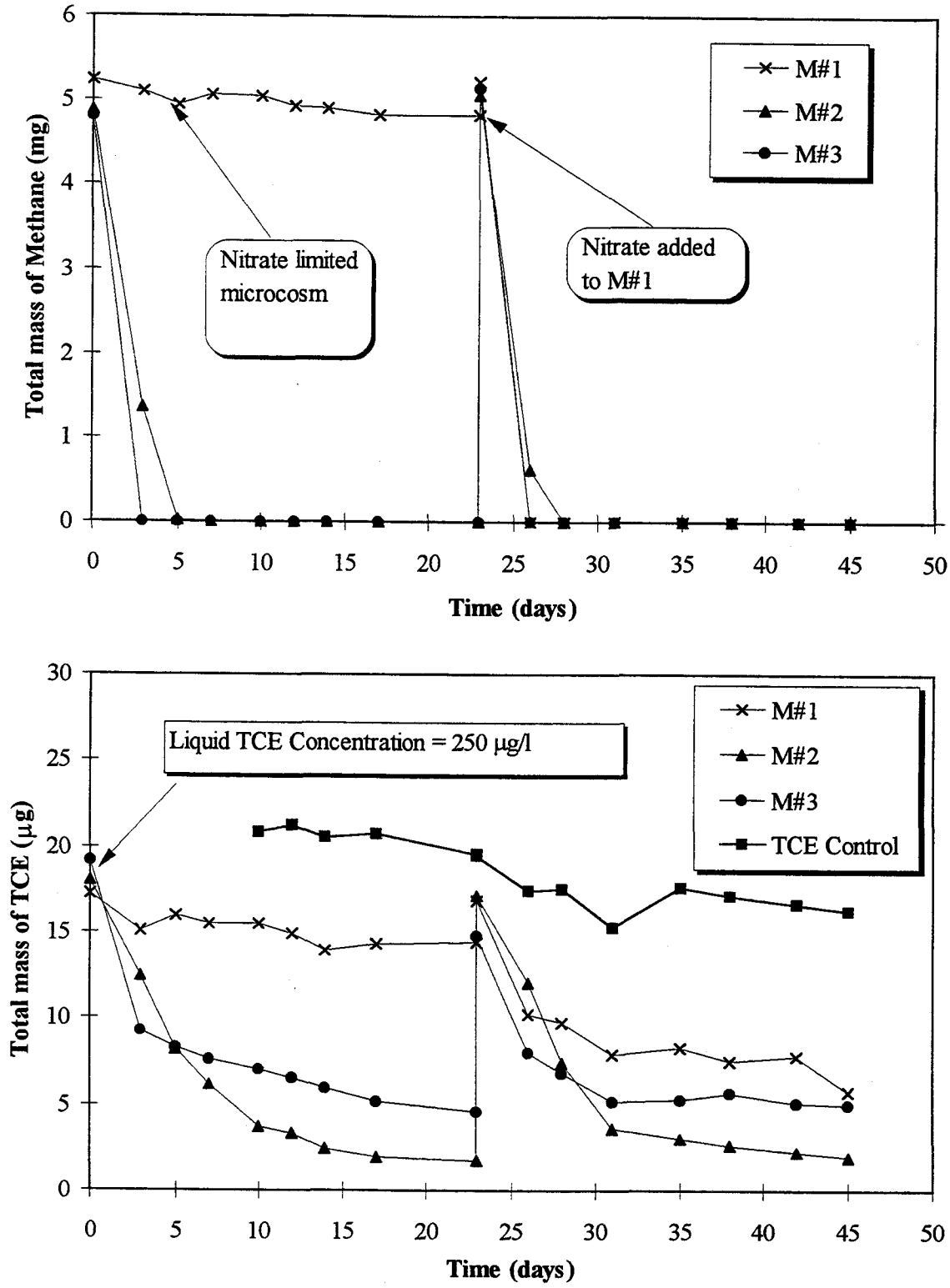


Figure 5.1 The effect of nitrate addition on methane degradation and TCE transformation.

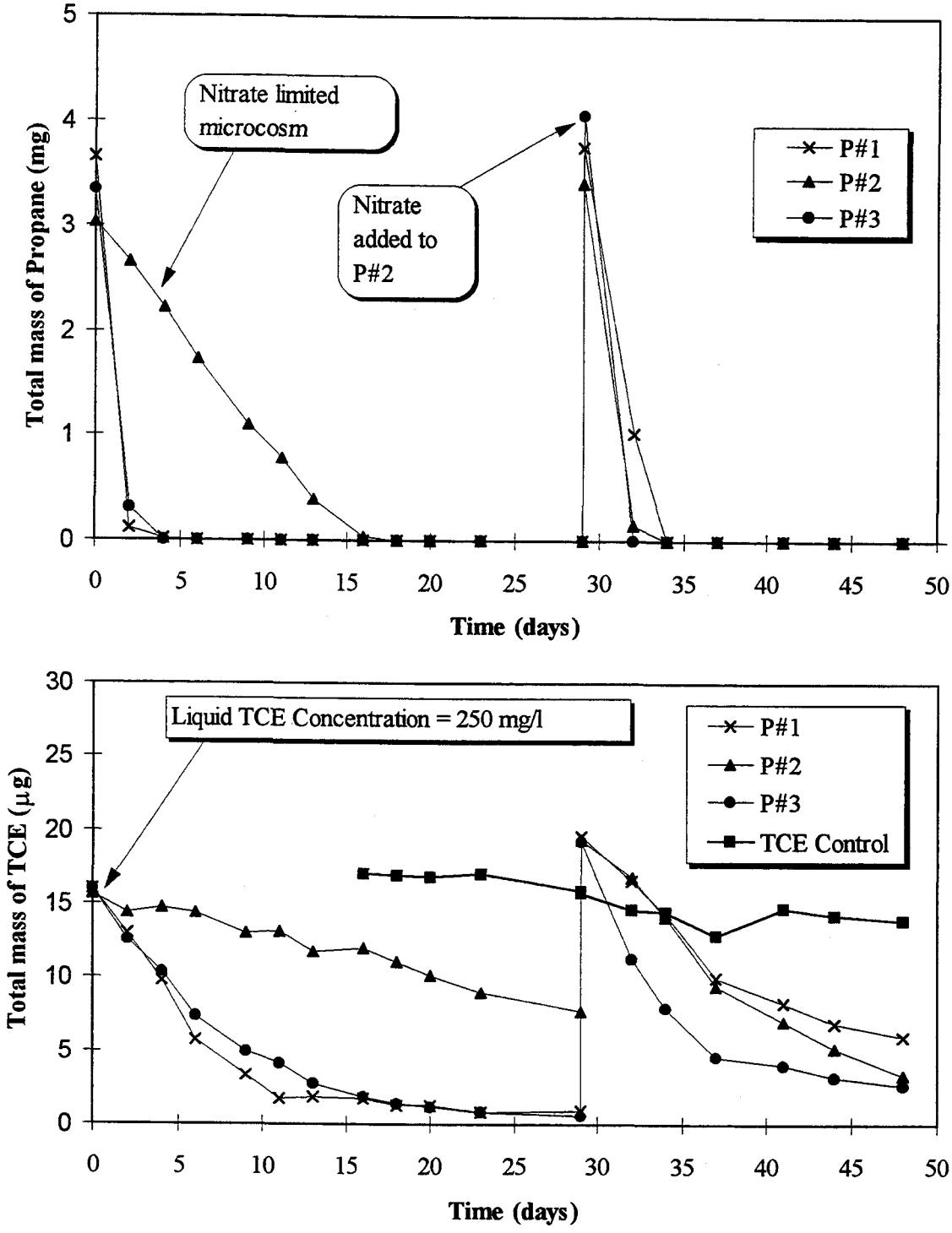


Figure 5.2 The effect of nitrate addition on propane degradation and TCE transformation.

utilization of propane and TCE transformation. Nitrate was amended into M#1 and P#2 upon second additions of methane, propane, and TCE. More rapid removal of methane, propane and TCE was observed, with similar methane and propane removal rates achieved in all the microcosms. TCE transformation was greatly enhanced in microcosm M#1 and P#2 that were initially nitrate limited. The results indicated that nitrate was a limiting nutrient in the site groundwater and the nitrate addition is necessary for effective TCE transformation. The results also indicated that enhancing rates of methane and propane uptake were associated with enhanced TCE transformation.

Figure 5.3 shows the effect of nutrient addition on butane utilization and TCE cometabolism. None of the butane microcosms transformed TCE, with or without nitrate addition. Butane utilization, however, was also enhanced with nitrate addition. Nitrate addition therefore increased butane utilization, but not TCE transformation. No TCE transformation occurred even after several readditions of butane, confirming TCE was not being transformed by butane utilizers.

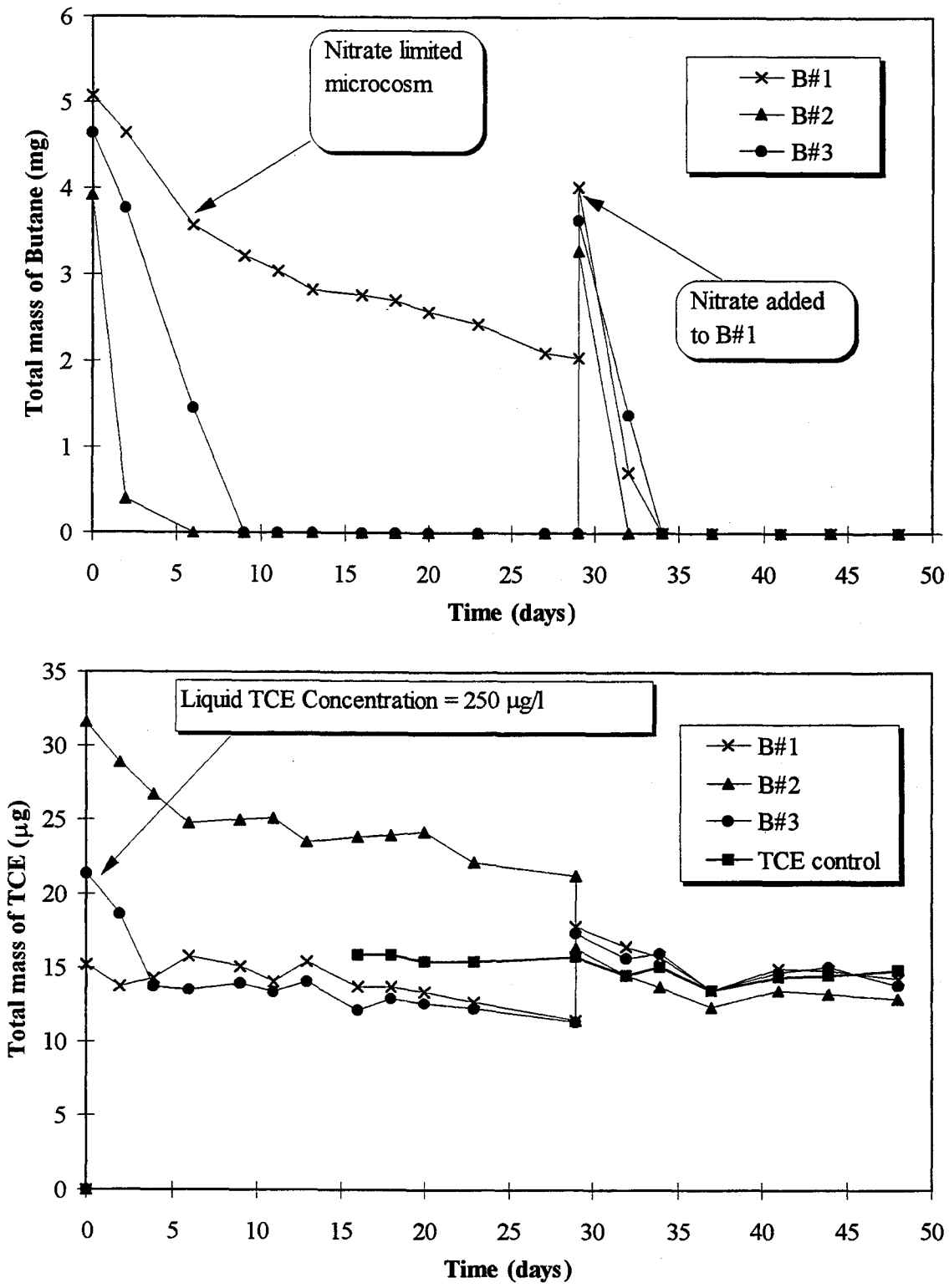


Figure 5.3 The effect of nitrate addition on butane degradation and TCE transformation.

Microcosm studies with methane and propane enrichment cultures

Methane and propane enrichment cultures were used to study the effects of nutrient limitations on TCE cometabolism. In the study, successive addition of substrate and TCE were made without exchanging groundwater or media for over 150 days. The study was conducted in batch microcosms without aquifer solid materials being present. Figure 5.4 shows methane degradation and TCE transformation achieved by methane-utilizers after inoculation into the microcosm containing media. The media contained 0.1 μM of $CuCl_2.2H_2O$ and 23.5 mM of $NaNO_3$ (2000 mg/l of $NaNO_3$) initially. Methane microcosm (MM) showed an increase in TCE transformation ability over 150 days of incubation without adding or exchanging of media. During the first 60 days of incubation, limited TCE transformation was observed in this microcosm. However, the rate and extent of TCE transformation gradually increased and complete removal of TCE was observed after 75 days of incubation. Competitive inhibition was not observed with increasing of TCE concentration. Population shifts in the microcosm may have occurred resulting in an increase TCE transformation ability. This methane microcosm achieved the highest TCE transformation yield of up to 0.21 g TCE/ g methane.

Figure 5.5 and 5.6 present methane degradation and TCE transformation achieved by methane-utilizers after inoculation of the microcosm with groundwater and 50 % media/ 50% groundwater, respectively. The nitrate concentration of groundwater microcosm was initially 33 mg $NaNO_3$ /L, resulting from 1 ml of media being present in the inoculation of batch suspended cultures. The concentration of nitrate in 50% media / 50% groundwater was initially 1000 mg $NaNO_3$ /L. The results shows that during the first 60 days of incubation, complete TCE transformation was observed in both methane microcosms (MG and MMG). Methane microcosm MG, containing 100 % GW, was the most effective to transform TCE followed by methane microcosm MMG, containing with 50% media / 50% groundwater. Effective TCE transformation continued with increasing of TCE concentrations. However, unlike methane microcosm (MM), competitive

