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Abstract approved:

Lewis Semprini

This thesis focused on using microcosms to better understand the aerobic cometabolic processes of TCE and *cis*-DCE transformation that occurred during a Cometabolic Air Sparging (CAS) demonstration at McClellan Air Force Base. The microcosms were created with groundwater and aquifer materials from the demonstration site. Concentrations of compounds in the microcosms were maintained to mimic conditions where the demonstration was performed. Propane was used as the primary substrate to stimulate indigenous propane-utilizers present in the McClellan subsurface. The microcosms were used to test the potential of the propane-utilizers to transform the CAHs of interest, and determine their nutrient requirements while transforming these compounds. Vadose zone microcosms were also created and used to compare the cometabolic processes and nutrient requirements of the propane-utilizers under these different conditions.

After the addition of propane a ten-day lag period was observed before the propane-utilizers were stimulated in all the microcosms. The presence of CAHs

and excess nitrogen did not have any effect on the lag period required to stimulate these microorganisms. Microcosms that received nitrogen amendments maintained effective transformation of TCE and c-DCE with successive additions. The rate of c-DCE transformation was observed to be faster than TCE transformation. Complete removal of the CAHs occurred in these microcosms. No other nutrients, such as phosphorous, were observed to cause any nutrient limitations. However, the microcosms that only had limited amounts of nitrogen present were only able to maintain transformation ability for a short time. Propane utilization rates gradually decreased with each addition, and CAH transformation eventually ceased. This was also observed during the CAS field demonstration after successive additions of propane. Ammonia gas was added to the sparge gas in the field and propane utilization and CAH transformation resumed. Ammonia gas was added to the nitrogen-limited microcosms, and like the field demonstration, propane utilization and CAH transformation resumed. Nitrogen was found to be a critical nutrient for effective cometabolism of CAHs. Nitrogen supplied either as ammonia or nitrate was required for the propane-utilizers to maintain effective rates of propane utilization and CAH transformation ability. By comparing different sets of microcosms under different conditions, estimates were made to the amount of nitrogen required by the propane-utilizers with and without CAHs transformed. The transformation of CAHs significantly increased the propane-utilizers requirements for nitrogen. A 2.0-3.8-fold increase in was observed for nitrogen

consumption when CAHs were transformed, possibly resulting from toxic effects caused by the transformations.

The sparge gas used at the CAS demonstration also contained ethylene at a low concentration (1% vol/vol). The microcosm experiments with this concentration of ethylene were found not to have any negative effects on CAH transformation. The propane-utilizers were also able to maintain propane utilization and CAH transformation at high CAH concentrations.

The vadose zone microcosms showed that propane utilization in the vadose zone was an order of magnitude lower than what was observed in the saturated microcosms. Also bioavailable nitrogen was required to maintain propane utilization rates. However, higher CAH concentrations were found to inhibit the stimulation of the propane-utilizers under these conditions.

Aerobic Cometabolism of Trichloroethylene and *cis*-Dichloroethylene in Propane-Fed Microcosms from the McClellan Air Force Base

by

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Brian Timmins, Author

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Aerobic Cometabolism of Trichloroethylene and *cis*-Dichloroethylene in Propane-Fed Microcosms from the McClellan Air Force Base.

CHAPTER 1
INTRODUCTION AND THESIS OVERVIEW

1.1 History of McClellan AFB and Field Demonstrations

McClellan Air Force Base (AFB) in Sacramento, California is a site where long term disposal of TCE has occurred. McClellan AFB was established in 1936 as an aircraft repair depot and supply base. Prior to 1936, the land on which McClellan AFB was constructed had been devoted to agricultural use. Base operations expanded significantly during World War II and in subsequent years. The primary mission of McClellan AFB has been to provide logistics and maintenance support for various types of aircraft, as well as maintenance support for several communications and electronics systems (EPA ROD, 1995). Fulfilling this mission has involved the use of a wide range of toxic and hazardous substances, including industrial solvents and caustic cleaners, electroplating wastes contaminated with heavy metals, oils contaminated with polychlorinated biphenyls (PCBs), low-level radioactive wastes, aviation fuels, and a variety of oils and lubricants containing solvents (EPA ROD, 1995). Hazardous wastes from operations at McClellan AFB have historically been discharged to land on the base. The waste was discharged to burial pits, landfills, sludge/oil pits or burn pits, or piped through a subsurface industrial wastewater line (IWL) to two industrial wastewater treatment plants (EPA ROD, 1995). Sludges from

these former IWTPs were then discharged on Base to the land. Most of these former disposal areas were located on the west side of the Base. These land disposal practices were discontinued in the late 1970s.

Due to these practices of waste disposal there are a number of chlorinated aliphatic hydrocarbon (CAH) contaminated sites. Operable Unit A (OU A) is one of those sites, and is located at the southern end of the Base. Relatively high concentrations of TCE and *cis*-dichloroethylene (c-DCE), exceeding 500 μg/L, have been detected in the groundwater at the site (Tovanabootr et al., 2000; Tovanabootr et al., 2001). A deep vadose zone exists at the A site, with the contaminated groundwater level being approximately 108-ft bgs. TCE concentrations differed in the upper and lower zones of the A aquifer. 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 the upper and lower A zone, respectively (Tovanabootr et al., 2000).

Tovanabootr and Semprini (1998) conducted a microcosm study constructed with aquifer materials and groundwater from the OU A site. These microcosms were used to investigate the potential to stimulate indigenous microorganisms for aerobic cometabolism. Aerobic cometabolism is the process by which microorganisms utilize a primary substrate as a carbon and energy source using non-specific enzymes that also fortuitously oxidizes CAHs. Microbes grown on three substrates (methane, butane, and propane) were tested for their potential to cometabolically degrade TCE, CF, and 1,1,1-TCA. Propane-utilizers were found to transform TCE, CF, and TCA, indicating they were better suited for cometabolizing CAH mixtures in the McClellan subsurface (Tovanabootr and Semprini, 1998). As a result of these studies this site was chosen for

a demonstration of in-situ Cometabolic Air Sparging (CAS) using propane as the primary substrate. CAS is an innovative form of conventional air sparging, and is designed to degrade or remove CAHs in groundwater and to reduce off-gas CAH emissions during air sparging. As with traditional air sparging, CAS also involves air injection directly into an aquifer. However, CAS is unique in that it also includes the addition into an aquifer and the overlying vadose zone of a gaseous cometabolic substrate, such as propane, to promote the in-situ cometabolic degradation of CAH compounds.

Results from the demonstration showed that stimulation of propane-utilizing microorganisms in the contaminated groundwater occurred after a lag period of 22 to 59 days. After six weeks of repeated additions of propane to the aquifer, TCE and c-DCE concentrations decreased in proportion to propane usage (Tovanabootr et al., 2000). During this demonstration propane utilization rates decreased due to nitrogen limitations in the site groundwater, which led to a decrease in the CAH transformation rates. Ammonia was added to the sparge gas to provide bioavailable nitrogen. Upon addition of the ammonia gas, propane utilization rates increased, and CAH transformation resumed. The study was less successful in demonstrating the stimulation of propane-utilizers in the vadose zone. Overall propane concentrations tended to increase during the demonstration as more propane was sparged into the zone. Several locations showed decreases in propane concentrations after ammonia was added, suggesting the nitrogen may have been limiting in the vadose zone. Decreases in TCE and c-DCE were observed in the vadose zone monitoring wells, and appeared to be coincident with propane utilization at these locations.

Further microcosm studies were needed to better understand the cometabolic processes that were occurring during this demonstration. Previous microcosm studies did not mimic the concentrations of propane, c-DCE and TCE found at the site. Experiments needed to be conducted to find out how much propane could be utilized and CAHs transformed with the background levels of nitrate found at the site. Also the effects of the addition of ammonia gas and the nitrogen requirements of the microorganisms had not been thoroughly investigated. The utilization of propane and CAH transformation in the vadose zone was not as effective as the saturated zone, so vadose zone microcosms were required to attempt to understand and optimize the processes in this zone.

1.2 Chemical Properties of the CAHs and Gases

Trichloroethylene and cis-dichloroethylene has specific chemical properties that are of interest for this study. TCE has been used in industry primarily as a degreaser and extraction agent, because it dissolves most organic compounds and does not react with steel, copper, zinc, or other metals. It is a highly volatile compound that more favorably partitions into air than water (Montgomery, 1991). Due to TCE having a higher density than water it tends to sink under gravitational forces into groundwater. This property, combined with TCE being slightly soluble in water, classifies it as a Dense Nonaqueous Phase Liquid (DNAPL). Both TCE and c-DCE are considered non-flammable and non-explosive at ambient temperatures. *Cis*-DCE is often found with TCE in groundwater as a product of the anaerobic transformation of TCE. This substitution of the chlorine atom with a hydrogen atom changes the chemical

properties, with c-DCE having a greater solubility in water and a lower boiling point and Henry's air/water partition coefficient (Table 1.1). A literature review found the reported water solubility for c-DCE varied considerably from 3500-6300 mg c-DCE/L (SRC, 2001; NTP, 2001; Speclab, 2001). An experiment was conducted on the water-saturated solution used in these experiments and a c-DCE concentration of 6000 mg/L was measured.

Table 1.1 Chemical properties of TCE, c-DCE, propane, and ethylene.