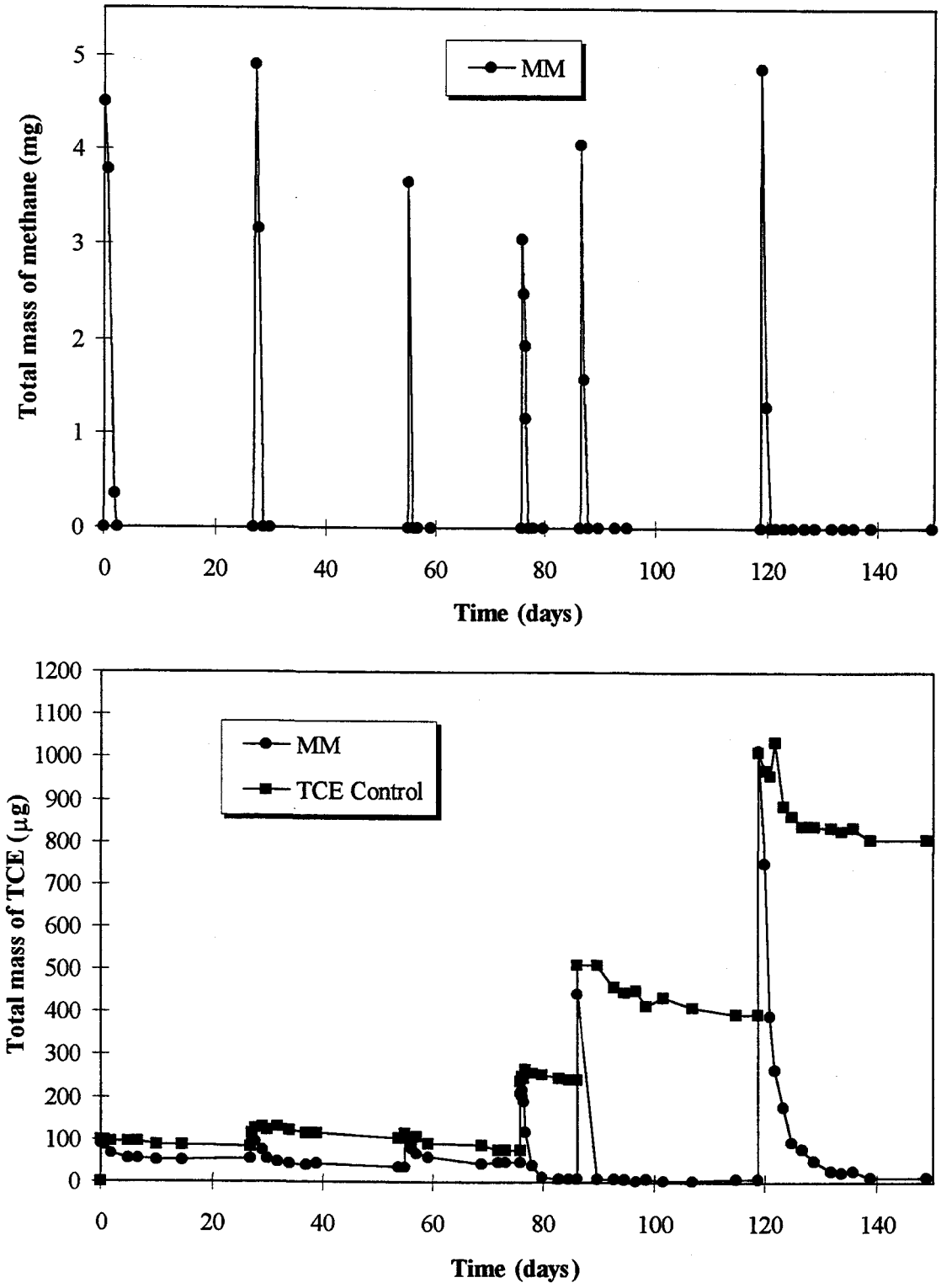
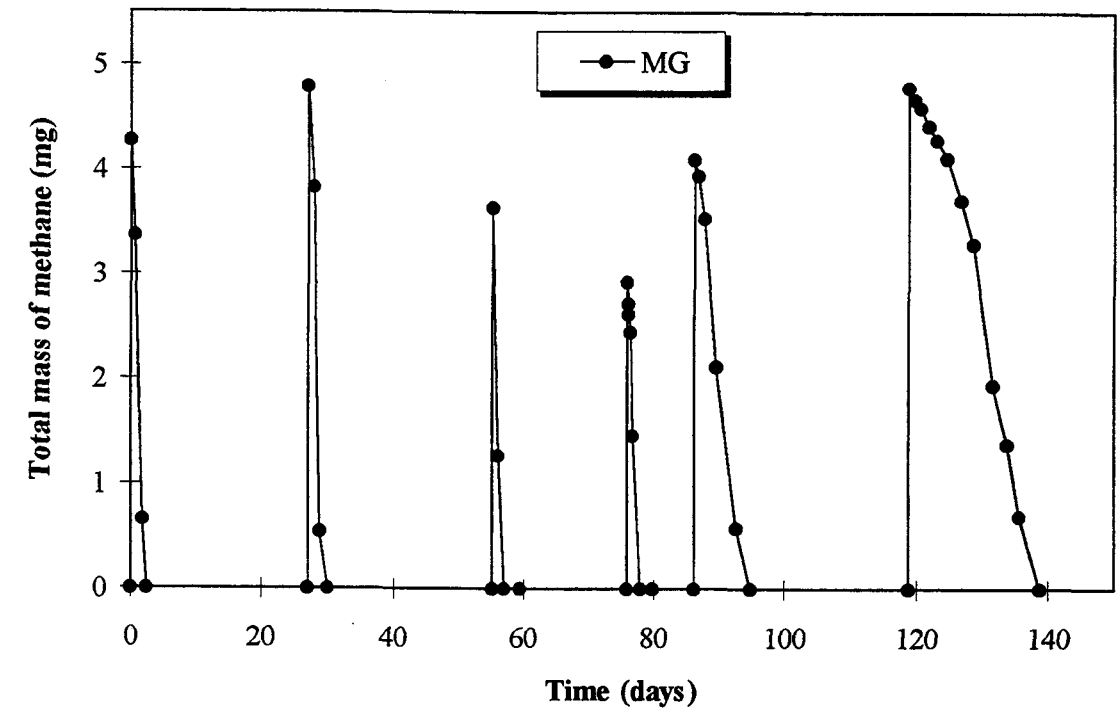


Figure 5.4 Methane degradation and TCE transformation in the microcosm containing 100% media.



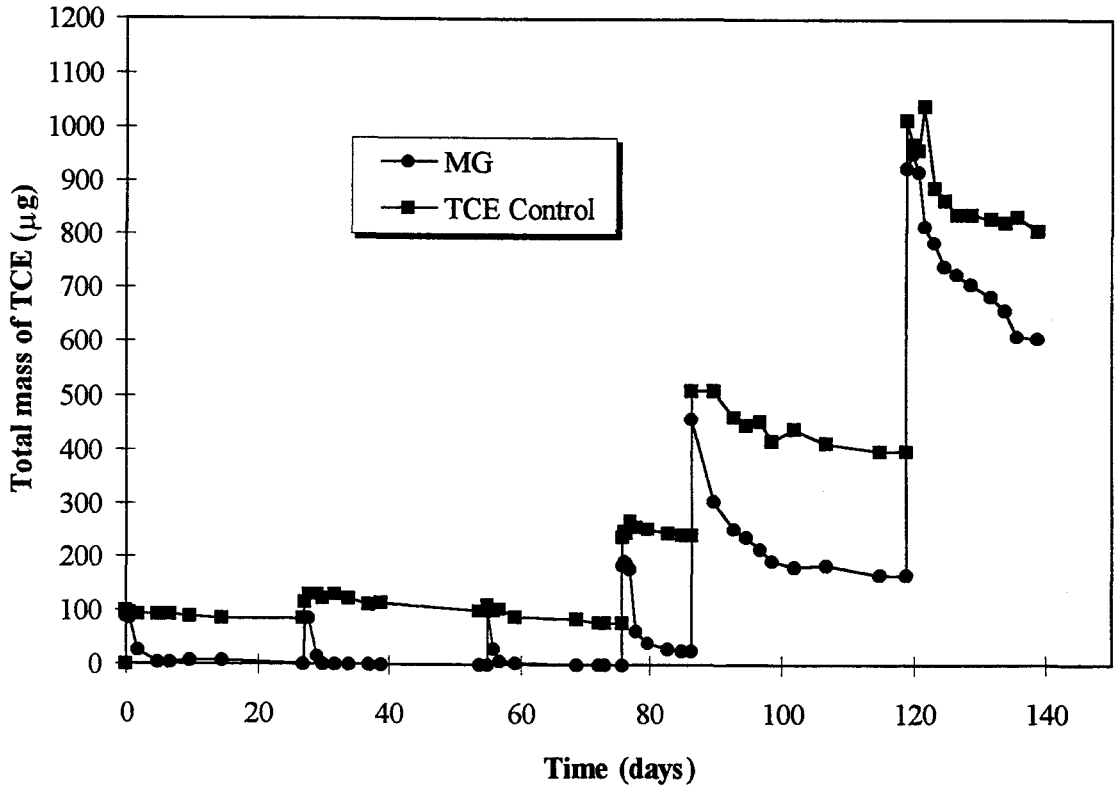
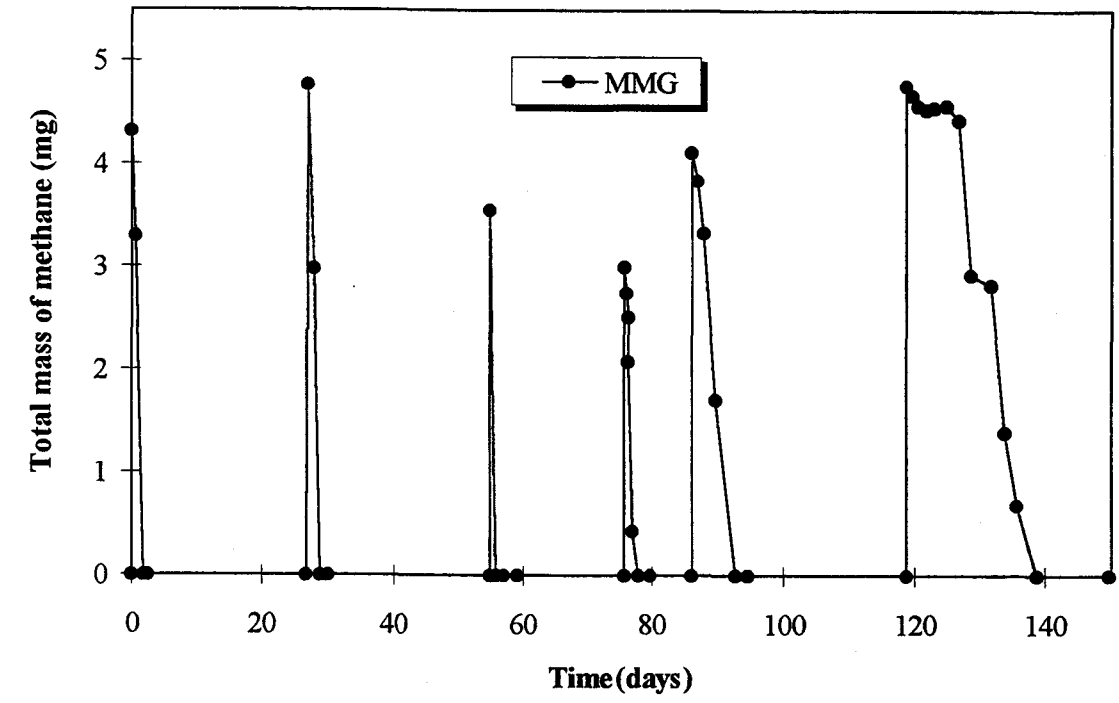


Figure 5.5 Methane degradation and TCE transformation in the microcosm containing 100% groundwater.



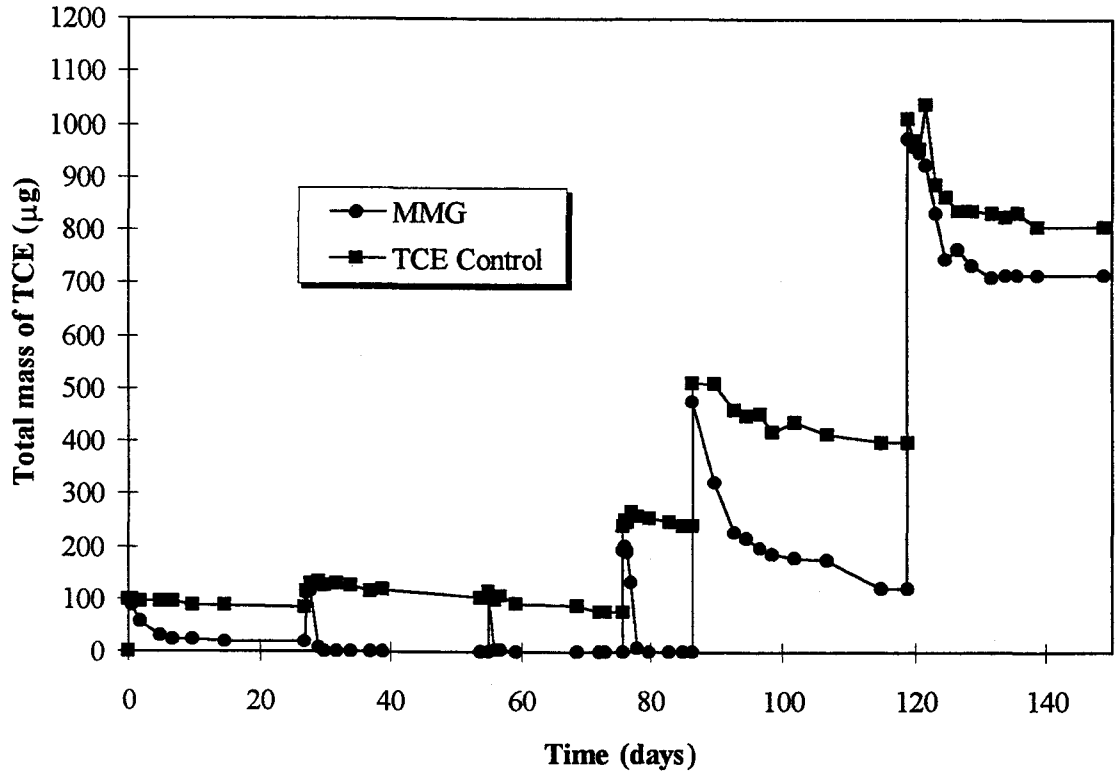


Figure 5.6 Methane degradation and TCE transformation in the microcosm containing 50/50% (media / groundwater).

inhibition between TCE and methane were observed at high TCE concentration in both microcosms. Maximum TCE transformation yields obtained from methane microcosms (MG and MMG) are less than those of methane microcosm (MM).

At the end of the 150 days of incubation, nitrate was found to be completely utilized in all the microcosms. An interesting observation was that effective TCE removal coincided with the formation of bubbles in the microcosms upon shaking. The high accumulation of bubbles was associated with increasing TCE transformation abilities upon depletion of nitrate. The formation of bubbles is possibly related to accumulation of PHB contents in the microcosm as time proceeded.

Figure 5.7 and 5.8 are the results from the control microcosms to which TCE was added after 85 days of incubation in order to confirm the different TCE transformation abilities on media and groundwater. The results shows that TCE transformation in media and groundwater control microcosms were similar to those observed in methane microcosms MM and MG, respectively. This confirmed that the microcosms originally having nutrient rich conditions more effectively transform TCE. The results also indicate that earlier exposure to TCE was not causing the differences in the microcosms. As will be discussed, we feel that the improved TCE transformation ability in the methane-utilizers grown in media may have resulted from nitrogen limitations that occurred as time proceeded. In addition, the formation of bubbles associated with high TCE removal were also observed in these microcosms.

The transformation yields for TCE by methane microcosms containing the different media formulations are presented in Figure 5.9. All microcosms exhibit an increase in TCE transformation yields with increasing TCE concentrations and incubation time. The early time transformation yields were limited by the mass of TCE present. The microcosms containing 100% media exhibited lower transformation yields during the first 60 days of incubation than the microcosms containing 100% groundwater or 50% media/ 50% groundwater. After 75 days of incubation, the situation was reversed, with the highest TCE transformation yield (0.21 g TCE/g methane) was observed in the methane microcosm containing 100% media.

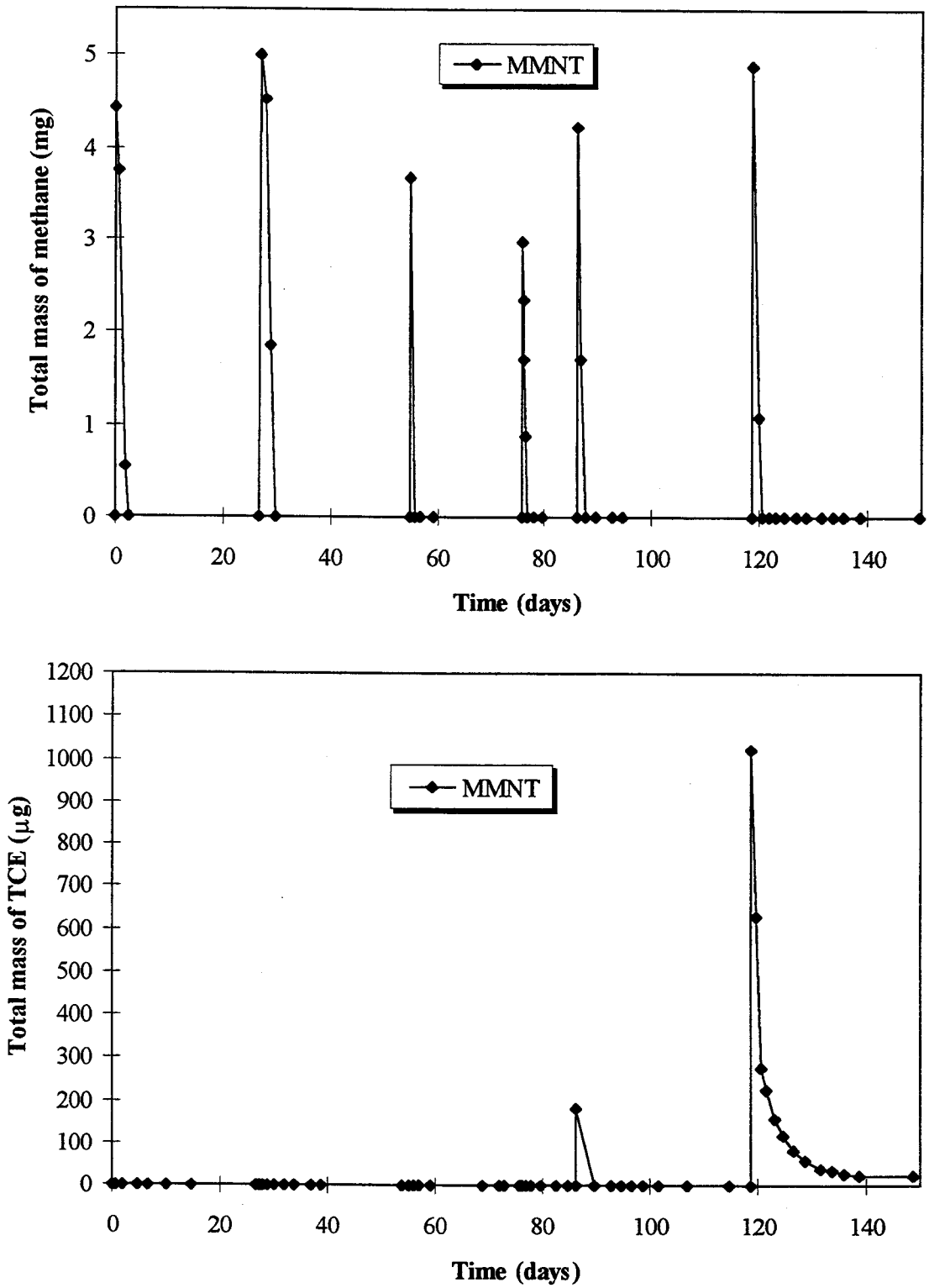


Figure 5.7 Methane degradation and TCE transformation in the control microcosm containing 100% media.

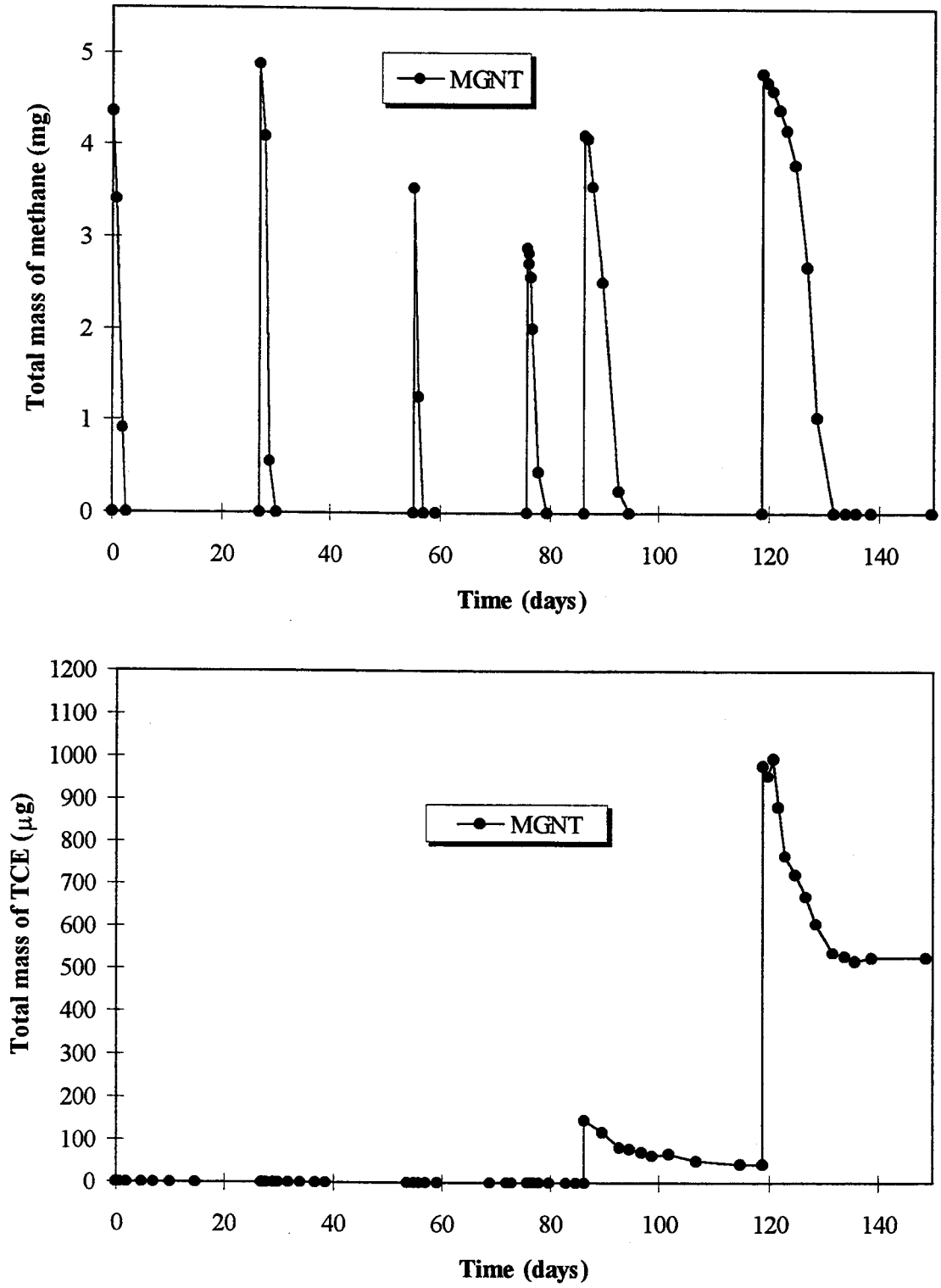


Figure 5.8 Methane degradation and TCE transformation in the control microcosm containing 100% groundwater.

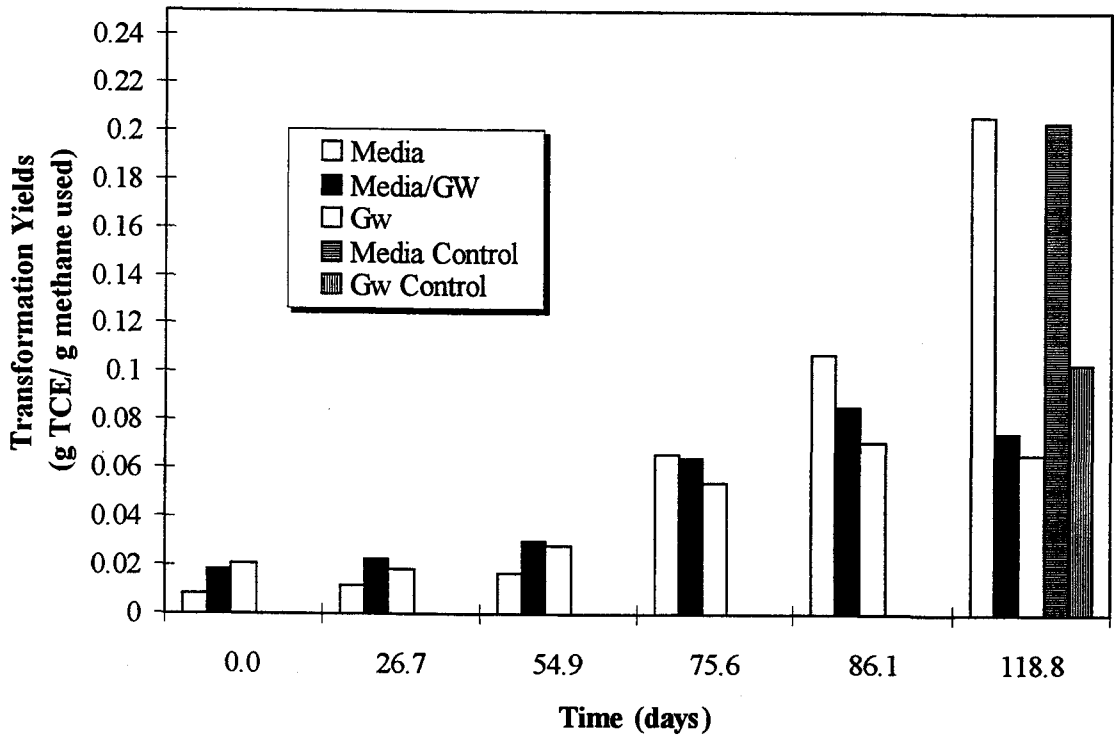


Figure 5.9 Transformation Yields for TCE by methane-utilizers on different medium conditions.

Figure 5.10, 5.11, and 5.12 present the TCE transformation and propane degradation in the propane microcosms with the different medium conditions. Unlike methane microcosms, all propane microcosms showed similar TCE transformation abilities over 120 days of incubation among different medium conditions. Transformation yields for TCE among propane microcosms were less than those observed in methane microcosms. TCE transformation yields of 0.013 g TCE/ g propane were observed in all propane microcosms. This result differs from those of propane-utilizers that stimulated in the microcosms constructed with groundwater and aquifer solids. TCE transformation yields observed in propane microcosms without aquifer solids were less than the propane microcosms with aquifer solids. Prolonged TCE transformation activity after propane was consumed was not observed in propane enrichment studies. It is interesting that long term TCE transformation activity was achieved with aquifer solids and the background groundwater chemistry.

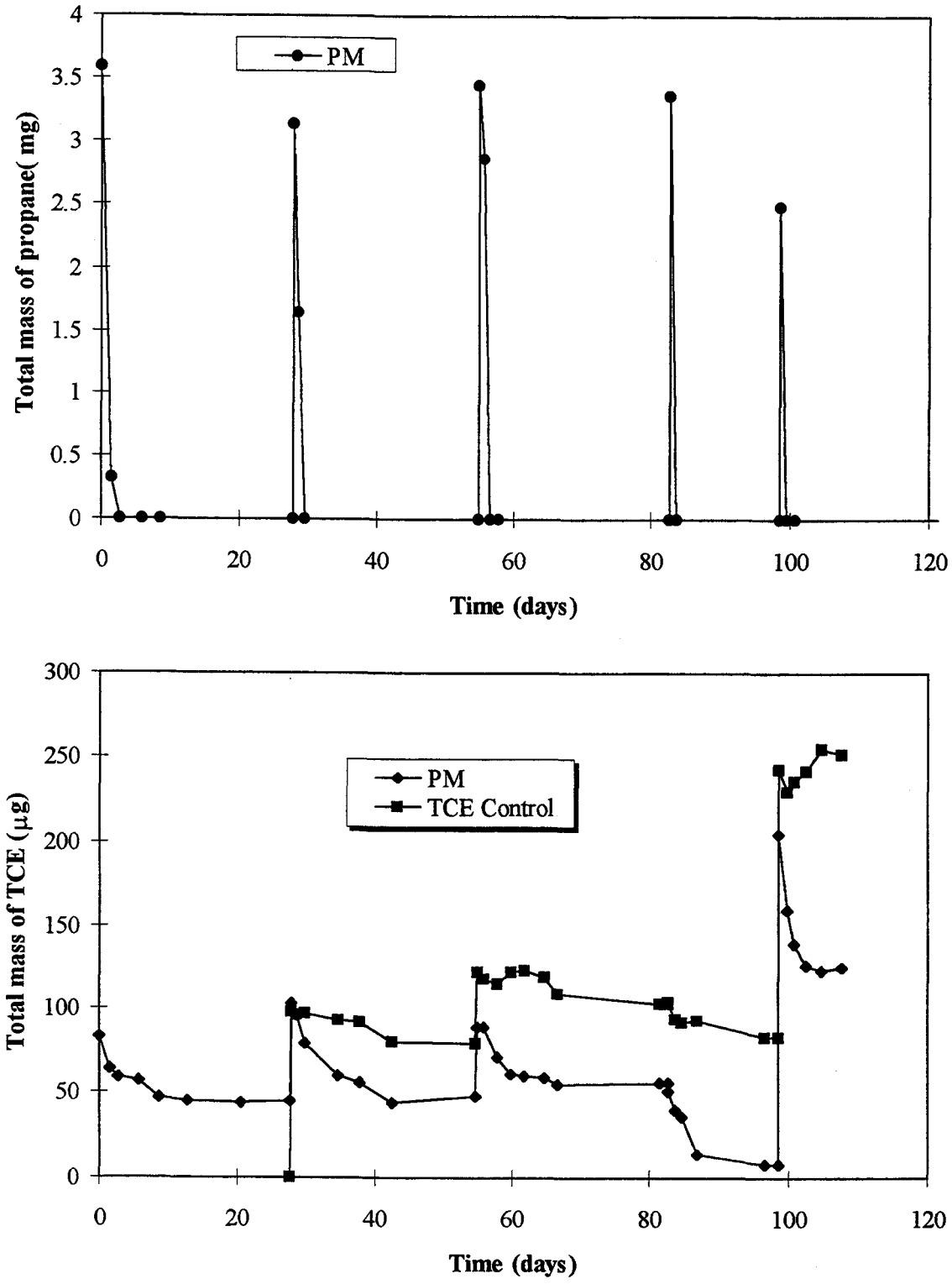


Figure 5.10 Propane degradation and TCE transformation in the microcosm containing 100% media.

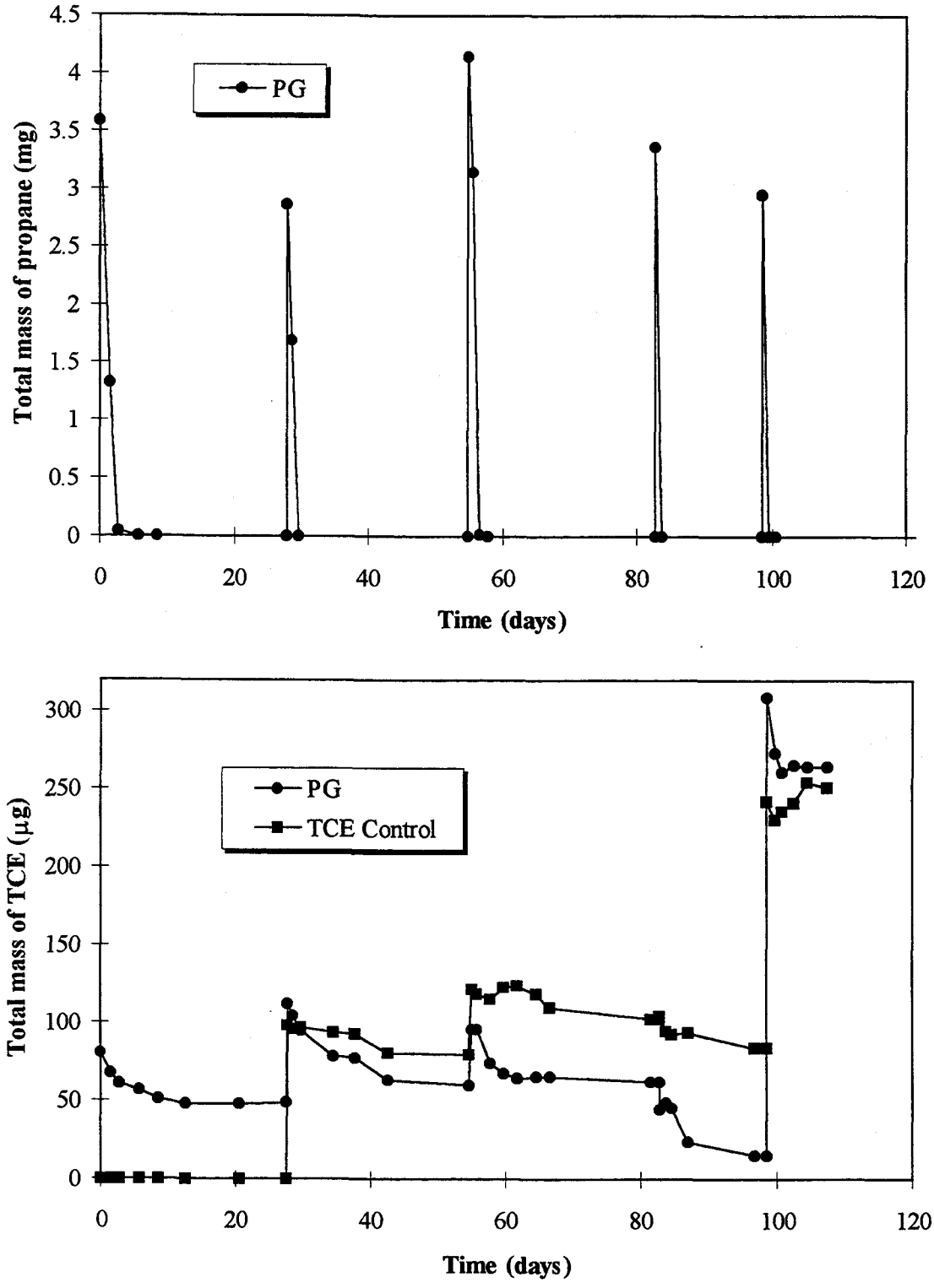


Figure 5.11 Propane degradation and TCE transformation in the microcosm containing 100% groundwater.

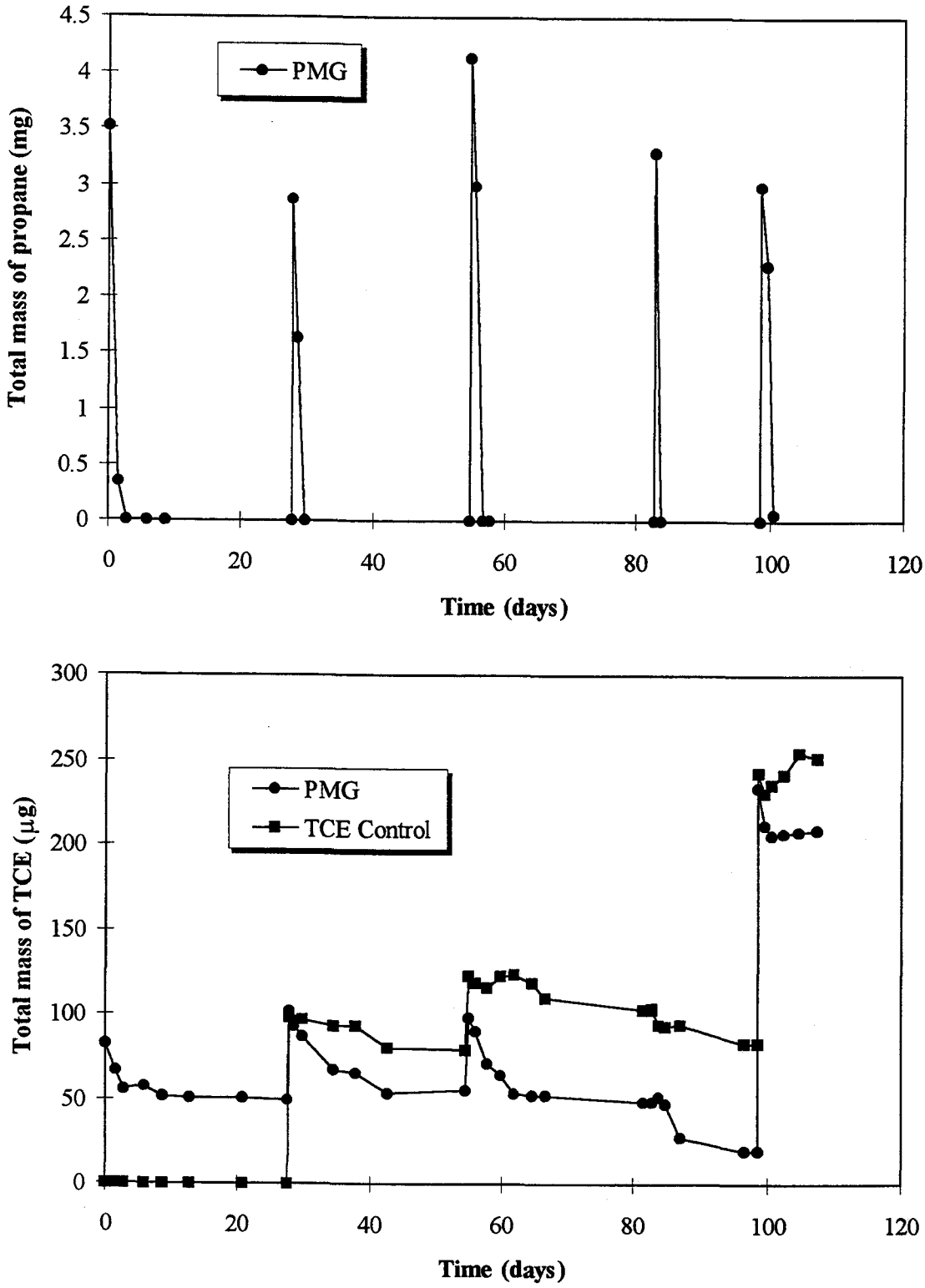


Figure 5.12 Propane degradation and TCE transformation in the microcosm containing 50/50 % (media and groundwater).