Contaminant Properties	**TCE**[1,2,3,4]	**c-DCE**[1,2,3,4]	**Ethylene**[1,2,3]	**Propane**[1,2,3]
Formula	$CHClCCl_2$	CHClCHCl	CH_2CH_2	C_3H_8
Molecular Weight (g/mol)	131.4	96.95	28.05	44.11
Boiling Point (°C)	86.7	55	-103.7	-42.1
Aqueous Solubility (mg/L)	1100	6000*	131	62.4
Henry's Law constant (Hcc), @ 20°C dimensionless	0.342	0.123	7.64	28.9
Log Octanol/Water Partition Coefficient	2.3	1.86	1.13	2.36
U.S. Drinking Water MCL (µg/L)	5	70	**	**

*** Calculated**

**** Not reported**

1) **NTP Chemical Repository, 2001**
2) **Speclab, 2001.**
3) **SRC PhysProp Database, 2001**
4) **Montgomery, 1991**

The properties provided in Table 1.1 were used in the analysis and mass calculations for the microcosm experiments. The boiling point indicates when the compounds are expected to elude in the gas chromatography analysis. For example, if a gas sample was analyzed with all the compounds in Table 1.1 ethylene would be expected to have the shortest retention time, while TCE would have the longest retention time. For the microcosm mass calculations the Henry's constant was used to quantify the mass balance between the liquid and gas phases. Microcosms were constructed having both a liquid and gas phase in order to be able to sample the headspace for analysis. Gas phase samples were taken from the headspace of the microcosms and a gaseous concentration was determined. The liquid concentration was then calculated using the Henry's Law constant assuming equilibrium partitioning. The aqueous solubility was used to determine the concentrations of water-saturated solutions that were added to the microcosms to obtain the desired concentrations.

1.3 Objectives of this Study

Previous microcosm studies evaluated the ability of methane-, propane-, and butane-utilizers to transform TCE, CF, and TCA in McClellan microcosms. This microcosm study focused on the stimulation of indigenous propane utilizing microorganisms using McClellan aquifer material and groundwater from the site of the CAS demonstration. Propane-utilizers stimulated in the microcosms were tested for their ability to transform TCE and c-DCE since they are the major in the groundwater and vadose zone at the site of the CAS field demonstration. This study evaluated the ability to use microcosms as a tool to better understand the cometabolic processes being

induced in the field demonstration. The effects of nitrogen limitations and nitrogen addition on propane utilization and CAH transformation were examined. The potential of using ethylene as a surrogate compound to study c-DCE and TCE cometabolism was also examined. The specific objectives of this study were to determine:

1) if the indigenous microorganisms present at the site could be biostimulated using propane as a substrate.

2) the lag period before propane stimulation occurred.

3) how effectively these indigenous microorganisms transformed the CAHs of interest, and to calculate the transformation yields (μg CAH/μg substrate).

4) how long the background levels of nutrients present in the groundwater from the site could sustain propane utilization and CAH transformation.

5) the effects of ethylene present as a component of the propane used in the CAS demonstration on propane utilization and CAH transformation, and whether processes such as competitive inhibition or toxicity are of concern.

6) if ammonia can be used as a bioavailable nitrogen source, and compare the rates of propane utilization and CAH transformation to the rates obtained using nitrate as a nitrogen source.

7) the nitrogen requirements for cell growth and maintenance in microcosms with and without CAHs present using nitrate and ammonia as nitrogen sources.

8) the effects of higher concentrations of CAHs on propane utilization and CAH transformation.

9) if propane utilizers could be stimulated in the vadose zone with varying CAH concentrations and ammonia additions.

CHAPTER 2
LITERATURE REVIEW

2.1 History of TCE in Industry and the Environment

Trichloroethylene (TCE), a chlorinated aliphatic hydrocarbon (CAH), has been produced commercially since the 1920's in many countries by the chlorination of ethylene or acetylene. Its use in vapor degreasing began in the 1920's. In the 1930's, it was introduced for use in the dry cleaning industry, but it has had limited use in this industry since the 1950's. Currently 80-90% of TCE worldwide is used to degrease metals. Use for all applications in Western Europe, Japan, and the United States in 1990 was estimated to be 225 thousand tones (IARC). Historically TCE has also been used as a refrigerant, a heat exchange liquid, in organic synthesis reactions, as a fumigant, an anesthetic, in cleaning and drying electronic parts, as a diluent in paints and adhesives, as an industrial solvent, as a chain terminator in PVC production, and as an extractant for decaffeinating coffee (NTP). The many applications of TCE for industrial uses increased the demand for the solvent, which during those years the average production was over 200 million pounds per year (Vogel et al., 1987). In 1976 the US Environmental Protection Agency (EPA) placed TCE on the list of hazardous substances under the Resource Conservation and Recovery Act (RCRA), and in 1982 it was determined to be a suspected carcinogen (Infante and Tsongas, 1982). A proposed Maximum Contaminant Level (MCL) of 5 ppb was set for drinking water by the US EPA in 1986 (U.S. EPA, 1993). Due to TCE classification as a suspected carcinogen and hazardous substance its use in industry became restricted. The EPA Toxic Release

Inventory (TRI) program reported a total on and off-site release of 10,853,820 pounds TCE from US industries for 1999. Figure 1.1 shows the decrease in the amount of TCE released from 1989 to 1999 as reported by the TRI program. The main industries TCE was released from in 1999 were fabricated/primary metals, textile, plastics, transportation equipment, machinery, electrical equipment, and chemical industries (TRI).

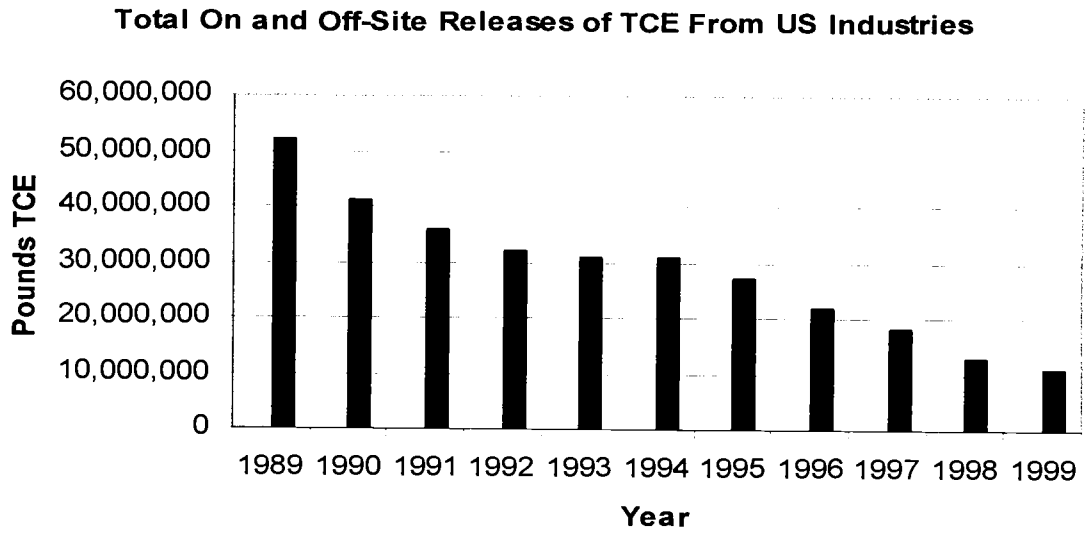

Figure 1.1 Total releases of TCE in pounds from US industries from 1988-1999.

Although the use of TCE has been decreasing, its previous use and improper disposal has led to extensive environmental contamination of sediments and groundwater throughout the United States. Prior to the enactment of RCRA in 1976,

most of the TCE used by industries and military installations was carelessly discharged into surface waters and on land. Military installations practiced direct disposal of TCE and dumped it into waste pits, buried it in drums, or placed it in leaky underground storage tanks.

TCE is slightly soluble in water (1100 mg/L) and has a moderate octanol/water coefficient (Montgomery, 1991). These properties cause TCE to migrate quickly in groundwater and to partition onto the organic matter in the subsurface. TCE is considered a recalcitrant compound, which means it exhibits slow breakdown rates in the subsurface (Munter and DeVries, 1987). In recent years the EPA and other federal agencies have published several documents, which provide a detailed inventory of the numbers and types of contaminated sites in the US. Of the 1,235 sites on the National Priorities List (NPL) a total of 64% of those sites had halogenated Volatile Organic Compounds (VOCs) in the soil or groundwater (Glass, 2000). The U.S. Department of Energy (DOE) has 15% of their 137 installations, which contain some 10,500 sites that need remediation in the United States, contaminated with TCE (Glass, 2000).

2.2 Role of Microorganisms

Bioremediation of contaminated sites is a developing alternative to traditional remediating technologies. It works by microbially altering the structure of the organic compounds, and the degree of alteration determines whether biotransformation or mineralization has occurred. Biotransformation is the simplification of an organic compound into a daughter compound, while mineralization is the complete breakdown of organic molecules into cellular mass, carbon dioxide, and water (LaGrega et al.,

1994). Bioremediation depends on the presence of the appropriate microorganisms in the correct amounts and combinations, and on the appropriate environmental conditions. In a typical gram of sediment, there are thousands of species of microorganisms that play an integral role in the cycling of carbon and nitrogen between the atmosphere and the terrestrial environment. Microorganisms have been found at great depths in the subsurface, and recently have been discovered in the harsh environment of the deep sea where life was previously thought not to exist.