Discussion

The results from microcosm studies with aquifer solids and groundwater indicated that the availability of nitrogen (as nitrate) strongly influenced TCE transformation. When nitrate was a limiting nutrient earlier in the incubations, TCE transformation by both methane and propane-utilizers was greatly reduced, even under conditions where the primary substrate was utilized, but at a reduced rate. The results from butane microcosms showed nitrate addition increased butane utilization but not TCE transformation. The addition of nitrate is necessary during biostimulation for effective in-situ cometabolism, when a nitrogen source is limiting in the subsurface.

The results from methane enrichment cultures grown under different medium conditions show that nutrient availability had a great impact on TCE transformation ability of methane-utilizers. All methane microcosms continued their ability to transform TCE over 150 days without exchanging media or groundwater. TCE transformation yields in all methane-utilizers increased with time, however TCE transformation yields at early time were limited by the mass of TCE present. Nutrient availability, possibly nitrate or copper, may have played major roles on methane-utilizer's ability to transform TCE. Low copper concentration (0.1 μM) in the microcosms established the copper-limited conditions that may be favorable to the expression of sMMO. This results were consistent with previous study by Graham, et al., 1993. They revealed that copper limitations favored Type II *M. trichosporium* OB3b. The Type II organisms were dominated under conditions that induced sMMO expression. sMMO activity appeared at a measurable soluble copper concentration level of 0.15 μM.

We also suspect that nitrogen fixation occurred in the microcosms resulting in an increase TCE transformation ability. Depletion of nitrate in the microcosms presumably resulted in nitrogenase activity. This was consistent with the observations of bubble formation in the microcosms. The formation of bubbles was associated with high TCE removal in all methane microcosms. It may be that the bubbles were an indicator of high PHB contents in the microcosms as a result of nitrogen-limiting conditions. Type II methanotrophs (sMMO form) which were capable of fixing nitrogen may have been

selected when nitrate was limited in the microcosms. These results are consistent with the prior studies. Type II methanotrophs that efficiently transformed TCE accumulated high PHB contents under copper and nitrogen-limited conditions (Graham et al.,1993; Chu and Alvarez-Cohen, 1996; Shah et al., 1996). It is also interesting that our maximum TCE transformation yields of 0.21 (g TCE/g methane) observed in methane microcosms upon depletion of nitrate were higher than the prior studies with resting cells of nitrogen-fixing methanotrophs. TCE transformation yields of 0.020, and 0.11 (g TCE/ g methane) (0.25 g TCE/ g dry cell wt, assuming net cell yield of 0.4 g dry cell wt / g methane), were observed by Chu and Alvarez-Cohen, 1996 and Shah etal., 1995, respectively.

This study suggests that TCE transformation may be enhanced by methane-utilizers for in-situ bioremediation by first growing them under rich nutrient conditions and then limiting nutrients such as nitrogen. Further research is required to test this hypothesis.

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CHAPTER 6

Future Research

Based on this research, the following recommendations for future research are as follows:

1). Determine what is causing of the prolonged TCE activity of propane-utilizers.
2). Determine whether intermediate by products from propane degradation serve as an alternative energy source for enhancing TCE cometabolism.
3). Study the competitive inhibition and toxicity effects of TCE transformation on propane-utilizers
4). Evaluating the product toxicity and inhibitory effects from transformation of chlorinated aliphatic hydrocarbons including TCE by indigenous methane and propane-utilizers.
5). Determine kinetic parameters from the batch microcosm methods and develop an effective kinetic model for TCE and CAHs transformation in the presence of methane or propane as growth substrates.
6). Evaluate strategies for nutrient addition that help optimize cometabolic transformation of TCE and other CAHs.
7). Develop the bioremediation system that looks most promising for in-situ bioremediation of CAHs at McClellan Air Force Base.
8). Determine whether more effective transformation of CAH mixtures can be achieved using mixtures of cometabolic substrates.
9). Study the affect of long term CAHs bioremediation using continuous flow column microcosms studies.

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APPENDICES

Appendix A

Experimental Protocol for Microcosms Studies

Materials

- 125 ml amber serum bottles
- Teflon™ butyl rubber cap
- 1.5 cm crimp seals
- Groundwater from McClellan AFB (Site 22) FD-GW-02
- McClellan aquifer solids (Upper A zone, 105A and 108.5C, Site 22)
- Pure O_2 cylinder with regulator
- Pure N_2 cylinder with regulator
- Pure CH_4 cylinder with regulator
- 10 % propane and butane cylinder with regulator
- 25 ml gas-tight syringes
- Sieve No. 8 (2.38 mm opening)
- Laminar flow hood
- Mechanical shaker table
- Sterilized filters (0.45 μm)
- Mechanical centrifuges
- 10 μl, 100 μl and 50 μl gas-tight microsyringes
- 5 ml and 500 μl liquid microsyringes
- 500 ml volumetric flasks
- 15 ml and 100 ml beakers
- 10 ml and 50 ml volumetric pipettes
- Vortex mixer
- Pipette bulb
- Autoclave apparatus

Chemicals

- High grade trichloroethylene, 1,1,1-trichloroethane, and chloroform
- High grade methanol
- Sodium nitrate
- Yeast extract
- K_2HPO_4 + NaH_2PO_4 (a buffer 7.5 solution)
- $MgSO_4$
- $CaCl_2$
- $(NH_4)_2SO_4$
- Trace element solution

Microcosm Preparation and Operation

Indigenous microcosm studies with aquifer solids

The studies were performed in batch microcosms constructed with aquifer material and groundwater from McClellan Air Force Base. Methane, propane and butane were used as growth substrates for each of microcosm studies. The microcosm method was adapted from Broholm et. al., (1990) and Yi Mu and Scow, (1994). Duplicate microcosms were prepared for each of substrates tested. The microcosms were constructed using 125 ml amber serum bottles (Wheaton Class Co., Millville, NJ.). Aquifer material from the McClellan Air Force Base, Sacramento, CA, was wet sieved with site groundwater under a laminar flow hood using a No. 8 sieve (2.38 mm opening) to remove large particles. The site groundwater was filtered (0.45 μm sterilized filter) before use. 15 ml of wet solids and 50 ml of filtered ground water were added to each batch microcosm, leaving a 60 ml air-filled headspace as a source of oxygen. The headspace permitted sampling of the gaseous substrate, oxygen, and CAHs. The microcosms were crimp sealed with a Teflon™ butyl rubber cap (Kimble Co., IL), then inverted and incubated at room temperature on a shaker table at 100 rpm. The microcosms were maintained for a one year period, with periodic groundwater exchanges and readdition of growth substrates and CAH.

Enrichment microcosms with media and groundwater

Mixed methane and propane enrichment cultures used in this study were obtained from batch microcosm studies on McClellan AFB. Methane and propane enrichment cultures which are capable of TCE degradation from previous microcosm experiments were separately grown in the growth medium containing with : 15 mM. K_2HPO_4 + NaH_2PO_4 (a buffer 7.5 solution), 0.5 mM. $MgSO_4$, 0.1 mM. $CaCl_2$, 23.5 mM. $NaNO_3$, 0.796 mM. $(NH_4)_2SO_4$, 0.1 g of yeast extract, and 1ml of trace element solutions. Each of suspended cultures in 500 ml serum bottles were then shaken and incubated at 30 °C on a shaker table at 200 rpm. Each of 500 ml serum bottles was supplied with methane or propane to sustain the microbial growth before use in the experiments.

Enrichment cultures were used to study the effect of nutrient addition on TCE cometabolisms without exchanges of groundwater. The effect of nutrient addition were investigated in 125 ml serum bottles. The serum bottles were inoculated with 1 ml of each of batch suspended cultures before addition of growth substrate and TCE. Three serum bottles were constructed for each of substrate contained with 60 ml of different medium. The medium contents of three different serum bottles were 100 % media, 100 % groundwater, and 50/50 % media /groundwater. The serum bottles were then crimp sealed with a Teflon^{TM} butyl rubber (Kimble Co., IL) then inverted and incubated at room temperature on a shaker table at 100 rpm. The serum bottles were maintained for a period of 200 days without groundwater exchange and nutrient addition.

Control microcosms

Control microcosms included: 1) CAH control microcosms containing with aquifer solids, groundwater, and CAH, but lacking the growth substrate; 2) sterilized control microcosms prepared in the above manner but exposed for 11 hours to a Cobolt 60 gramma irradiation source. After irradiation, filtered (0.45 μm) ground water was added to the microcosm under a laminar flow hood.

Sampling Procedure

The pressure of headspace microcosms were equalized to the atmospheric pressure using pure oxygen before gas and liquid sampling was performed. All gas samples were withdrawn from microcosm headspace using a 25 or 100 µl gas-tight microsyringes. A dry syringe was needed to ensure sample integrity. The syringe was ringed five times with methanol and stored in the oven overnight before use. The gas samples were used to quantified methane, propane, butane and CAH concentrations on gas chromatography (FID and ECD). Liquids samples were obtained using 100 µl syringe. The sampling syringe were rinsed with ten times with methanol and water between each sampling. The liquid samples were used for analysis of aqueous CAH concentrations and nitrate concentrations using gas chromatography (ECD, purge and traps) and anionic analyses, respectively.

Groundwater Amendment

The microcosms were maintained for a one year period, with periodic groundwater exchange and substrate readdition. The exchange of 25 ml of groundwater was performed in the batch microcosm prior to readditions of the growth substrate and TCE. The groundwater was amended with nitrate to 30 mg/L, since nitrogen was found to be limiting in the groundwater. The microcosms were centrifuged for 20 min at 1000 rpm to keep the microorganisms in the microcosms. The serum caps were then removed under a laminar flow hood. 25 ml of groundwater was replaced with new ground water and the microcosms were then resealed.

Media Content

Growth medium containing with : 15 mM. K_2HPO_4 + NaH_2PO_4 (a buffer 7.5 solution), 0.5 mM. $MgSO_4$, 0.1 mM. $CaCl_2$, 23.5 mM. $NaNO_3$, 0.796 mM. $(NH_4)_2SO_4$, 0.1 g of yeast extract, and 1ml of trace element solutions. Each of suspended cultures in 500 ml serum bottles were then shaken and incubated at 30 °C on a shaker table at 200 rpm. Each of 500 ml serum bottles was supplied by methane or propane to sustain the microbial growth before use in the experiments.

Appendix B

Analytical Methods for CAHs

Protocol for Preparing Saturated Aqueous Stock Solutions

This method was adapted from Niemet, 1995 and Kim, 1996. The following procedures were used to meet CAH concentrations requirements for aerobic microcosm studies.

Materials

- 125 ml amber serum bottle, including Teflon™ butyl rubber cap
- Small beaker
- High grade CAH of interest

Procedure

1. Autoclave the beaker, serum bottle containing RO/DI water and cap
2. A saturated CAH stock solution was prepared by adding 4 ml of pure TCE in a 125 ml capped serum bottle containing with RO/DI water. For TCE, CF and 1,1,1-TCA, the chemical and physical properties are shown in Table B.1.
3. Cap bottle, label and date.
4. The bottle was shaken vigorously and allowed to settle for at least 24 hours before use.

Table B.1. Physical and Chemical Properties of TCE, CF, and 1,1,1-TCA.

CAH Properties	TCE	1,1,1 TCA	CF
Formular	$CHCl=CCl_2$	CCl_3CH_3	$CHCl_3$
Boiling point (°C)	87.2	74.1	61.7
Aqueous Solubility (mg/L)	1080 @ 20° C	1550 @ 20° C	9300 @ 25° C
Molecular weight	131.4	133.42	119.38
Henry's law constant (Hpc), 20 °C ($atm.m^3/mol$)	9.9×10^{-3}	1.5×10^{-2}	3.39×10^{-3}
Henry law constant (Hcc), 20 °C (dimentionless)	0.342	0.642	0.109

GC Standard Curve Protocol for CAHs

Materials

- High grade CAHs
- Three 10 ml volumetric flasks
- Three 125 ml amber serum bottles with Teflon™ butyl rubber cap
- 10, 50 and 100 µl liquid microsyringes
- 10 and 50 ml volumetric pipettes
- Automatic Pasteur pipettor with pipettes
- Cap crimper
- Vortex mixer

Stock Solution Preparation (Standard Methods, 1992)

1. Fill a 10 ml volumetric flasks nearly to the meniscus with methanol.
2. Place the uncapped flask on balance and allow 20 minutes for the methanol on the sides of the meniscus to dry.
3. Add 1 drops of solvent from a 10 µl microsyringe to volumetric flask, being careful to prevent the drops from touching the sides of the flask.
4. Read the values from the balance and quickly recorded the added weight.
5. Fill a 10 ml volumetric flasks completely to the meniscus with methanol.
6. Cap the flask and mix completely on mixer votex.
7. Store in refrigerator for later use. Discard stocks after 1 month.

Gas Standard Preparation of CAHs

1. Fill five 125 ml amber serum bottle with 65 ml autoclaved DI water and cap completely.
2. Add appropriate amount of the stocks, using the appropriate microsyringe, to obtain combined concentrations of CF, TCE and 1,1,1-TCA in the amber serum bottles. Table B.2 presented the gas standard concentration of CF, TCE and 1,1,1-TCA in the amber serum bottles.
3. Number and record concentrations.

4. Place on the mechanical shaker for 4 hours before use

Table B.2. Gas standard concentration of TCE, CF, and 1,1,1-TCA

CF standard concentration (μg/L)	TCA standard concentration (μg/L)	CF standard concentration (μg/L)
0	0	0
40.73	123.79	83.67
81.46	247.58	167.35
122.19	371.38	251.03
162.92	295.17	334.71
203.65	618.97	418.30

Analysis

TCE, 1,1,1-TCA, and CF were determined from analysis of the headspace of the serum bottles. A Hewlett Packard (Wilmington DE) 5890 gas chromatograp equipped with a 3393 A integrator and a ^{63}Ni electron capture detector was used to quantified CAH concentrations. Separation was obtained by a capillary (HP-624; 19091v-433; 1,4 μm length; Hewlett Packard, Wilmington, DE.) operated isothermally at 80 °C. An argon/methane (95/5) mixture at head pressure of 60 psi was used as the carrier gas. Injections of 100 μl were used for CAH headspace analysis.

Sample Chromatograms

A set of chloromatograms for a typical standard curve are included below. Standard curves were obtained by plotting the signal output (Area) vs gas standard concentration for each of compound and fitting an equation to the data. Over the range considered of CAH concentrations, the area was linear, with an $r^2 > 0.099$ obtained by fitting to linear square fit using Microsoft ® Excel 95.

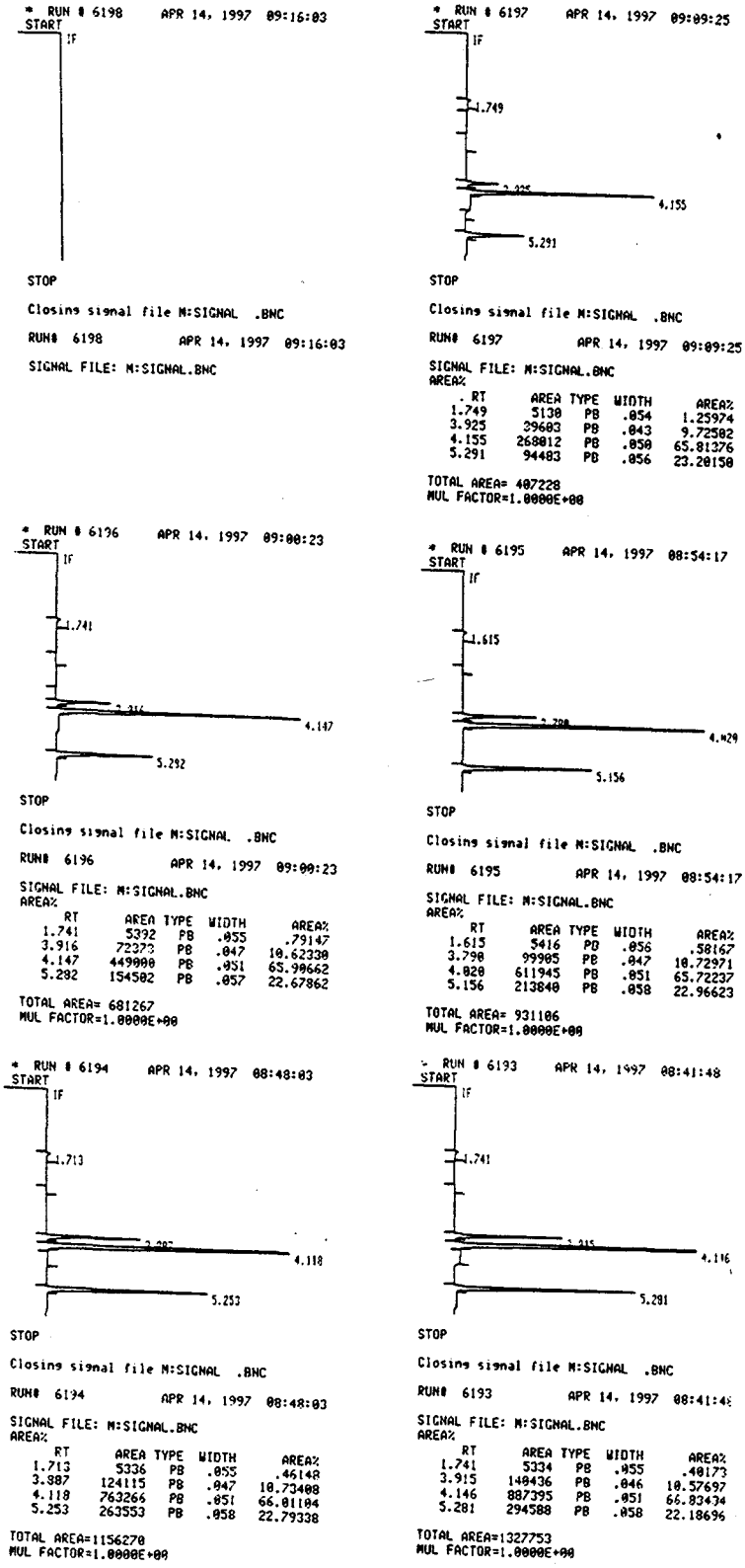


Figure B.1 GC chromatograms for gas standard concentration of CF, 1,1,1-TCA and TCE.

Liquid Standard Preparation of CAHs

1. Fill five 125 ml amber serum bottle with 65 ml autoclaved DI water and cap completely.
2. Add appropriate amount of the stocks, using the appropriate microsyringe, to obtain combined concentrations of CF, TCE and 1,1,1-TCA in the amber serum bottles. Table B.2 presented the aqueous standard concentration of CF, TCE and 1,1,1-TCA in the amber serum bottles.
3. Number and record concentrations.
4. Place on the mechanical shaker for 4 hours before use

Table B.3. Aqueous standard concentration of TCE, CF, and 1,1,1-TCA

CF standard concentration (μg/L)	TCA standard concentration (μg/L)	CF standard concentration (μg/L)
0	0	0
5.77	3.96	4.83
11.55	7.93	9.67
17.33	11.90	14.51
23.10	15.87	19.34
28.88	19.83	24.18

Analysis

The analysis of TCE, 1,1,1-TCA, and CF concentrations in liquid phase were quantified by the purge and trap method using a modified version of standard EPA Method 8010. A Hewlett Packard Purge and Trap model 7695 was used in conjunction with a Hewlett Packard 5890 gas chromatography equipped with an Hall conductivity detector. A 100 μl sample was diluted in 5 ml of glass distilled water and then transferred into the trap of the purge and trap unit. Separation were obtained by a capillary column (HP-624; 19091v-433; 1.4 μm length; Hewlett Packard, Wilmington, DE.).

Sample Chromatograms

A set of chloromatograms for a typical standard curve are included below. Standard curves were obtained by plotting the signal output (Area) vs liquid standard concentration for each of compound and fitting an equation to the data. Over the range considered of aqueous CAH concentrations, the area was linear, with an $r^2 > 0.099$ obtained by fitting to linear square fit using Microsoft ® Excel 95.

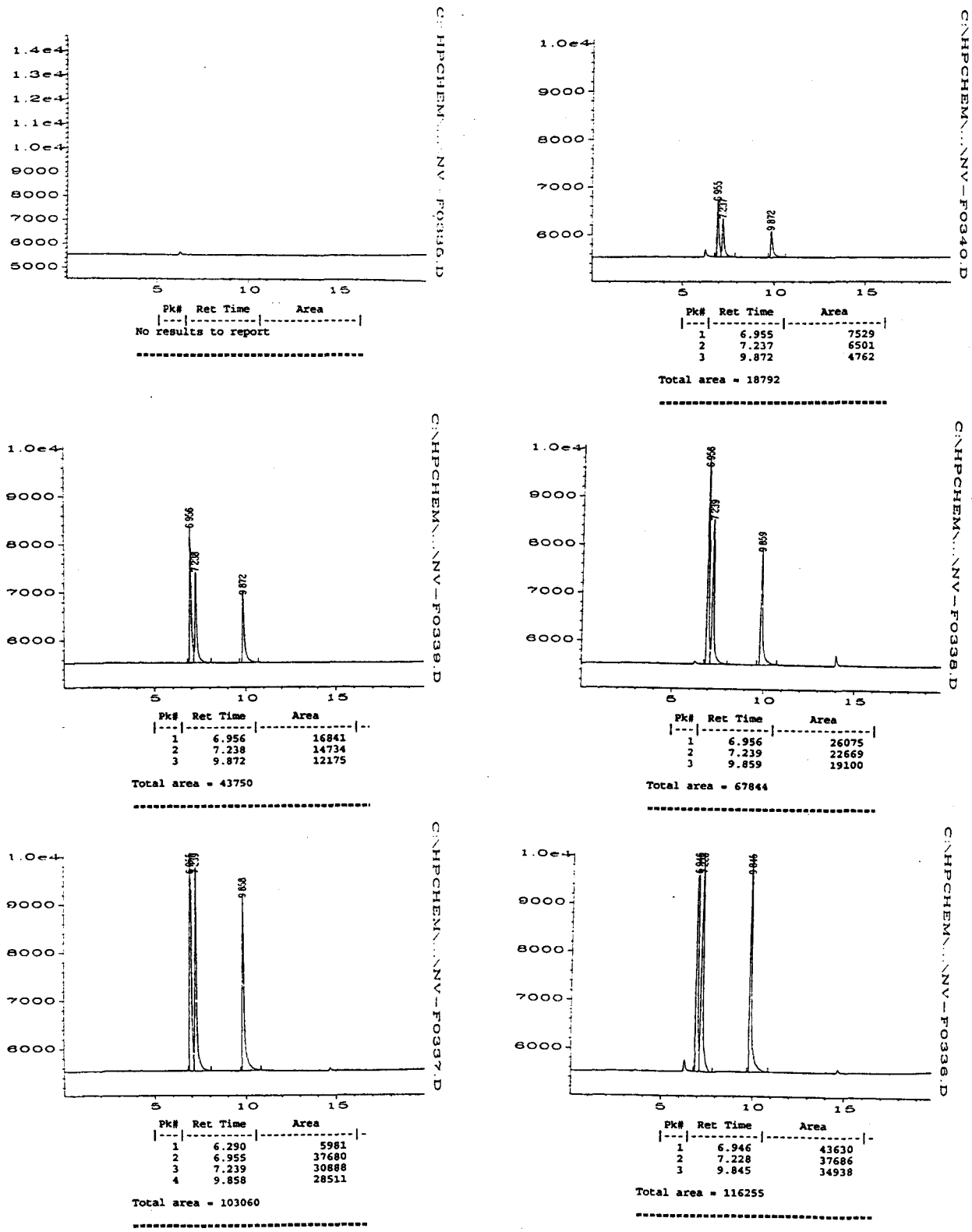


Figure B.2 GC chromatograms for aqueous standard concentration of CF, 1,1,1-TCA and TCE.

Appendix C

Analytical Methods for Oxygen, Methane, Propane and Butane

Materials

- Methane (99.9%), Propane (10% in introgen), and butane (10% in introgen)
- Three 125 ml amber serum bottles with Teflon™ butyl rubber cap
- Three 100 µl liquid microsyringes
- 10 and 50 ml volumetric pipettes
- Cap crimper
- Mechanical shaker
- Regulator
- Needle

Gas Partitioner Standard Curve Protocol for Oxygen

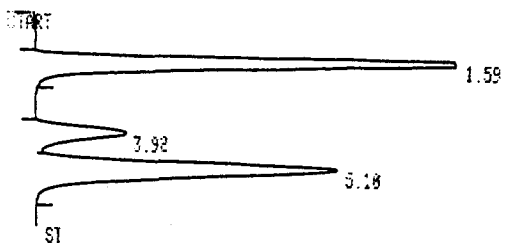
1. Calculate amount of oxygen required to obtain a concentration of 20% of air headspace
2. Fill the 125 ml amber serum bottles with 50 ml of DI water
3. Cap and shake thoroughly
4. 100 µl gas-tight syringe containing with a lure-lock tip and a septum were used to obtain concentration dilution.

Analysis

Headspace oxygen concentrations were determined on a Fisher Model 25V gas partitioner using nitrogen as a carrier gas. A 100 µl headspace from 125 ml amber serum bottles were obtained with a Pressure-Lok gas tight syring (Hamilton Co., Reno, NEV.). Separation were obtained by a stainless steel packed column (Supelco, INC., Bellefonte, PA).

Sample Chromatograms

A set of chromatograms for a oxygen standard curve is included below. Standard curves were obtained by plotting the signal output (area) of oxygen at the retention time of 3 minutes versus oxygen concentrations. Reasonable modeling of the data ($r^2 > 0.99$) was obtained by linear regression using Microsoft ® Excel 95.

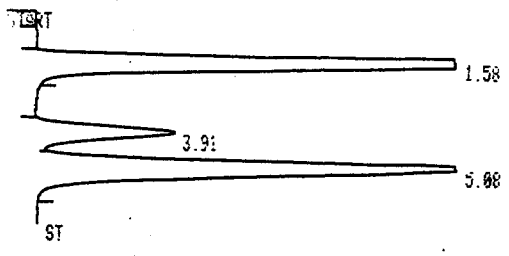


RUN # 1028 MAY/30/96 17:45:55

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.59	216330	PB	0.372	47.789
3.92	49556	BV	0.474	10.947
5.10	186800	VB	0.541	41.264

TOTAL AREA= 452690
MUL FACTOR= 1.0000E+00

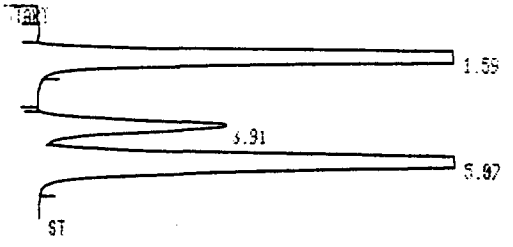


RUN # 1027 MAY/30/96 17:34:17

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.58	328700	PB	0.372	47.832
3.91	75286	BV	0.471	10.956
5.08	283210	VB	0.540	41.212

TOTAL AREA= 687190
MUL FACTOR= 1.0000E+00

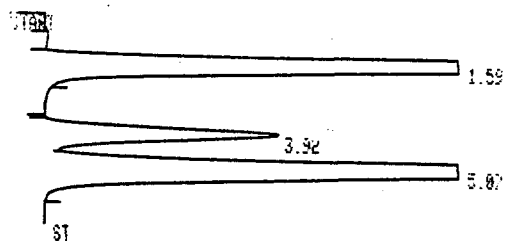


RUN # 1026 MAY/30/96 17:24:09

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.59	442180	PB	0.372	47.827
3.91	101270	BV	0.470	10.953
5.07	381100	VB	0.541	41.220

TOTAL AREA= 924550
MUL FACTOR= 1.0000E+00



RUN # 1025 MAY/30/96 17:16:01

AREA%

RT	AREA	TYPE	AR/HT	AREA%
1.59	555720	PB	0.377	47.915
3.92	126450	BV	0.472	10.903
5.07	477640	VB	0.543	41.182

TOTAL AREA= 1159800
MUL FACTOR= 1.0000E+00

Figure C.1 Chromatograms for headspace concentration of oxygen.

Standard Curve Protocol for Methane, Propane and Butane

1. Calculate amount of methane, propane, and butane required to obtain a headspace concentration of 100 mg methane/L, 100 mg propane/L, and 100 mg butane/L.
2. Fill the 125 ml amber serum bottles with 90 ml of DI water
3. Cap and shake vigorously and label.
4. Calculated amount of methane, propane, and butane were transferred from gas containers to each of 125 ml serum bottles by direct volume additions with 25 ml gas-tight syringes (Hamilton Co., Reno, NEV.).
5. 100 µl gas-tight syringe were used to obtain dilution concentrations.

GC Standard Curve Protocol for Methane, Propane and Butane

Methane, propane and butane concentrations were quantified by headspace analysis using a Hewlett Packard 5890 gas chromatography equipped with a flame ionization detector coupled with a 1.0 m - Hayesep Q stainless steel micropacked column (Restek Corporation, Bellefonte, PA). A 100 µl sample was used.

Sample Chromatograms

A set of chromatograms for methane, propane and butane standard curve are included below. All standard curves were obtained by plotting the signal output (area) of gas versus the concentrations. Reasonable modeling of the data ($r^2 > 0.99$) was obtained by linear regression using Microsoft ® Excel 95.

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*  RUN # 6187      APR 14, 1997  07:42:46
START; not ready
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STOP

Closing signal file M:SIGNAL  .BNC

RUN#  6187          APR 14, 1997  07:42:46

SIGNAL FILE: M:SIGNAL.BNC
NO RUN PEAKS STORED
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STOP

Closing signal file M:SIGNAL  .BNC

RUN#  6156          APR 11, 1997  12:52:03

SIGNAL FILE: M:SIGNAL.BNC
AREA%
      RT      AREA TYPE  WIDTH      AREA%
    .271   9265914   PB   .044  100.00000

TOTAL AREA=9.2659E+06
MUL FACTOR=1.0000E+00
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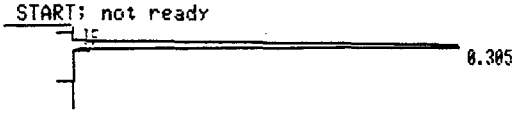


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Closing signal file M:SIGNAL  .BNC

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SIGNAL FILE: M:SIGNAL.BNC
AREA%
      RT      AREA TYPE  WIDTH      AREA%
    .305  17421104   BB   .064  100.00000

TOTAL AREA=1.7421E+07
MUL FACTOR=1.0000E+00
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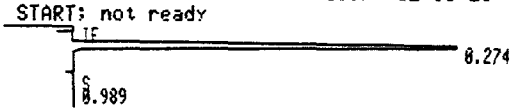


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STOP

Closing signal file M:SIGNAL  .BNC

RUN#  6158          APR 11, 1997  12:55:28

SIGNAL FILE: M:SIGNAL.BNC
AREA%
      RT      AREA TYPE  WIDTH      AREA%
    .274  25809744  SBB   .053  100.00000

TOTAL AREA=2.5810E+07
MUL FACTOR=1.0000E+00
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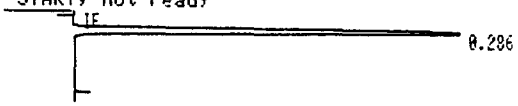


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STOP

Closing signal file M:SIGNAL  .BNC

RUN#  6159          APR 11, 1997  12:57:57

SIGNAL FILE: M:SIGNAL.BNC
AREA%
      RT      AREA TYPE  WIDTH      AREA%
    .286  34067136  SBB   .072  100.00000

TOTAL AREA=3.4067E+07
MUL FACTOR=1.0000E+00
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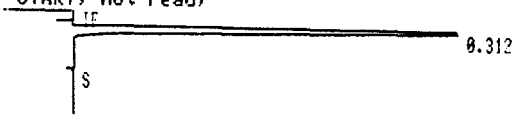


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STOP

Closing signal file M:SIGNAL  .BNC

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SIGNAL FILE: M:SIGNAL.BNC
AREA%
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    .312  42895296  SBB   .045  100.00000

TOTAL AREA=4.2895E+07
MUL FACTOR=1.0000E+00
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Figure C.2 GC chromatograms for headspace standard concentration of methane.