Many different applications have been created to apply bioremediation technologies to manage waste. The word bioremediation is fairly new and first appeared in 1987. In the last twenty years an increase in the use of this technology shows its potential for future applications. Remediation methods, such as pump and treat, have been used for remediating groundwater contaminated with CAHs. However, with a TCE drinking water standard of 5 ppb, pump and treat remediation is an inefficient and expensive method for removal of the CAHs from groundwater. It may require a time scale of decades to clean up a contaminated aquifer to the drinking water standards using the pump and treat method (Mackay and Cherry, 1989). In-situ bioremediation has good potential to clean up contaminates without bringing the groundwater to the surface. This technology may be capable of reducing the time required for restoring contaminated aquifers and the remediation costs incurred by companies. Technologies being applied today are biostimulation and bioaugmentation, bioreactors, phytoremediation, intrinsic bioremediation, slurries and sediment washing (LaGrega et al., 1994). For these technologies to become more efficient and cost

effective more research is needed to better understand the complex reactions and relationships of the microbial communities.

2.3 Aerobic Cometabolism of CAHs

This study focuses on aerobic cometabolism of chlorinated solvents. Cometabolism is when a secondary compound is fortuitously transformed by enzymes routinely degrading the primary substrate (Dalton, 1982). The enzymes for this type of process often have a low level of specificity, and react with many different types of compounds. Aerobic microorganisms that can grow on highly chlorinated ethenes, such as TCE, as a sole carbon or energy source have not yet been isolated. However, several types of commonly occurring aerobic soil microbes that use vinyl chloride (VC) as a sole organic carbon and energy source have been identified (Hartmans et al., 1992).

2.3.1 Co-Metabolism by Methane-Utilizers

The first report of aerobic cometabolism was in 1984 when natural gas was used to stimulate methanotrophs in a soil column to transform TCE aerobically (Wilson and Wilson, 1985). Since this observation many studies have been conducted to investigate the potential of different substrates to aerobically co-metabolize CAHs. Studies have focused on methane as the primary substrate to co-metabolize TCE (Alvarez-Cohen and McCarty, 1991; Broholm et al., 1990; Brusseau et al., 1990; Dolan and McCarty, 1995; Fogel et al., 1986; Henson et al., 1988; Little et al., 1988, Oldenhuis et al., 1989; Tsien et al., 1989). Methane utilizing microorganisms, methanotrophs, are widespread in the transition zone between aerobic and anaerobic zones in the subsurface where methane

and oxygen are present (Hanson, 1980). In the first step of methane utilization, methane is oxidized to methanol by the methane monooxygenase (MMO) enzyme. This oxidation step requires $NADH_2$ as an electron donor, which is generated in the last two steps of the methane reactions. Methanol is transformed to formaldehyde, formaldehyde is then oxidized to formate, and formate is oxidized to carbon dioxide. The electrons produced in these reactions regenerates the NAD^+ to $NADH_2$, required for the initial oxidation of methane (Dalton and Stirling, 1982).

Fogel et al. (1986) found that methane-utilizing bacteria could transform vinyl chloride, 1,1-DCE, cis- and trans-1,2-DCE, and TCE. However, tetrachloroethylene (PCE) was not degraded. They found that the presence of acetylene, a specific inhibitor or MMO, inhibited the degradation of TCE. In 1988 the first observed TCE biodegradation by pure cultures of methanotrophic bacteria were reported (Little et al., 1988). Water-soluble breakdown products of TCE epoxide, glyoxylic acid and dichloroacetic acid, were shown to accumulate reflecting an inability of the pure culture to metabolize TCE fully. The accumulation of these breakdown products led to the proposal of a possible TCE epoxide biodegradation pathway. Also this pure cultures' inability to fully metabolize TCE gave insight into the complexity of mixed cultures, where by-products produced by methanotrophs appear to be further metabolized to CO_2 by heterotrophic bacteria (Little et al., 1988).

Methanotrophs produce two types of MMO, soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO), that are responsible for the utilization of substrates and transformation of CAHs. All methanotrophic bacteria produce pMMO, while a limited number produce sMMO. SMMO is produced under

copper limiting conditions (Oldenhuis et al., 1989). It was found that sMMO had a much wider substrate range than pMMO, but neither MMO could biodegrade fully chlorinated carbon tetrachloride (CT) or PCE (Oldenhuis et al., 1989). It has been shown that the rapid degradation of TCE by *Methylosinus trichosporium* OB3b occurred only when sMMO was detectable (Tsien et al., 1989). Further investigations with a purified sMMO system showed that halogenated alkenes were oxidized predominately by epoxidation (Fox et al., 1990). Transformation yields, which represent the mass of CAH degraded per mass of growth substrate utilized, have been used to estimate the efficiency of microorganisms to degrade CAHs. A more recent study found that pMMO was able to biodegrade VC, t-DCE, c-DCE, TCE, and 1,1-DCE; with transformation yields for VC and t-DCE being 20 times greater than the yields reported by others for cells expressing sMMO (Anderson and McCarty, 1997). This study suggests that pMMO expression may be advantageous for degrading VC and t-DCE. It may also be easier to maintain pMMO expression in treatment systems, because pMMO is expressed by all methanotrophs whereas sMMO is expressed only by type II methanotrophs under copper-limiting conditions (Anderson and McCarty, 1997).

TCE transformation by-products have been found to inhibit TCE degradation (Alvarez-Cohen and McCarty, 1991; Henry and Grbic-Galic, 1991; Oldenhuis et al., 1991; Tsien et al., 1989). However, hydrolysis products of the TCE epoxide and trichloroacetaldehyde did not inactivate MMO (Fox et al., 1990). Henry and Grbic-Galic stated that neither the TCE itself nor the aqueous intermediates were responsible for the toxic effect, but was suggested that TCE oxidation toxicity may have resulted

from reactive intermediates that attacked cellular macromolecules. It has been found during the conversion of [^{14}C] TCE that some of the various proteins in *M. trichosporium* became radiolabeled; indicating that TCE-mediated inactivation of cells was caused by nonspecific covalent binding of degradation products to cellular proteins (Oldenhuis et al., 1991). Other studies reported a decrease in the cell's ability to consume methane after transformation of TCE, and the toxicity effect increased with increasing amounts of TCE transformed (Alvarez-Cohen and McCarty, 1991). Chu and Alvarez-Cohen proposed TCE oxidation exerts a broad range of toxic effects that damage both specific and nonspecific cellular functions (1999). In their experiment TCE oxidation caused sMMO-catalyzed activity and respiratory activity to decrease linearly with the amount of substrate degraded. During severe TCE oxidation toxicity methane oxidation ceased and there was a 95% decrease in respiratory activity. Cells failed to recover even after seven days of incubation with methane, suggesting that cellular recovery following severe TCE product toxicity is not always possible (Chu and Alvarez-Cohen, 1999).

The MMO enzyme system of methanotrophs requires electrons (reducing power) to carry out its catabolic function. During growth on methane, reducing power is regenerated by the mineralization of methane to carbon dioxide. During the oxidation of a cometabolite such as TCE, however, the reductant supply is not regenerated (Henry and Grbic-Galic, 1991). When no substrate is available to regenerate the reductant cells can be depleted of reducing power. The supply of reductant plays a significant role in the TCE transformation rate and capacity (Alvarez-Cohen and McCarty, 1991). Studies on competitive inhibition also revealed that high

methane concentrations have a negative effect on TCE transformation (Broholm et al., 1992; Odenhuis et al., 1991; Semprini et al., 1991). Since MMO is responsible for both methane oxidation and TCE epoxidation, methane and TCE are considered to be competitive substrates such that in the presence of both compounds TCE transformation rates are reduced.

2.3.2 Cometabolism by Toluene and Phenol-Utilizers

Aromatic primary substrates, toluene and phenol, have also been used to transform TCE (Chang and Alvarez-Cohen, 1995; Folsom et al., 1990; Hopkins and McCarty, 1995; Hopkins et al., 1993; Landa et al., 1994; McCarty et al., 1998; Nelson et al., 1988; Wackett and Gibson, 1988). Nelson et al. (1987) first reported an aromatic degradative pathway in the cometabolism of a CAH, indicating the *meta* fission pathway being responsible for TCE transformation. In 1988 evidence was found that a toluene dioxygenase was involved in the TCE degradative ability of one organism (Nelson et al., 1988). A monooxygenase, produced by the TCE degrading bacteria G4, was reported by Shields et al. (1989) to degrade toluene via the *ortho* and *meta* fission pathways. Two more enzymes expressed by JMP134, phenol hydroxylase and 2,4-dichlorophenol hydroxylase, were reported as monooxygenases that were also able to degrade TCE (Harker and Kim, 1990).

Toxic effects of TCE oxidation were shown in toluene utilizers that were similar to the methane utilizers. Using *Pseudomonas putida* F1, cytotoxicity was indicated by growth inhibition and by the covalent modification of cellular molecules exposed to TCE (Wackett and Householder, 1989). In this study 17% of the radioactivity that had

been added to the culture, as [^{14}C] TCE, was accounted for in the total cell fractions. However, a chemostat with *P. cepacia* G4 was capable of a wide range of volumetric TCE loading rates without showing toxic effects. It took a high load of TCE to cause inhibition of toluene and TCE conversion, but once the TCE levels were decreased back to the non-toxic level the organisms recovered within two days (Landa et al., 1994). These results show that *P. cepacia* is more resistant to high concentrations and shock loading than the methane utilizers. Some toluene utilizers may be more resistant to TCE, either because less reactive products are formed or because the organism is less sensitive to the damage caused by TCE degradation products.

A field evaluation of in-situ biotransformation of CAHs with phenol and toluene as primary substrates showed positive results (Hopkins and McCarty, 1995). Removal efficiencies for TCE ,c-DCE, and VC were greater than 90%, and trans-DCE was 74%. Only 1,1-DCE showed low removal efficiencies at 50%, along with a high degree of transformation product toxicity (Hopkins and McCarty, 1995). Removal efficiencies for phenol and toluene were similar. The potential to use toluene and phenol compounds in field applications is limited due to the regulations and toxicity involved with these compounds. However, phenol and toluene never exceeded their detection levels of 0.5 μg/L of 1 μg/L, respectively. This toluene concentration is well below the EPA MCL of 1 mg/L. Another field evaluation demonstrated the viability of toluene injection by removing 97-98% of TCE in regional groundwater plumes, and biodegrading toluene to an average of 1 μg/L at the boundaries of the study zone (McCarty et al., 1998).

Other factors, such as feeding patterns and competitive inhibition effect TCE transformation rates. Several reactors that had different structures of enrichments over a 24-hour period showed dramatic differences in TCE transformation. The reactors with fewer enrichments over the 24-hour period had a 10-fold increase in TCE transformation compared to the reactors that were fed continuously and semi-continuously (Shih et al., 1996). From these results they concluded that cometabolic activity depends upon microbial community structure and that the community structure can be manipulated by altering the growth substrate feeding pattern. Another factor when using toluene is the potential to inhibit TCE transformation by using a high concentration of toluene that out competes TCE for the enzymes. When toluene concentrations went above 1mg/L TCE transformation was strongly inhibited (Arcangeli and Arvin, 1997).

2.3.3 Biological Oxidation Pathways of TCE

The University of Minnesota using sMMO and two types of toluene oxygenases has proposed three oxidation pathways (Figure 1.2). The first speculation of the possible formation of a TCE epoxide by sMMO was by Fogel in 1986. Newman and Wackett detected 16% of the TCE oxidized in their experiments was transformed into chloral hydrate, which was biologically transformed into 2,2,2-trichloroethanol and trichloroacetic acid. These are the two main pathways for TCE transformation using sMMO.

The other two enzymes in Figure 1.2 are the toluene mono- and dioxygenase that have been detected in several studies (Harker and Kim, 1990; Jenal-Wanner and

McCarty, 1997; Nelson et al., 1988; Shields et al., 1989; Wackett and Householder, 1989). The monooxygenase enzyme produces the TCE epoxide intermediate, and the dioxygenase produces the glyoxylate and formate intermediates.

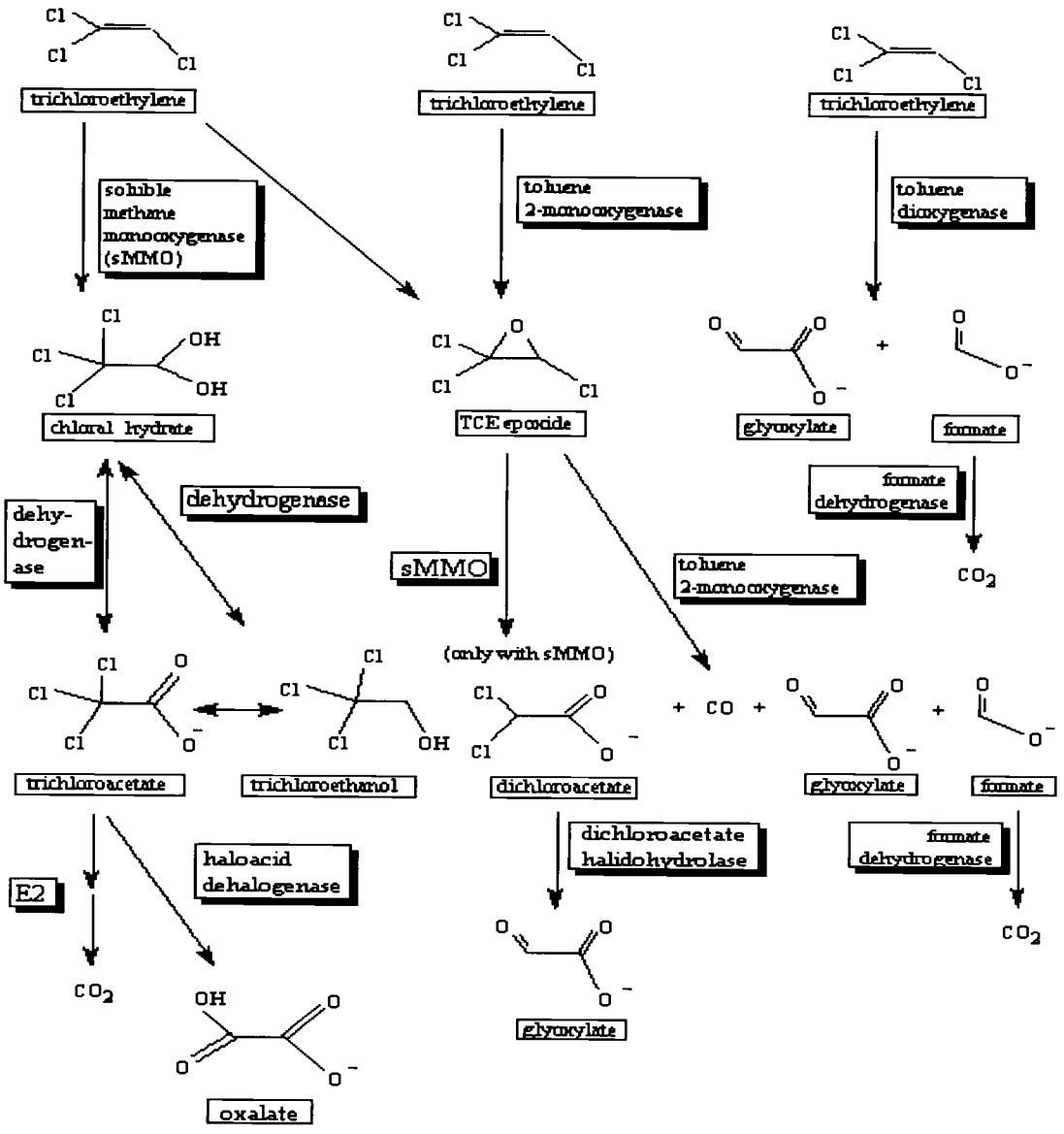

Figure 2.1 Proposed oxidation pathways of TCE by the University of Minnesota, 2001

2.3.4 Cometabolism by Butane-Utilizers

Butane utilizing microorganisms were found to contain an alkane-type monooxygenase with broad substrate specificity that was able to grow on several saturated, straight-chain hydrocarbons but not on 1-alkenes (VanGinkel et al., 1987). Similar to MMO, this nonspecific enzyme driving butane oxidation was capable of CAH oxidation. Butane also has a higher solubility and transfer rate in water than methane making more suitable to use in bioremediation field experiments. Kim (1996) reported the effective cometabolic transformation of chloroform induced by the oxygenase enzymes of butane-utilizing bacteria from the Hanford DOE site, Washington. In addition, these butane-utilizing microorganisms also had abilities to degrade a broad range of CAHs including 1,1,1-TCA, 1,1-DCE, c-DCE, VC, chloroethane, di- and trichlorethane and their abiotic transformation products (Kim et al., 1997; Kim, 2000). The complete transformation of 1,1,1-TCA concentrations as high as 2400 μg/L in aqueous solution was observed. Resting cells were not significantly affected upon exposure to 1,1,1-TCA. Compared to 1,1-DCE and other chlorinated ethanes, the transformation of 1,1,1-TCA was relatively slow. No transformation of fully chlorinated CAHs (CT and PCE) was observed by the butane utilizers (Kim, 2000). Another study used microcosms bioaugmented with a selected culture maintained effective transformation of 1,1,1-TCA, and mixtures of 1,1,1-TCA, TCE, and 1,1-DCE (Rungkamol, 2001). Little 1,1,1-TCA transformation was found in microcosms where indigenous microorganisms were stimulated. The transformation of 1,1-DCE in the bioaugmented microcosms was the fastest, followed by 1,1,1-TCA and TCE (Rungkamol, 2001). Tovanabootr (1997) found that indigenous microorganisms

form the McClellan subsurface, when stimulated on butane, were not able to transform TCE, CF, or 1,1,1-TCA.

2.3.5 Cometabolism with Propane-Utilizers

Most of the cometabolism research with gaseous alkanes has focused on the use of methane; very little research has been done with propane as the primary growth substrate. Propane utilizers that were enriched from soil and water samples were found to degrade a broad range of aliphatic hydrocarbons (Hou et al., 1983). Propane-oxidizing organisms have a propane monooxygenase (PMO) as evidenced by their ability to grow with propane as the sole source of carbon and energy (Vestal and Perry, 1969). This broad-specificity oxygenase initiates the oxidation of propane by inserting an oxygen molecule, converting it to 2-propanol, which is further oxidized to acetone (Perry, 1980). Also propane can undergo terminal oxidation and be oxidized to 1-propanol (Stephens and Dalton, 1986).