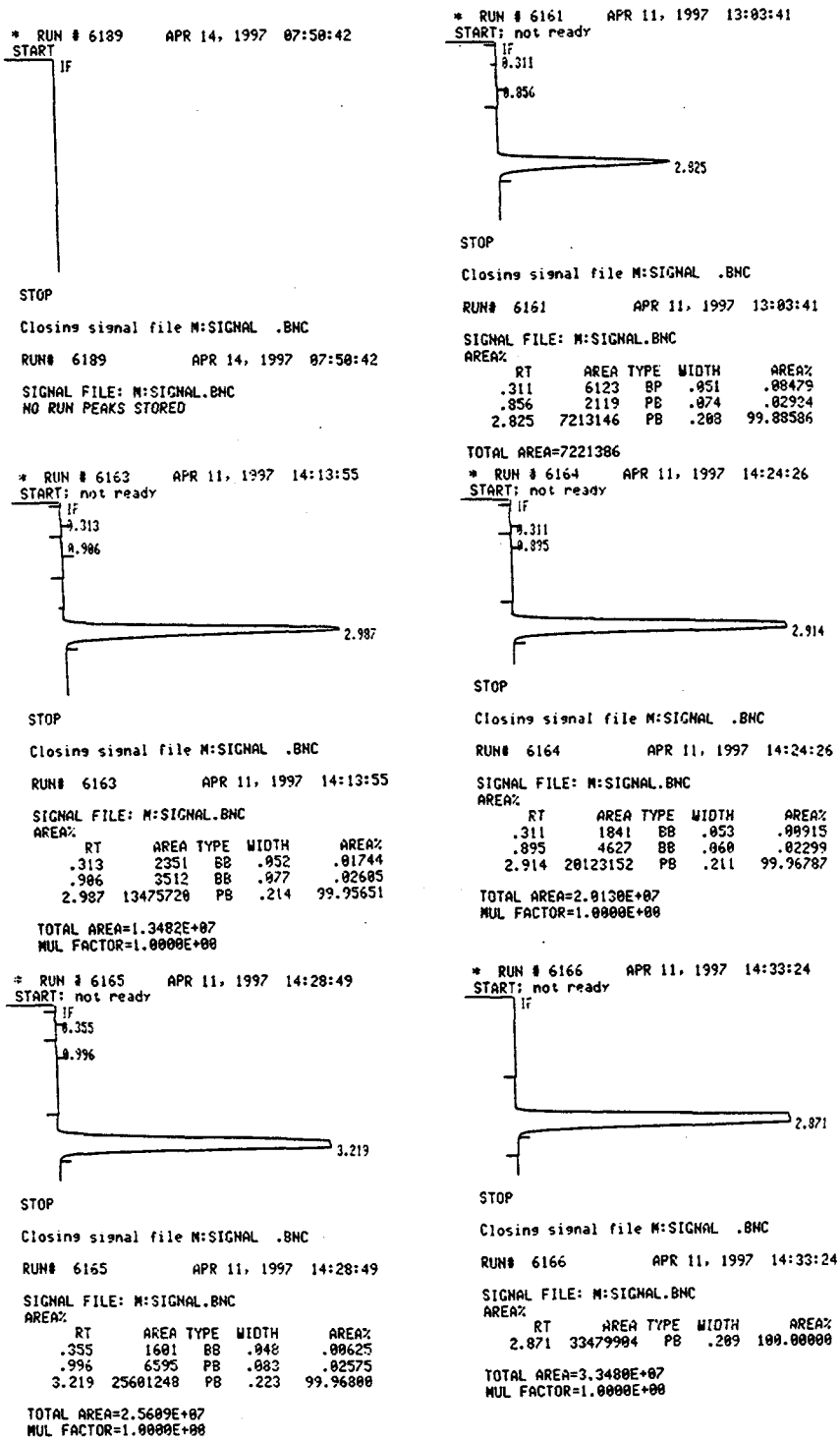


Figure C.3 GC chromatograms for headspace standard concentration of propane.

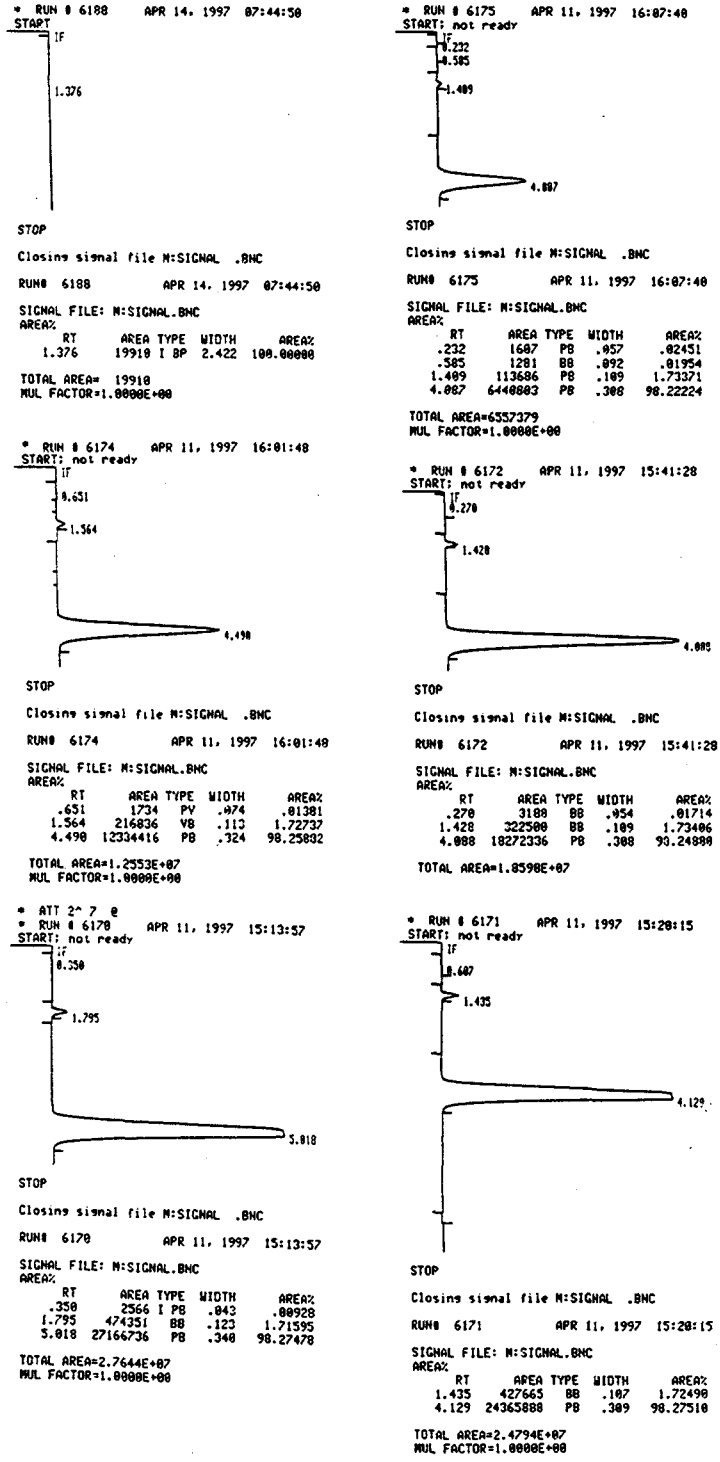


Figure C.4 GC chromatograms for headspace standard concentration of butane.

Appendix D

Analytical Methods for Anions (Nitrate)

Materials

- High grade sodium nitrate salts
- 1,000 ml volumetric flask
- Five 100 ml volumetric flasks
- 1 and 10 ml volumetric pipettes
- Automatic Pasteur pipettor with pipettes
- Sex Dionex Polyvials TM with filter caps

Stock Solution Preparation

1. Calculate the amount of sodium nitrate required to obtain a concentration of 50 mg nitrate/L of the respective anions in 1,000 ml volumetric flask.
2. Add the calculated amounts of sodium nitrate to the 1000 ml flask.
3. Fill flasks with deionized water.
4. Shaker vigorously and label.

Aqueous Standards Preparation

1. The dilution of the stock solution were made in the 100 ml volumetric flasks to obtain concentration of 2, 6, and 10 mg nitrate/L.
2. Shake and label.
3. Add 2 ml of each standard to a labeled Polyvials TM and cap.
4. Fill one Polyvial TM with deionized water for a blank.

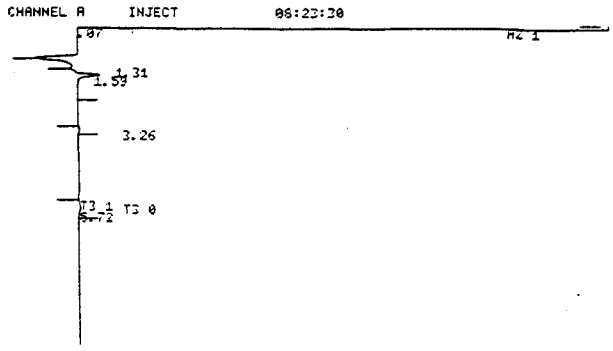
Analysis

Nitrate concentrations were determined on a Dionex 4000I ion chromatography. A Dionex Ionpac AS4A column, which utilizes a regenerant containing H_2SO_4 and Na_2CO_3 and $NaHCO_3$ eluent composition, was used for the chromatography separation.

A 50 µl aqueous sample was analyzed. Samples were run with the blank first in the sequence.

Sample Chromatograms

A set of chromatograms for a typical nitrate standard curve is included below. Standard curves were obtained by plotting the output signal (area) vs nitrate concentration for each sample. Over the range considered (0-10 mg nitrate/L) the area was linear with concentration of nitrate. Reasonable linear line of the data ($r^2 = 0.99$) was obtained by linear regression using Microsoft® Excel 95.



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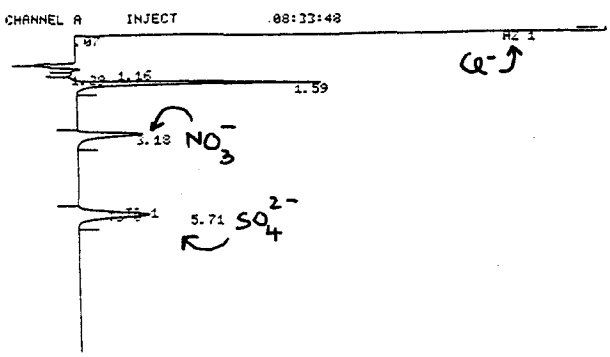
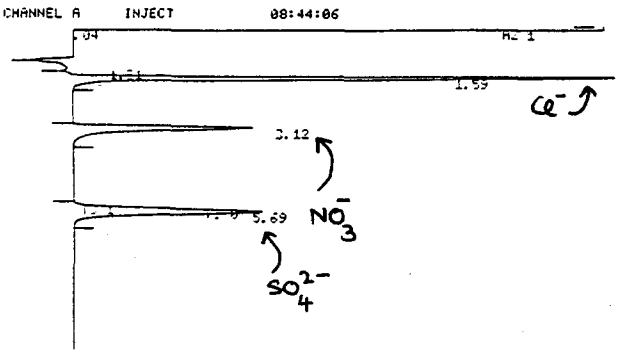
FILE 1. METHOD 5. RUN 2 INDEX 2

NAME	CONC	RT	AREA BC	RF
1	0.	0.07	2523921 01	
2	0.	1.31	707085 02	
3	0.	1.59	1659777 03	
4	0.	3.26	12733 01	
5	0.	5.72	38481 01	
TOTALS	0.		4943997	



ANIONANAL YSIS 08:33:48 CH= "A" PS= 1.
FILE 1. METHOD 5. RUN 3 INDEX 3

NAME	CONC	RT	AREA BC	RF
1	0.	0.07	2461460 01	
2	0.	1.16	427742 02	
3	0.	1.29	330858 02	
4	0.	1.59	2708872 03	
5	0.	3.18	963351 01	
6	0.	5.71	1508617 01	
TOTALS	0.		8400100	



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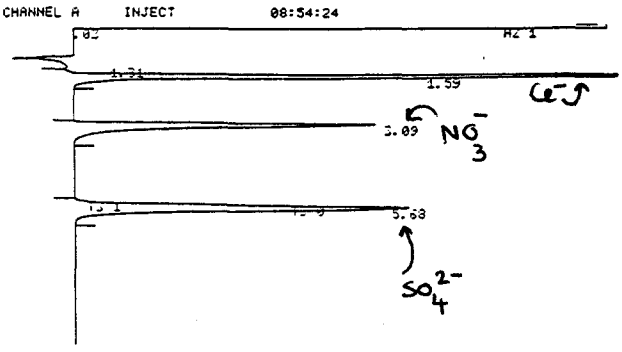
FILE 1. METHOD 5. RUN 4 INDEX 4

NAME	CONC	RT	AREA BC	RF
1	0.	0.04	2462037 01	
2	0.	1.31	589642 02	
3	0.	1.59	6450537 03	
4	0.	3.12	2797970 01	
5	0.	5.69	3978725 01	
TOTALS	0.		16278911	



ANIONANAL YSIS 08:54:24 CH= "A" PS= 1.
FILE 1. METHOD 5. RUN 5 INDEX 5

NAME	CONC	RT	AREA BC	RF
1	0.	0.03	2436988 01	
2	0.	1.31	556964 02	
3	0.	1.59	10744054 03	
4	0.	3.09	4859640 01	
5	0.	5.68	6976333 01	

INTERNAL STANDARD NOT LOCATED!

Figure D.1 IC (nitrate) chromatograms, 0, 2, 6, and 10 mg nitrate/L.

Appendix E

Laboratory Data

Chapter 3 : Comparison of TCE transformation on long-term batch microcosms by methane and propane utilizing microorganisms stimulated from McClellan Air Force Base

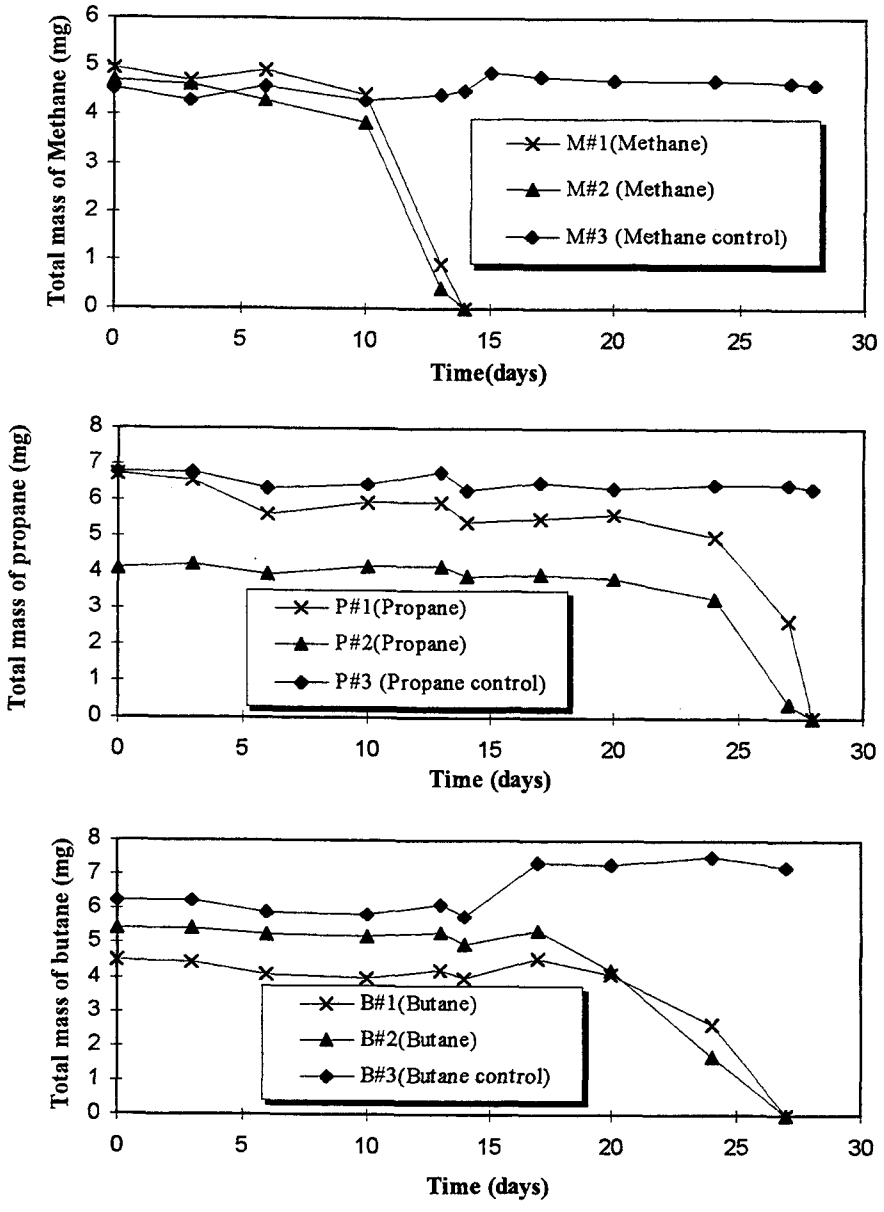


Figure E.1 Lag time of methane, propane, and butane-utilizers.