Several studies have shown that propane can be used to cometabolically transform a variety of CAHs and mixtures of CAHs. The PMO enzyme in *Mycobacterium vaccae* JOB5 was able to transform TCE, 1,1-DCE, *cis*- and *trans*-DCE, and VC (Wackett et al., 1989). A mixed culture was found capable of transforming TCE, CF, and 1,2-DCA, but not PCE or CT (Chang and Alvarez-Cohen, 1995). Intermediates formed in the biodegradation of TCE by *M. vaccae* were 2,2,2-trichloroethanol and 2,2,2-trichloroacetaldehyde (Vanderberg et al., 1995). Both trichloroethanol and trichloroacetaldehyde are known mutagens (Crebelli et al., 1985). These compounds may be highly reactive and causing covalent modification of cellular

components during oxidation of TCE. Cell inactivation and toxicity is probably not due to a buildup of these compounds in solution. Chang and Alvarez-Cohen (1995) found toxic products were rapidly depleted, leaving no toxic residues in solution.

Propane has also been shown to competitively inhibit TCE degradation (Chang and Alvarez-Cohen, 1995; Malachowsky et al., 1994; Tovanabootr, 1997). Both propane and TCE are competitors for the same PMO enzyme. If one of these compounds is at a large enough concentration it will inhibit the degradation of the other. Significant increases in TCE transformation rate have occurred due to the addition of low concentrations of growth substrate that do not inhibit degradation (Chang and Alvarez-Cohen, 1995).

Tovanabootr and Semprini (1998) used batch microcosms constructed using subsurface cores and groundwater from the McClellan AFB and stimulated indigenous microorganisms on propane. This study showed propane-utilizers to be effective in transforming TCE, TCA, and CF. Propane fed microcosms remained active for up to four weeks after propane was consumed, and were shown to effectively transform mixtures of CAHs (Tovanabootr and Semprini, 1998). From these results propane was chosen as the primary substrate in a cometabolic air sparging field demonstration at McClellan AFB.

In the CAS demonstration propane and air were pulse injected into the saturated zone once or twice per week for a duration of 5-10 hours after propane utilization was observed. After six weeks TCE, c-DCE, and DO levels decreased in proportion to propane usage. Propane and CAH concentrations began to increase after 160 days of operation due to nitrogen limitations in the groundwater (Tovanabootr et al., 2000).

Ammonia gas was added to the sparge gases and propane uptake rate increased and TCE and c-DCE removal resumed. Since the site of the CAS demonstration differed from the area where Tovanabootr obtained his core materials, new microcosm studies were needed using core material obtained during the installation of the CAS demonstration wells.

2.4 The Effects of Nutrients on Aerobic Cometabolism

Cells require nutrients in order to be able to carry out cellular functions such as growth and maintenance. Effective cometabolic transformation of CAHs depends on effective microbial growth in the subsurface environment. After carbon, the next most abundant element in the cell is nitrogen. A typical bacterial cell is composed of about 12 % nitrogen (dry weight), and nitrogen is a major element in proteins, nucleic acids, and several other constituents in the cell.

Most nitrogen found in the environment is in its' inorganic forms, either as NH_3 or NO_3^-. Microorganisms have two assimilatory metabolic pathways: the pathways necessary for utilization of nitrogen from the extracellular medium and the biosynthetic pathways for intracellular production of nitrogen-containing compounds (Merrick and Edwards, 1995). Assimilation of nitrate involves three pathway specific steps: uptake, reduction to nitrite, and further reduction to ammonium. Assimilatory enzymes are cytoplasmic, so the NO_3^- has to be transported into the cell and reduced to ammonium. Ammonia does not need to be transported across the cell wall; it rapidly diffuses across the cytoplasmic membranes and is invariably the preferred source of nitrogen for bacterial growth, in that it supports a higher growth rate than any other nitrogen source

(Merrick and Edwards, 1995). Once ammonia is inside the cell it is incorporated into two major pathways producing glutamate and glutamine, which serve as the key nitrogen donors for biosynthetic reactions. Without a sufficient source of nitrogen cells will not be able to grow efficiently and CAH transformation will decrease.

The addition of a nitrogen sources such as nitrate or ammonia to the nitrogen-deficient subsurface may be required to enhance CAH transformation. Few studies have compared the use of nitrate and ammonia as nitrogen sources for aerobic cometabolism. Chu and Alvarez-Cohen (1998) were able to grow cultures on three different nitrogen sources, ammonia, nitrate, and molecular nitrogen, and tested their ability to transform TCE. The nitrogen-fixing cultures showed the highest TCE oxidation rates. However, the ammonia-supplied cells had a higher TCE transformation rate than the nitrate-supplied cells (Chu and Alvarez Cohen, 1998). Nitrate was also tested on microcosms where TCE transformation and substrate utilization had ceased. The addition of nitrate was found to increase substrate utilization rates and TCE transformation in nitrogen limited microcosms (Tovanabootr, 1997). During the CAS demonstration at McClellan AFB the background nitrate levels were able to support propane utilization and CAH transformation for a limited time. Propane utilization and CAH transformation decreased, and nitrogen was found to be limiting during the demonstration. Ammonia gas was added to the sparge gas to supply bioavailable nitrogen. Upon addition of the ammonia gas propane utilization rates and CAH transformation resumed (Tovanabootr et al., 2000).

Nutrient limitations need to be considered for effective bioremediation decisions in the field. No experiments have been conducted on the nitrogen requirements of

microorganisms for cometabolic processes. It is unknown if the microorganisms require more nitrogen in the presence of CAHs due to the toxic effects that occur during transformation of CAHs. Also very little research has been done comparing the effects of nitrate and ammonia as sources of nitrogen. This study, the amounts of nitrogen required by the propane-utilizers in the presence and absence of CAHs is quantified using both nitrate and ammonia as the primary nitrogen sources.

2.5 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 provide a good screening method to determine whether indigenous microorganisms that can catalyze specific reactions of interest are present at a site. Many studies have used batch or column soil microcosms to evaluate the potential of indigenous microorganisms to transform CAHs (Broholm et al., 1993; Jenal-Wanner and McCarty, 1997; Kim et al., 1997; Rungkamol, 2001; Tovanabootr, 1997; Yi Mu and Scow, 1994). Most of these studies focused on determining the feasibility of promoting in-situ cometabolism by stimulating microorganisms on specific cometabolic substrates, which is basically a screening experiment. However, even if laboratory screening experiments suggest good bioremediation potential, a major concern remains, is to whether the laboratory test conditions apply and compare to the processes that will occur in the field. Jenal-Wanner and McCarty (1997) found that semicontinuous slurry microcosms were able to simulate in-situ biodegradation of TCE in a contaminated aquifer using toluene as a cometabolic substrate. TCE and

toluene removal efficiencies, oxygenase enzyme expression, and oxygen to primary substrate ratios were all similar between slurry microcosms and field results. The only difference was that the microcosms were more efficient at TCE removal (Jenal-Wanner and McCarty, 1997). The objective of this microcosm study was to examine if batch microcosms could be used to mimic what was observed at the McClellan field site during the CAS demonstration.

CHAPTER 3
AEROBIC TRANSFORMATION OF TCE AND C-DCE BY PROPANE-UTILIZING MICROORGANISMS STIMULATED FROM THE MCCLELLAN SUBSURFACE IN BATCH MICROCOSMS

3.1 Introduction and Objectives

Microcosm studies were performed for a site where cometabolic air sparging and push/pull biostimulation demonstrations were being conducted. The site is located on the southern end of McClellan Air Force Base in Sacramento, California. Microcosm studies for the McClellan site consisted of a series of tests to determine the potential for aerobic cometabolism using the growth substrate of interest (propane), and contaminants present (TCE and c-DCE). The selection of suitable microorganisms to transform specific CAHs in soil and groundwater are of interest for in-situ bioremediation. Microorganisms that catalyze the transformation of a significantly broad range of contaminant substrates are desirable for enhancing in-situ bioremediation.

Propane-utilizers that were stimulated from the McClellan aquifer material in batch microcosms have been shown to transform TCE, 1,1,1-TCA, CF, and mixtures of these CAHs (Tovanabootr, 1997). The cometabolic air sparging demonstration showed indigenous propane-utilizers transforming a mixture of TCE and c-DCE (Tovanabootr, 2000). No initial nitrogen source was added in the sparge gases, so the only available nitrogen source was the background levels found at the site. After several weeks of sparging propane utilization rates decreased, and CAH transformation ceased. Nitrate levels in the groundwater were low indicating the cause to be nitrogen limitation.

Ammonia gas was added to the sparge gas to provide bioavailable nitrogen to the treatment zone. Soon after the addition of ammonia the propane utilization rates and CAH transformation rates increased (Tovanabootr, 2000). The large propane gas tank used at the site for sparging was contaminated with ethylene gas (1% by volume). This was discovered after the demonstration was over and concerns arose regarding the effects ethylene had on the experiment at that concentration. Gas from the tank at the site containing the propane/ethylene mixture was shipped to the lab at Oregon State University. This gas was used in an experiment to test for any negative effects it might have on CAH transformation.

This microcosm study uses a combination of different nutrient and contaminant conditions to test the ability of the indigenous propane-utilizers to transform CAHs under different circumstances. The microcosm experiments were used to determine: 1) whether or not indigenous microorganisms were present at the site that are able to utilize propane; 2) the lag time required to stimulate these microorganisms, and compare it to the previous microcosm studies and field demonstration; 3) how effectively these microorganisms transform TCE and c-DCE at the concentration levels found at the field site; 4) the transformation yields and compare them with the previous microcosm studies; 5) the effects of the presence of ethylene in propane on CAH transformation; 6) the nitrogen requirements of the indigenous microorganisms when stimulated on propane; 7) and if ammonia can be used a nitrogen source.