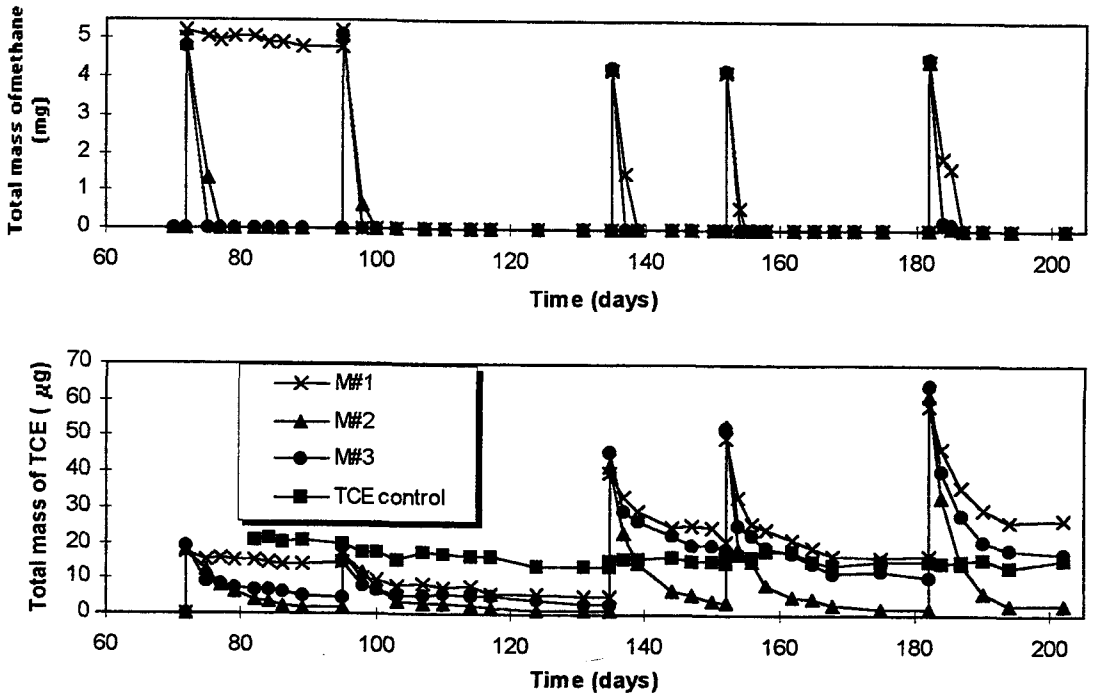


Figure E.2 Methane degradation and TCE transformation with increasing TCE concentration over 60 to 200 days of incubation.

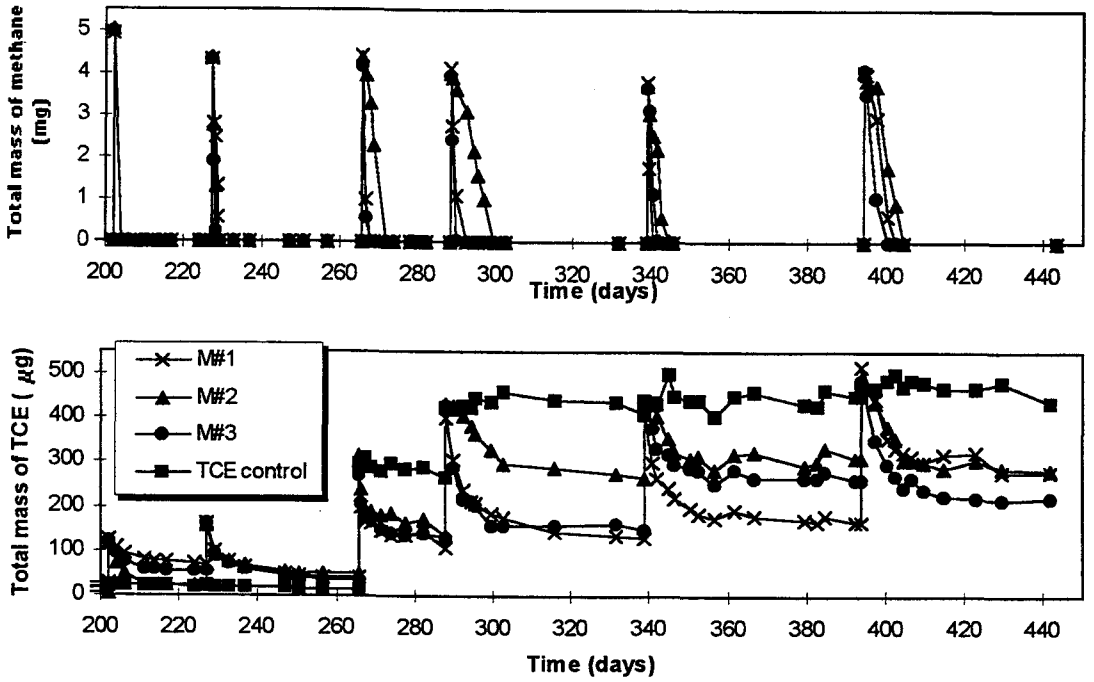


Figure E.3 Methane degradation and TCE transformation with increasing TCE concentration over 200 to 450 days of incubation.

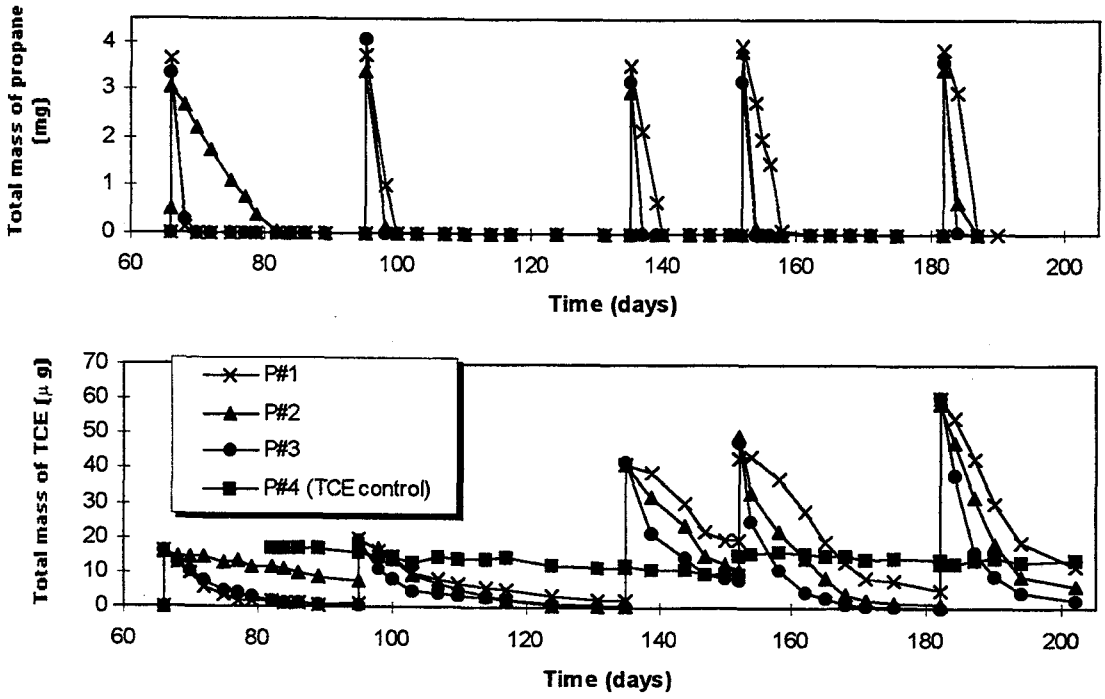


Figure E.4 Propane degradation and TCE transformation with increasing TCE concentrations over 60 to 200 days of incubation.

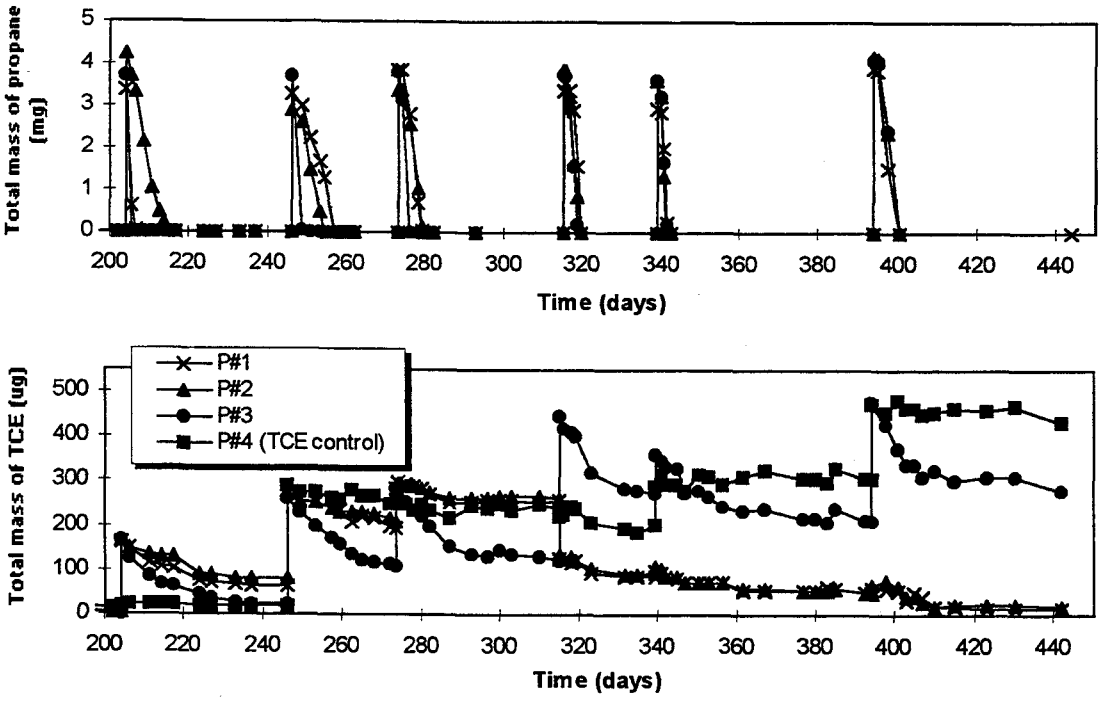


Figure E.5 Propane degradation and TCE transformation with increasing TCE concentrations over 200 to 450 days of incubation.

Chapter 4 : Long-term batch microcosms studies with CAHs treatment by methane, propane, and butane utilizing microorganism stimulated from McClellan Air Force Base

Transformation of TCE, CF, and 1,1,1-TCA by butane-utilizers

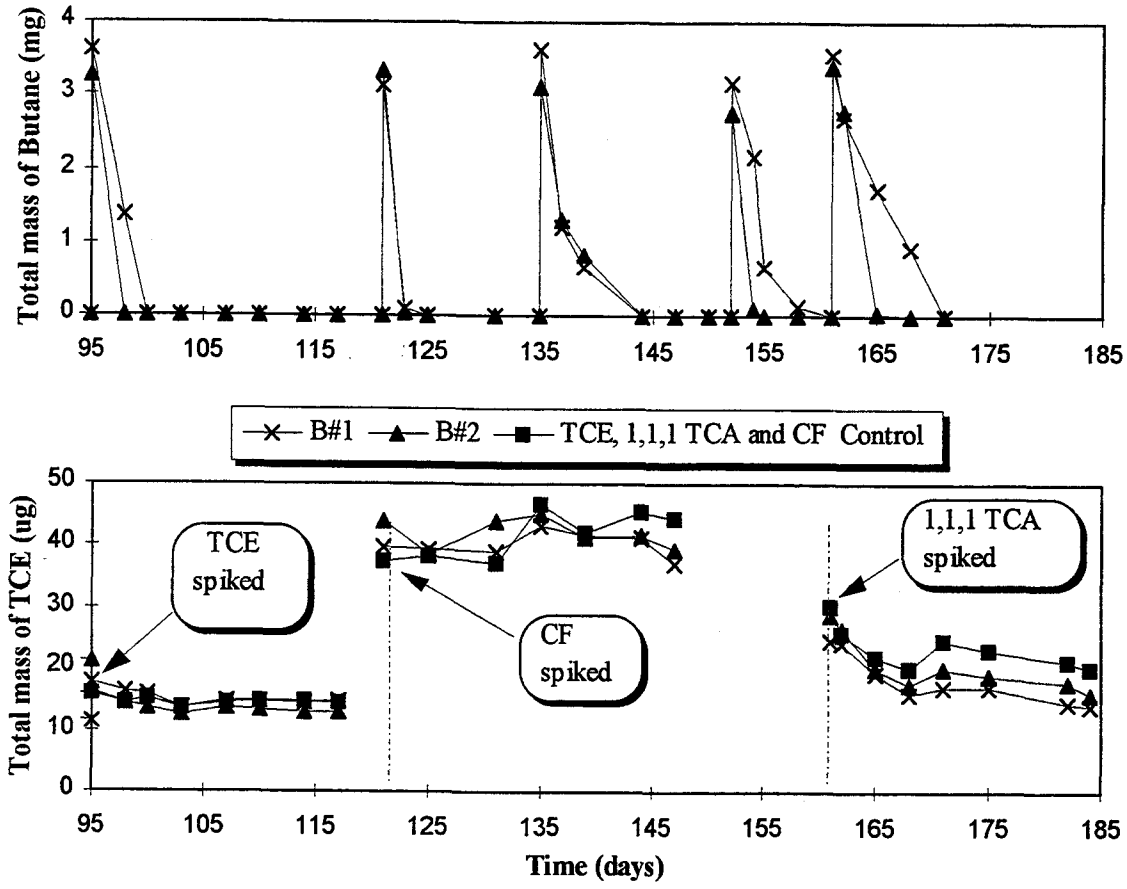


Figure E.6 Butane degradation and TCE, TCA, CF transformations.

Transformation of CAHs mixtures (TCE, 1,1,1 TCA, and CF) by methane (M#1 and M#2) and propane-utilizers (P#1 and P#2)

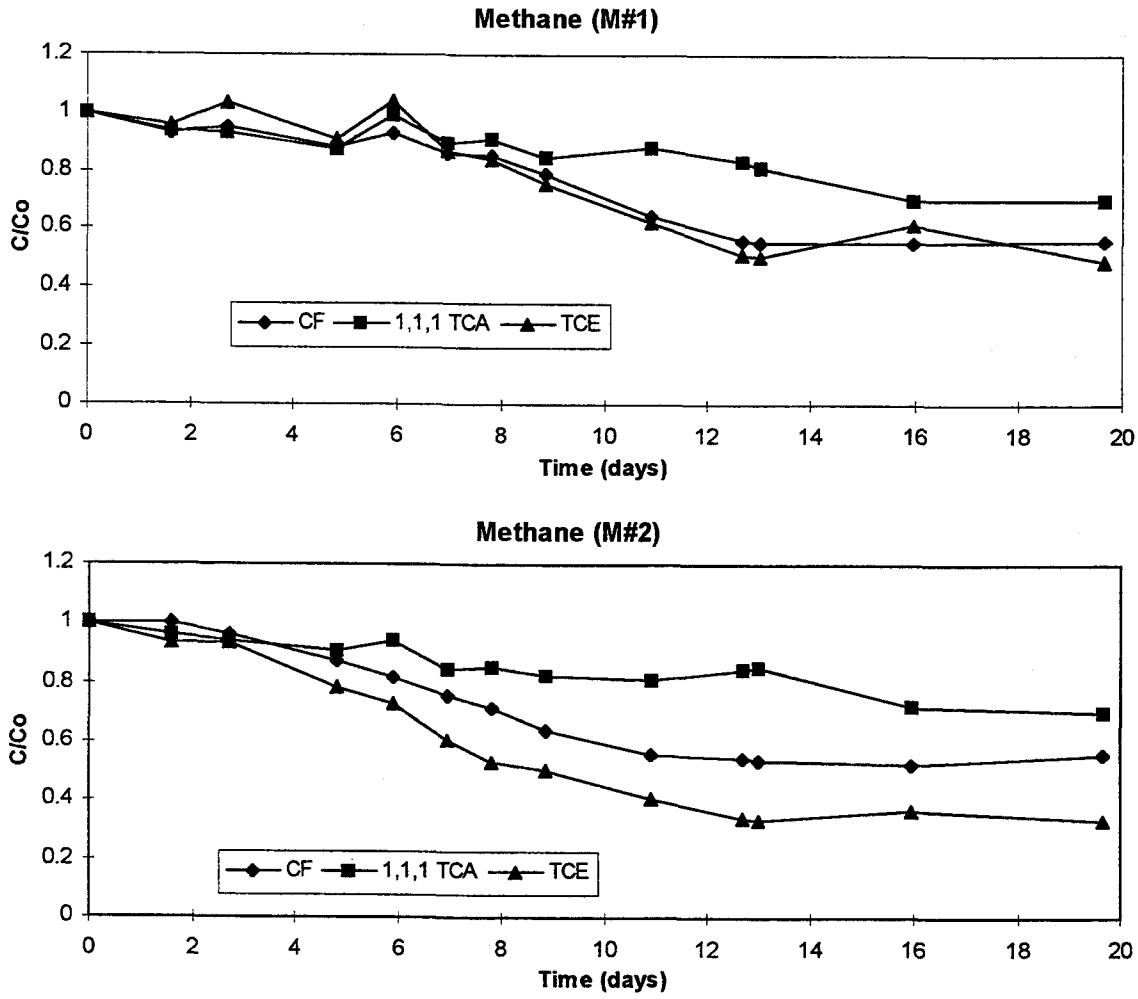


Figure E.7 CAHs transformation by methane-utilizers (M#1 and M#2).