3.2 Materials and Methods

3.2.1 Aquifer Material and Groundwater

Aquifer material and groundwater were collected at the field site to create the matrix of microcosms for the McClellan tests. The field site is located on the southern end of the AFB in an area with relatively high CAH concentrations, with TCE and *cis*-DCE concentrations exceeding 500 μg/L. Aquifer material was obtained from three cores taken March 19-24, 1999 during the construction of the monitoring wells for the CAS demonstration. Cores from monitoring wells C2, C3, and C4, at depths ranging from 112-113 ft-bgs, were used. Under a laminar flow hood the cores were aseptically removed from the brass sleeves, wet sieved through a No. 4 sieve to remove the large particles, and thoroughly mixed together before using. The core material was grayish-brown in color and had a sandy-loam texture. The groundwater for microcosm preparation was collected from monitoring well A1 at the CAS site. The groundwater was analyzed for nitrate and then purged with nitrogen to remove all the CAHs. Background nitrate levels were approximately 4.8 mg/L in the groundwater.

3.2.2 Microcosm Construction and Matrix

All materials used in microcosm construction were autoclaved (140 °C for 60 min), including batch media bottles, caps with septa, and all implements used during the construction. Aseptic construction methods help to ensure that the microorganisms that are stimulated come from the site of interest. An average of 40 g of aquifer material and 75 mL of groundwater was added to each microcosm bottle. Microcosm

bottles were 125 mL media bottles with gray butyl rubber septa and screw cap (Wheaton Glass Co., Millville, NJ). The aquifer material occupied a total volume of 15 mL, using the density of quartz 2.65 g/cm^3. This left a headspace volume of 60 mL in each bottle. The bottles have a total volume of 150 mL. The headspace permitted sampling of the gaseous substrate and CAHs.

The microcosm experimental matrix was as follows: Control MC 1 ($HgCl_2$ Poisoned), Control MC 2 (Autoclaved), Control MC 3 (No Substrate), Live MC 4-6 (CAHs, Propane), Live MC 7-9 (Propane), Live MC 10-12 (CAHs, Propane, Nitrate), Live MC 13-15 (Propane, Nitrate). For the microcosms amended with nitrate an average aqueous concentration of 125 mg/L was obtained by adding 0.15 mL of a 900 mmol/L $NaNO_3$ solution. Aqueous concentrations in the microcosms were calculated for propane, c-DCE and TCE using the dimensionless Henry's coefficients presented in Table 1.1 assuming equilibrium partitioning. Gaseous propane (98%) was added (0.5 mL) to all microcosms except control microcosm MC 3. A 2% mixture of propane in air (vol/vol) was used for the CAS demonstration. The initial headspace concentration for these microcosms was 0.83% propane by volume. This volume of propane converts to approximately 897 μg propane total mass using the gas law constant of 24.03 L/mol. Table 3.1 shows the initial total mass calculated for each microcosm using Henry's law constant. The average total mass of propane was 850 μg, which is 95% of the expected total mass added. Approximately 100 μL TCE and 7 μL c-DCE saturated water solutions (20 °C) were added to microcosms MC 1-6 and MC 10-12. The measured concentration of water saturated with TCE at 20 °C is 1100 mg/L, and 6000 mg/L for c-DCE. Literature for c-DCE saturated water at this temperature reports a value of 3500

mg/L. The solution used in these experiments was found to have a saturated concentration 1.7 times higher than the reported value (3500 mg/L). Using these saturation values the expected total mass of TCE and c-DCE added to each microcosm was calculated to be 110 μg and 42 μg, respectively. The average initial total mass calculated using Henry's coefficients for TCE and c-DCE was 101 μg and 35 μg, respectively. This represents a 7% difference for TCE and a 16% difference for c-DCE.

Table 3.1 Initial total mass and corresponding aqueous concentrations for microcosms.

Microcosm MC	Initial Total Mass (μg)	Initial Total Mass (μg)	Initial Total Mass (μg)	Aqueous Conc. (μg/L)	Aqueous Conc. (μg/L)	Aqueous Conc. (μg/L)
	Propane	c-DCE	TCE	Propane	c-DCE	TCE
1	830	35	110	430	360	1000
2	750	36	110	390	370	1000
3	0	38	120	0	390	1070
4	880	35	120	460	360	1040
5	850	35	110	440	350	970
6	790	32	100	420	330	890
7	850	0	0	440	0	0
8	850	0	0	440	0	0
9	880	0	0	460	0	0
10	790	32	100	420	320	910
11	790	32	100	410	320	910
12	800	32	100	420	330	890
13	880	0	0	460	0	0
14	830	0	0	430	0	0
15	880	0	0	460	0	0

Control microcosms 1-3 were created to evaluate abiotic loss processes. Control MC 1 was a killed control with 25 mg/L mercuric chloride added. Control MC 2 was autoclaved 3 times, with 24 hours between autoclaving, to kill any spore forming bacteria. The autoclaved control helps to determine if microbial contamination occurs during microcosm construction and operation. Control MC 3 did not have any substrate present, only TCE and c-DCE. This is a live control that will determine if the indigenous microorganisms can degrade the CAHs of interest without the presence of a substrate.

Live microcosms MC 4-6 did not have any nutrient amendments, and background levels of nitrate (4.8 mg/L), as a nitrogen source. These microcosms were used to evaluate if there are sufficient nutrients present to stimulate the indigenous microorganisms on propane, and to estimate how much substrate can be utilized and CAHs transformed with the ambient concentrations of nitrate present. Live microcosms MC 7-9 were not amended with nutrients, and had only propane added. Lag time to stimulate the microorganisms without any CAHs present, were determined in these microcosms. They were also used to compare the nutrient needs in the presence and absence of CAHs. Nitrate was added microcosms 10-12 to determine the rates of propane utilization and CAH transformation when nitrogen was in excess, and whether other nutrients besides nitrate limited propane utilization. Microcosms 13-15 also had nitrate added to determine if a higher concentration of nitrate would decrease the lag time required to stimulate the microorganisms. They also were used to compare the utilization rates with microcosms 7-9.

3.2.3 Chemical Sources, Saturated and Standard Solutions

Anhydrous ammonia (99.99%) was purchased from Scott Specialty Gases in Plumsteadville, PA. Trichloroethylene (TCE;>99%) and c-DCE (>97%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Propane (98%) and ethylene (99%) were also purchased from Aldrich Chemical Co. (Milwaukee, WI). Saturated stock solutions of CAHs were prepared at room temperature by adding specific amounts of the liquid to 125-mL serum bottles containing autoclaved deionized water. This procedure eliminated the use of carrier solvents, such as methanol. The bottles were shaken for 6 hours to ensure saturation, and were then allowed to settle for at least 24 hours before use.

Standard solutions were made for creating standard calibration curves for CAH Gas Chromatograph (GC) analysis. A given mass of the compound was dissolved in a known volume of methanol. A given volume of methanol solution was added to a capped 28 mL vial that contained 10 mL of deionized water. The solution was equilibrated on a shaker table at 20 °C. Headspace samples were taken and analyzed using GC methods. Mass balances using Henry's Law were made to determine the corresponding liquid concentrations, and to construct standard curves.

3.2.4 Sampling and Analytical Methods

The microcosm tests required the measurement of headspace concentrations of the gaseous substrate and CAHs of interest. Headspace samples were obtained using a 100-μL gas-tight syringe. Samples were manually injected onto the appropriate GC immediately after sampling. During the course of the microcosm incubation, headspace

sampling and oxygen and propane consumption created a vacuum inside the microcosms. After propane was consumed the headspace was equilibrated to atmospheric pressure under a laminar flow hood by adding pure oxygen. This maintained oxygen concentrations in the microcosms so that aerobic conditions were maintained throughout the experiment.

CAH and gaseous substrate concentrations were quantified by GC analysis. The HP 6890 GC was equipped with a photo ionization detector (PID) in conjunction with a flame ionization detector (FID). The CAHs with unsaturated bonds were detected with a PID, while the FID detected the gaseous cometabolic substrate (propane). The advantage of using PID/FID detectors in series was that the gaseous substrate and chlorinated ethenes could be determined in the same analysis. Separation was obtained by using a GS-Q capillary column (VWR Scientific Inc.) operated with a temperature gradient for better separation of the gaseous substrate and chlorinated ethenes. High-grade helium, at a head pressure of 60 psi, was used as the carrier gas. The method was calibrated by using the external standards. The total mass of compounds in the microcosms was determined by mass balance assuming Henry's law equilibrium partitioning.

Nitrate was measured by ion chromatography (IC) using a Dionex 40001 IC equipped with a conductivity detector. A Dionex Ionpac AS4A column was used for the chromatographic separation. The column uses a regenerate containing and H_2SO_4, Na_2CO_3, and $NaHCO_3$. A 0.7 mL aqueous microcosm sample was centrifuged to remove any suspended particulates, and a 0.5 mL sample was used in the analysis. External standards of $NaNO_3$ were used for calibration of this method.

3.3 Results and Discussion

3.3.1 Control Microcosms

Results from the control microcosms are shown in Figure 3.1, for 101 days of operation. Initial total mass and aqueous concentrations for these microcosms are presented in Table 3.1.

Propane concentrations remained essentially constant in the Controls 1 and 2 over the entire study period, while limited CAH losses were observed. An average TCE loss of 37% (std deviation = 2.2%) was observed in the three control bottles. Controls 1 and 2 averaged a 20% loss of c-DCE, while Control 3 showed a 40% c-DCE loss. The slow continuous loss of the CAHs was most likely due to slow leakage from the bottles, or partitioning into the butyl rubber septa or onto the aquifer solids. The limited loss of propane from the microcosms indicates leakage was very limited. The greater losses of TCE and c-DCE compared to propane likely resulted from partitioning processes. The greater c-DCE loss in the no-substrate control may have been partially due to intrinsic biotic c-DCE transformation. In any case, the CAH losses in the controls were quite small when compared to CAH removal observed in the active microcosms, as will be discussed.

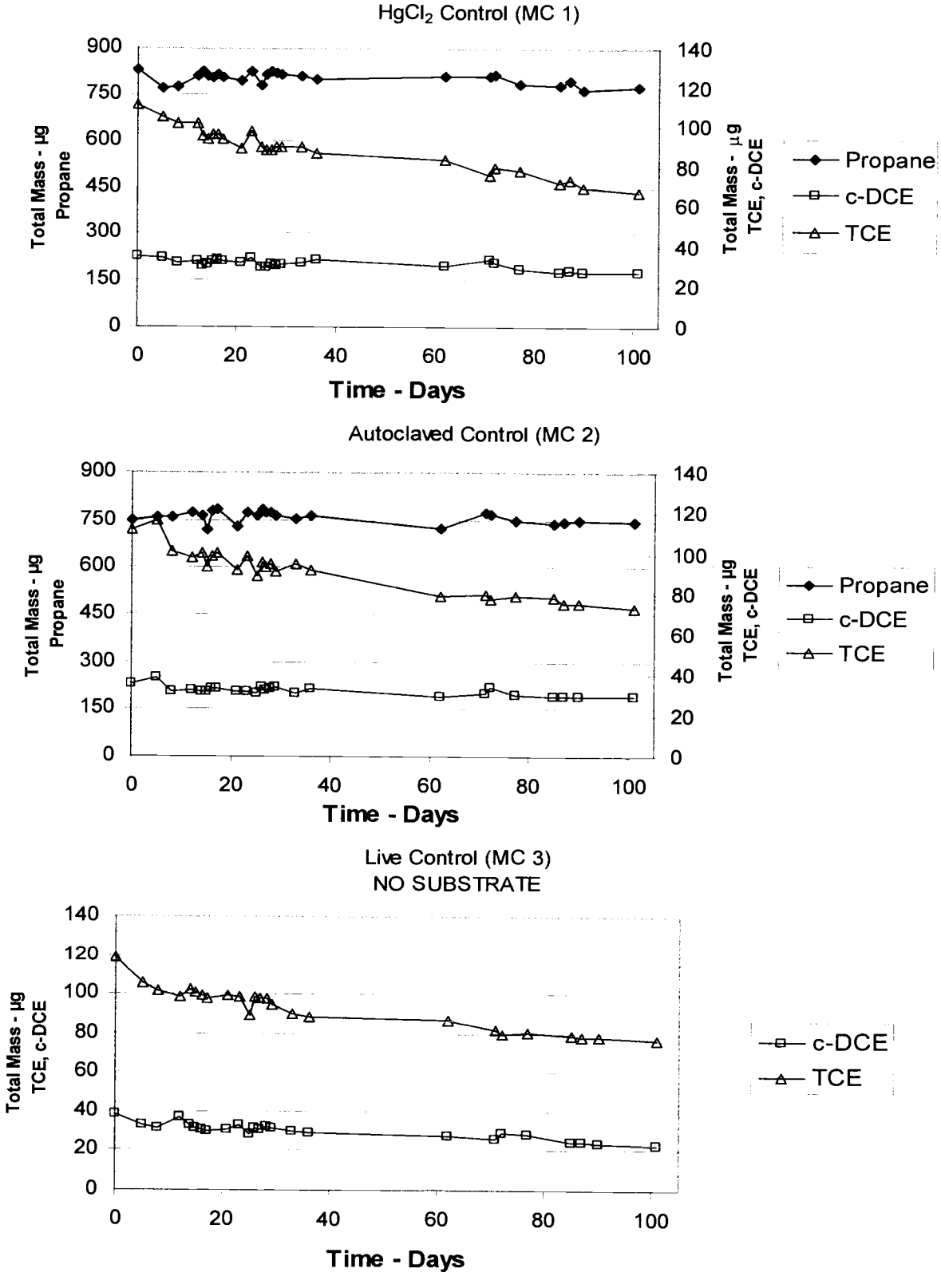

Figure 3.1 Propane, c-DCE, and TCE concentrations in control microcosms.

3.3.2 McClellan Live Microcosms MC 4-6

Live microcosms MC 4-6 were used to test the transformation ability of propane utilizers without nitrogen amendments. Only the background groundwater nitrate (4.8 mg/L) was available for microbial growth. These microcosm tests were used to determine: 1) the lag time of indigenous microorganisms in the presence of CAHs without any nutrient amendments; 2) the amount of propane utilized and CAHs transformed with only the background nutrient levels; 3) and the transformation yields and propane utilization rates.

The microcosms were operated for 37 to 41 days, through several successive additions of propane. Table 3.1 provides the initial mass of propane, c-DCE, and TCE added to the microcosms and corresponding aqueous concentrations. The lag times for propane consumption were very reproducible in all three microcosms with about ten days of incubation required before observable propane utilization occurred (Figure 3.2). After the initial lag time, propane was rapidly consumed in all the microcosms within a two-day period. Once the propane concentrations decreased, TCE and c-DCE concentrations also began to decline. By the time the second addition of propane was utilized all of the c-DCE had been completely transformed in the microcosms. All microcosms showed c-DCE being completely removed while TCE was partially removed. This could be due to a factor of three greater mass of TCE initially present than c-DCE in the microcosms, or because c-DCE is being transformed at a faster rate than TCE. Table 3.2 shows the amount of propane utilized and CAHs transformed after the first three additions of propane. Results were very reproducible in all three microcosms.

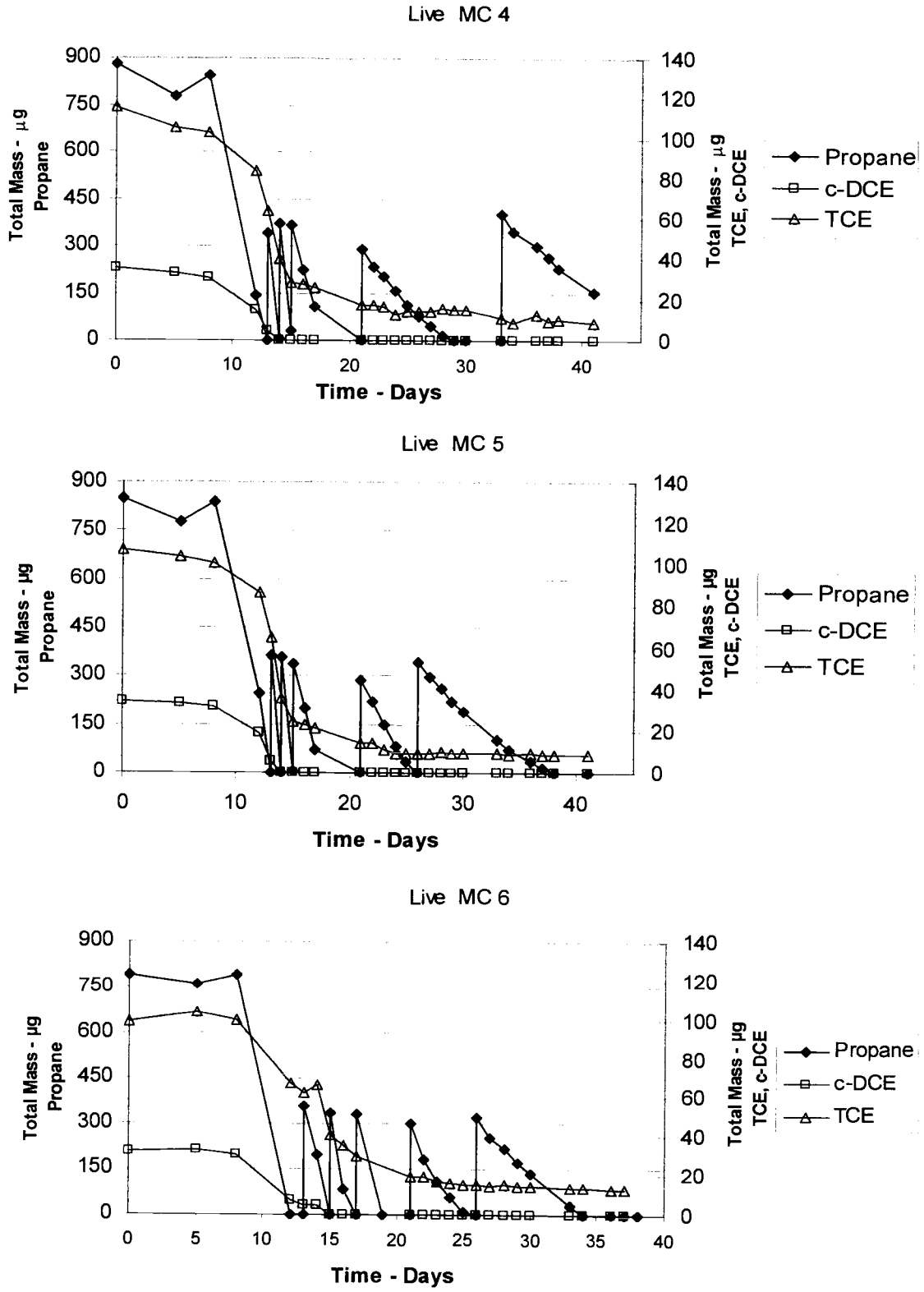

Figure 3.2 Propane, c-DCE, and TCE concentrations in microcosms MC 4-6 with no nitrate amendments.