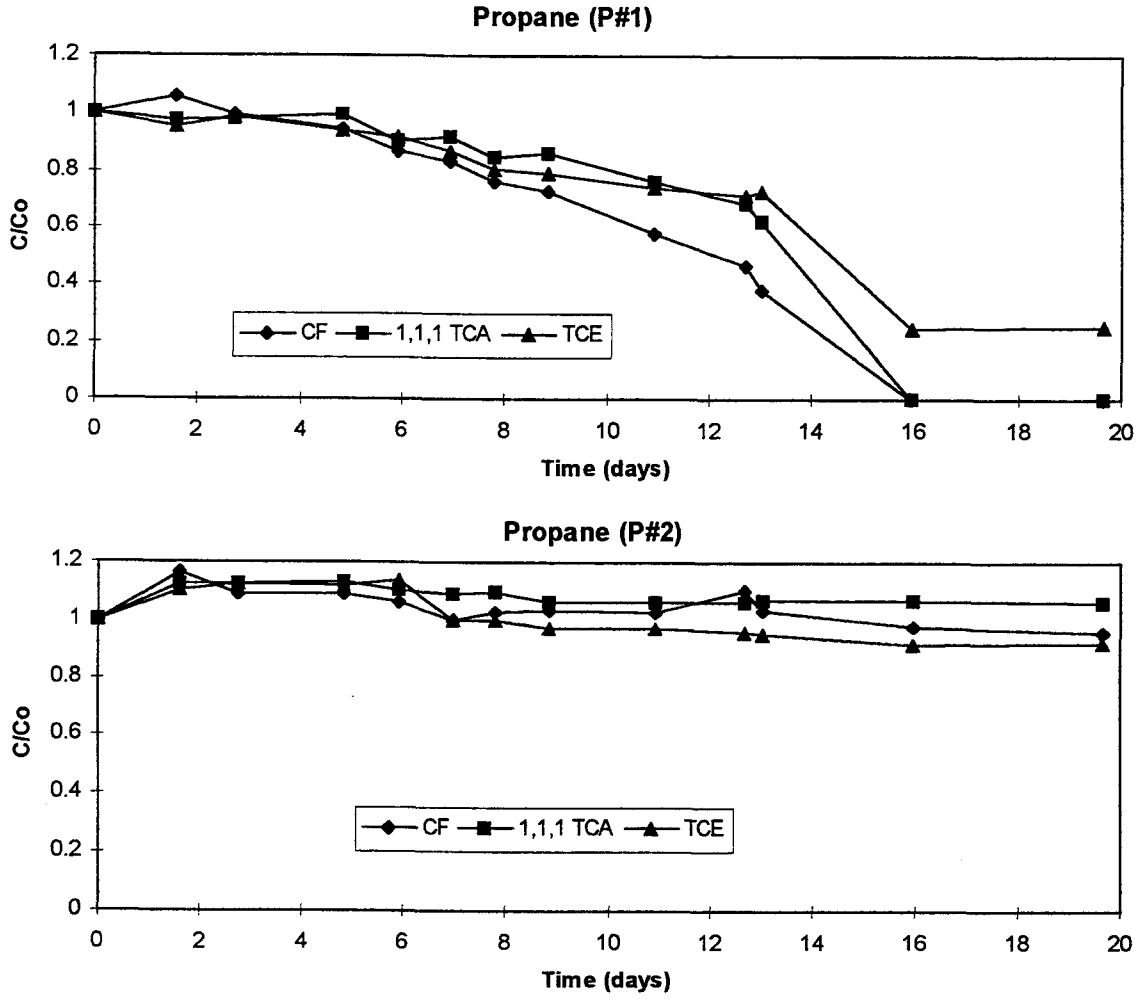


Figure E.8 CAHs transformation by propane-utilizers (P#1 and P#2).

Transformation of 1,1,1 TCA and TCE without exposure to CF by methane (M#1 and M#2) and propane-utilizers (P#1 and P#2)

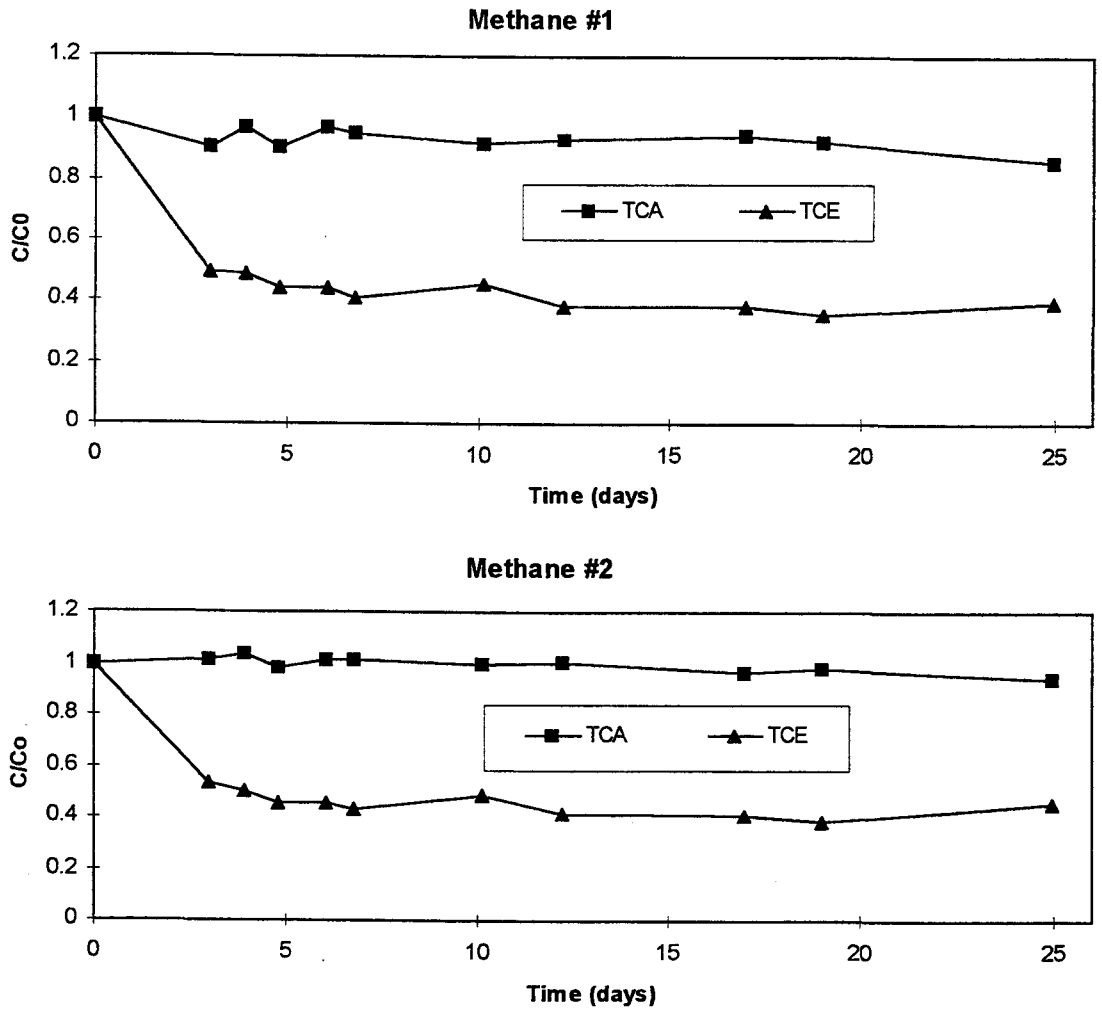


Figure E.9 TCE and TCA transformation in the absence of CF by methane-utilizers (M#1 and M#2).

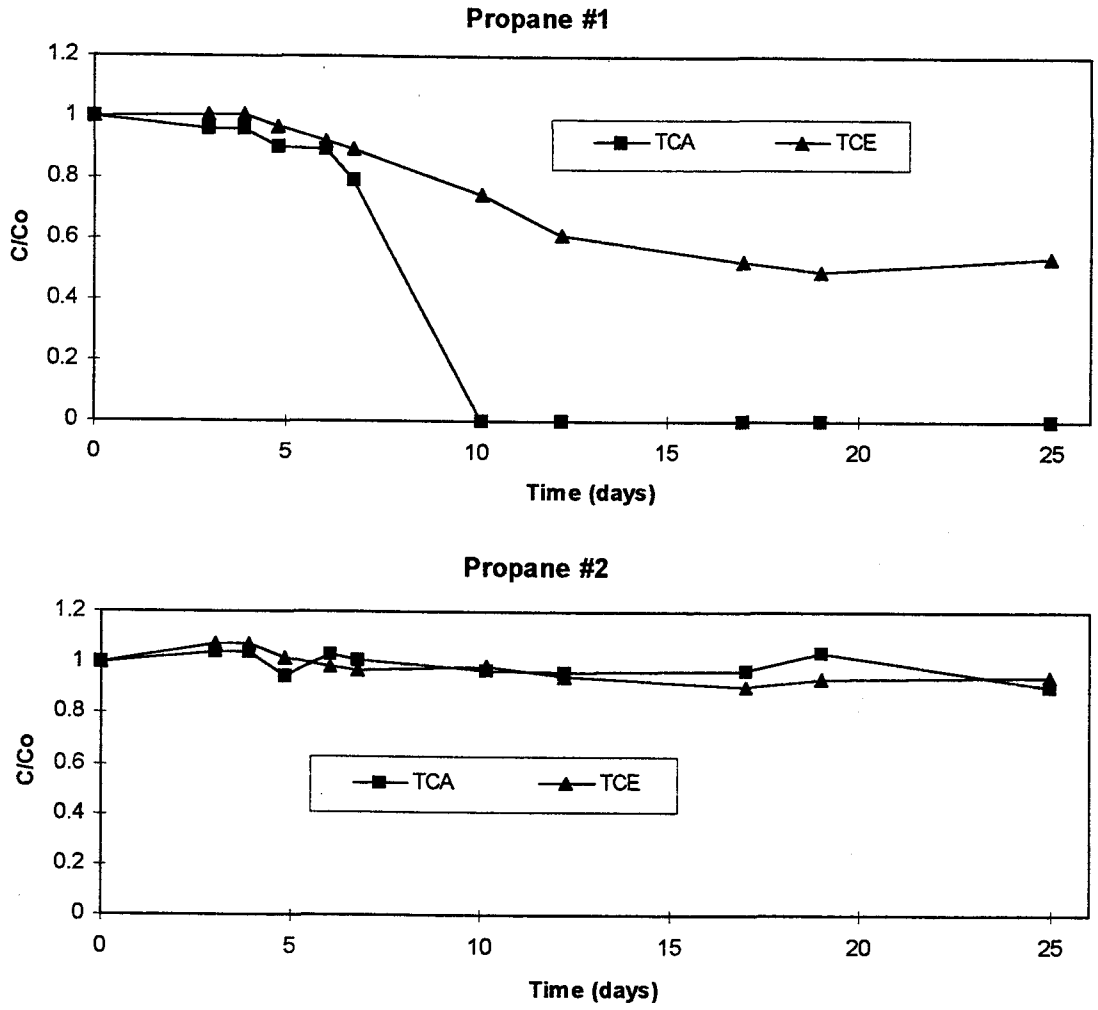


Figure E.10 TCE and TCA transformation in the absence of CF by propane-utilizers (P#1 and P#2).

Appendix F

Soil and Groundwater Samples at McClellan AFB

ISCB pilot test area was located nearby site 22 for analysis of hydrogeological characteristics and groundwater contamination (Figure F.1 and F.2). Upper and lower monitoring wells (EW312 and EW313), shown in Figure F.2, were installed to collect soil samples for laboratory testing on August 1994. The monitoring wells (PZ 40 to 43 and EW253 to 254) were used to determine the TCE contaminant distribution downgradient of TCE contaminant source.

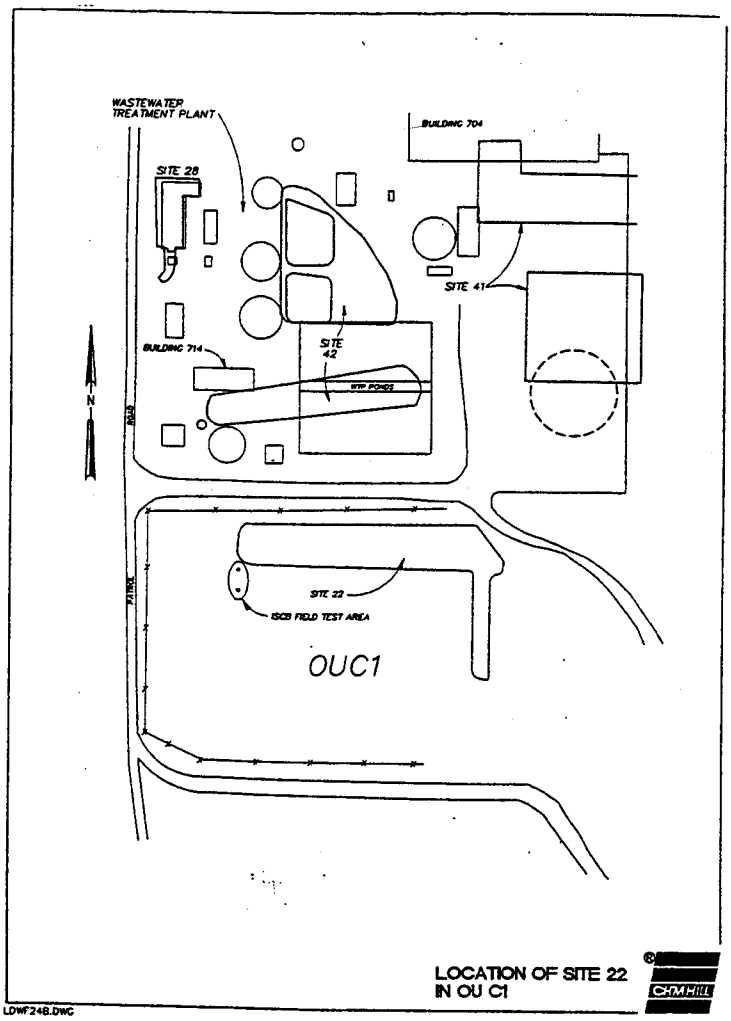


Figure F.1 ISCB pilot test area and the location of site 22 in the McClellan AFB.

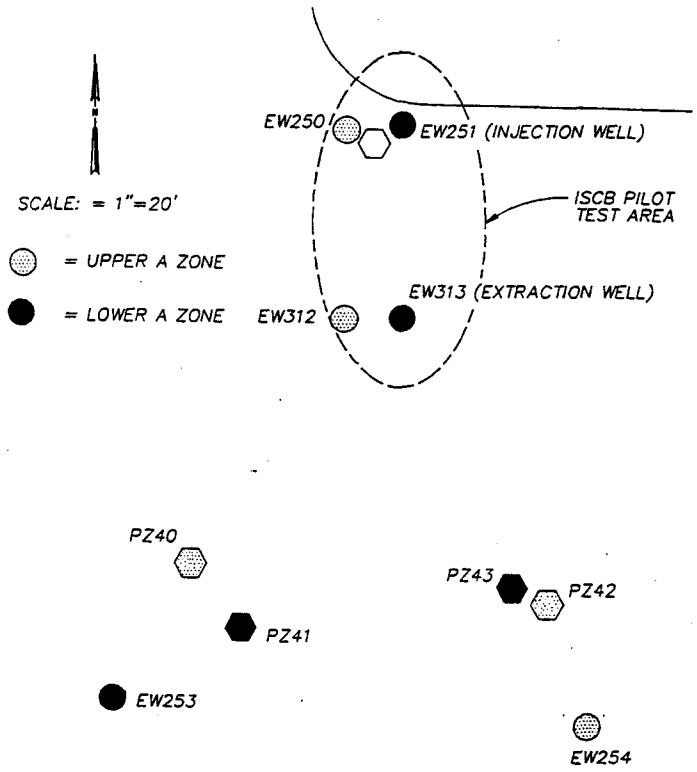


Figure F.2 ISCB pilot demonstration area.

The hydrogeologic conceptual model showed that a shallow aquifer (referred as A zone) was separated from lower aquifer (B zone) by a confining layer. Two different moderately permeable zones in the shallow aquifer were upper A zone (-33 to -45 ft MSL) and lower A zone (-55 to -68 ft MSL). Table F.1 shows the transmissivity, thickness, and hydraulic conductivity of two different shallow aquifer zones. The water table located at -37 ft MSL.

Table F.1 Hygrogeological characteristics of two different zone of shallow A aquifer.

Parameter	Lower A Zone	Upper A Zone
Transmissivity (ft^2/d)	20-100	2000
Thickness (ft)	12	12
Hydraulic Conductivity (ft/d)	1.7-8.3	170

The results of analysis of groundwater performed on July , 1994, indicated that groundwater needed to be amended with nitrogen. The summary of the laboratory data from the groundwater samples is shown in Table F.2.

Table F.2 Groundwater sampling and laboratory data from groundwater samples of McClellan Air Force Base.

ISCB-McClellan AFB				
Field ID	EW-253GW2	PZ40-GW1	FD-GW-02	EW-250GW2
Date Sampled	7/22/94	7/21/94	7/21/94	7/21/94
Alkalinity (mg/L)	119	360	364	174
Ammonia (mg/L)	0.07	0.06	0.05	0.09
Phosphate (mg/L)	0.03	0.12	0.11	0.11
Nitrate (mg/L)	1.1	1.3	1.3	2.5
Sulfate (mg/L)	9.8	18.7	18.2	14.8
Chloride (mg/L)	23.2	42.2	42	29.5
Bromide (mg/L)	0.69	0.833	0.81	0.856