Table 3.2 Amounts of propane utilized and CAHs transformed in microcosms MC 4-6 after the third addition of propane.

Microcosm	Lag Time (Days)	Propane Utilized (μg)	c-DCE Transformed (μg) (Percent Transformed)	TCE Transformed (μg) (Percent Transformed)
4	9-12	1590	35 (100%)	87 (75%)
5	9-12	1570	35 (100%)	84 (78%)
6	9-12	1490	32 (100%)	69 (70%)

After about 15 days of incubation the microcosms began to metabolize propane at slower rates (Table 3.3). Nitrate concentrations in these non-amended microcosms were below the analytical detection limit (25 μg/L) when sampled on day 22. Propane utilization continued in the absence of detectable nitrate, but at much slower rates. From day 21 to day 41, no measurable TCE transformation occurred and propane utilization rates decreased below 4 μg/hr.

The average transformation yields for these microcosms were 0.049 μg TCE/μg propane and 0.083 μg CAH/μg propane before propane utilization significantly decreased. The results indicated that as the propane utilization rate decreases, due to nitrogen limitations, TCE cometabolism slowed and eventually ceased.

Table 3.3 Propane utilization rates for microcosms MC 4-6.

Microcosm	Day 14-15 Utilization Rate (μg/hr)	Day 15-16 Utilization Rate (μg/hr)	Day 16-17 Utilization Rate (μg/hr)	Day 22-24 Utilization Rate (μg/hr)
4	14	5.9	4.9	1.6
5	15	5.7	5.5	2.8
6	8.2	11	6.9 *	2.1

* Rate from day 17-19

3.3.3 McClellan Live Microcosms MC 7-9

Microcosms MC 7-9 were fed propane in the absence of c-DCE and TCE, with only background levels of nitrate present. These microcosms were used to determine: 1) if the absence of CAHs would change the lag time required to stimulate the indigenous microorganisms; 2) the amount of propane utilization that occurred before showing the effects of nitrogen limitations were observed; 3) and the propane utilization rates.

The initial mass of propane and the corresponding aqueous concentration added into the microcosms are presented in Table 3.1. A lag time of 9-12 days occurred before propane utilization began, and propane was rapidly consumed within two days (Figure 3.3). Both the lag period and the time required to consume propane were similar to that observed in the CAH amended microcosms. The rate of propane utilization began to decrease after about 20 days of successive additions (Table 3.4). These microcosms without CAHs present consumed more propane than those with CAHs present (Table 3.5). Nitrate concentrations in the microcosms were below the analytical detection limit when sampled on day 31.

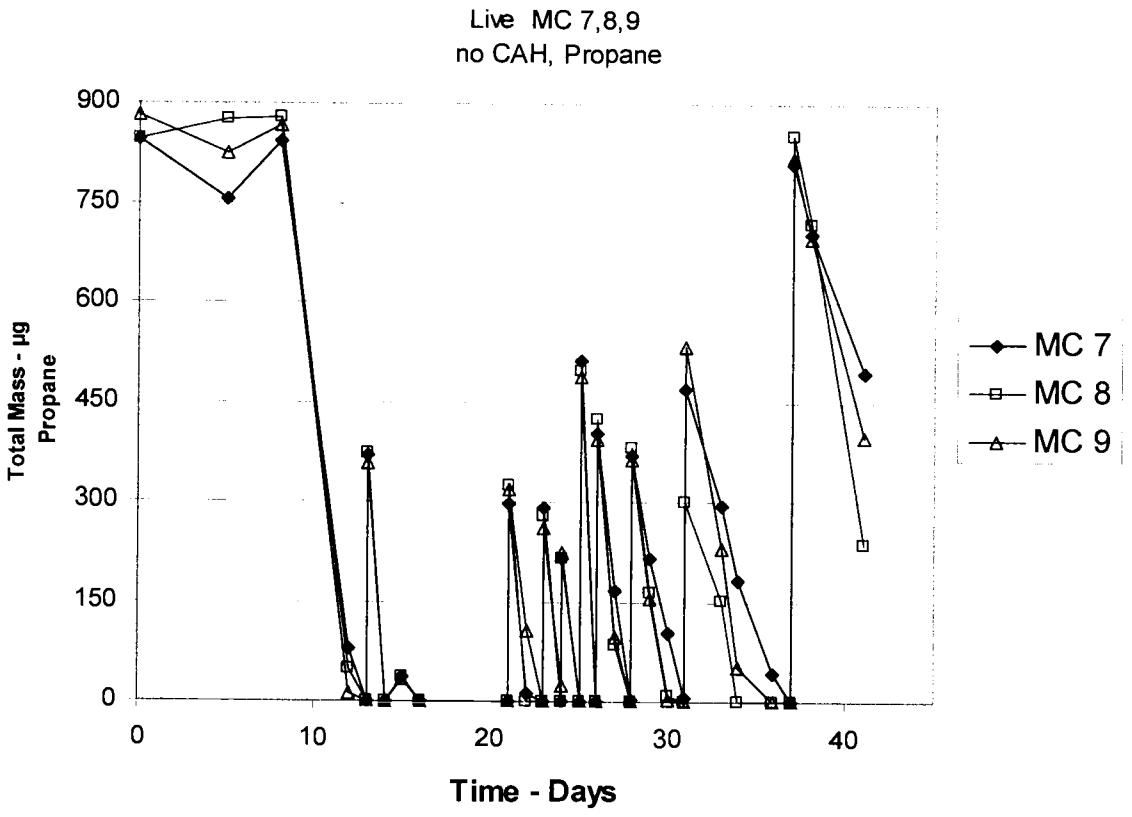

Figure 3.3 Propane utilization in live microcosms MC 7-9 without any nitrogen amendments or CAHs present.

Table 3.4 Propane utilization rates for live microcosms MC 7-9.

Microcosm Number	Day 8-12 Utilization Rate (µg/hr)	Day 26-27 Utilization Rate (µg/hr)	Day 28-29 Utilization Rate (µg/hr)	Day 38-41 Utilization Rate (µg/hr)
7	≥ 16	≥ 21	6.5	2.9
8	≥ 16	≥ 21	9.1	6.6
9	≥ 15	≥ 20	8.7	4.1

Table 3.5 shows the comparison of the amount of propane consumed before the effects of nitrate limitation occurred between microcosms with and without CAHs. The amount of propane utilized was estimated to be the amount used before a significant decrease (≤ 4 μg/hr) in propane utilization rate was observed. Utilization rates in Table 3.3 and 3.4 show the significant decrease in utilization rates. Microcosms MC 4-6 utilized 43 μmol propane before showing a propane utilization rate around 4 μg/hr, while microcosms MC 7-9 utilized 86 μmol propane. This result shows that the microcosms without any CAHs present utilized twice as much propane as the microcosms with CAHs present.

Table 3.5 Comparison of propane and nitrogen utilization under different conditions.

Microcosm Number (Time of nitrogen limitation)	Propane Utilized (μmol)	Nitrogen Utilized (μmol)	Ratio Nitrogen/ Propane Utilized (μmol/μmol)
4 (Day 21)	44	5.8	0.13
5 (Day 21)	43	5.8	0.13
6 (Day 21)	41	5.8	0.14
7 (Day 37)	87	5.8	0.07
8 (Day 37)	84	5.8	0.07
9 (Day 37)	88	5.8	0.07

Maximum utilization rates were significantly different between the microcosms exposed to CAHs compared to the unexposed microcosms. The initial rates for microcosms 7-8 ranged from 16-21 μg/hr, while microcosms 4-6 had a maximum range

of 8-15 μg/hr. Inhibition of TCE on propane utilization could be one factor causing the slower rates of utilization. The amount of nitrogen required per mole of propane consumed was 100 % higher and the maximum utilization rates were 38% lower in microcosms with CAHs present. More nitrogen may be needed when CAHs are present due to the effects of transformation product toxicity.

3.3.4 McClellan Live Microcosms MC 10-12

Microcosms MC 10-12 were amended with nitrate to achieve an average aqueous concentration of 125 mg/L as NO_3^-, thus nitrogen never became limiting in these microcosms. These microcosms were fed propane, c-DCE, and TCE. The initial total mass of CAHs, propane and corresponding aqueous concentrations are presented in Table 3.1. These microcosms were used to determine: 1) the lag time to stimulate the indigenous microorganisms in the presence of CAHs with nitrate amendments; 2) and propane utilization rates and CAH transformation yields in the absence of nitrogen limitations.

Figure 3.4 displays the results from the first 38 days of operation for the microcosms. For the first 15 days of incubation, these microcosms were very similar to microcosms 4-6. For example, having the same lag period prior to propane utilization, and most of the c-DCE was transformed upon the initial utilization of propane. Initial transformation yields were 0.036 μg c-DCE/μg propane, and 0.056 μg TCE/μg propane, with a combined transformation yield of 0.092 μg CAHs/μg propane, which is similar to the calculated CAH transformation yield in microcosms MC 4-6 (0.083 μg CAHs/μg propane). Three to four additions of propane were required to completely

remove the TCE from the microcosms. Since propane was successively added, its presence may have inhibited TCE transformation.

Residual TCE was left in microcosms MC 10 and 11 for four days before the fourth addition of propane. A 2-3 day lag period was observed in these two microcosms before propane utilization resumed (Figure 3.4). This may have been due to TCE transformation product toxicity.

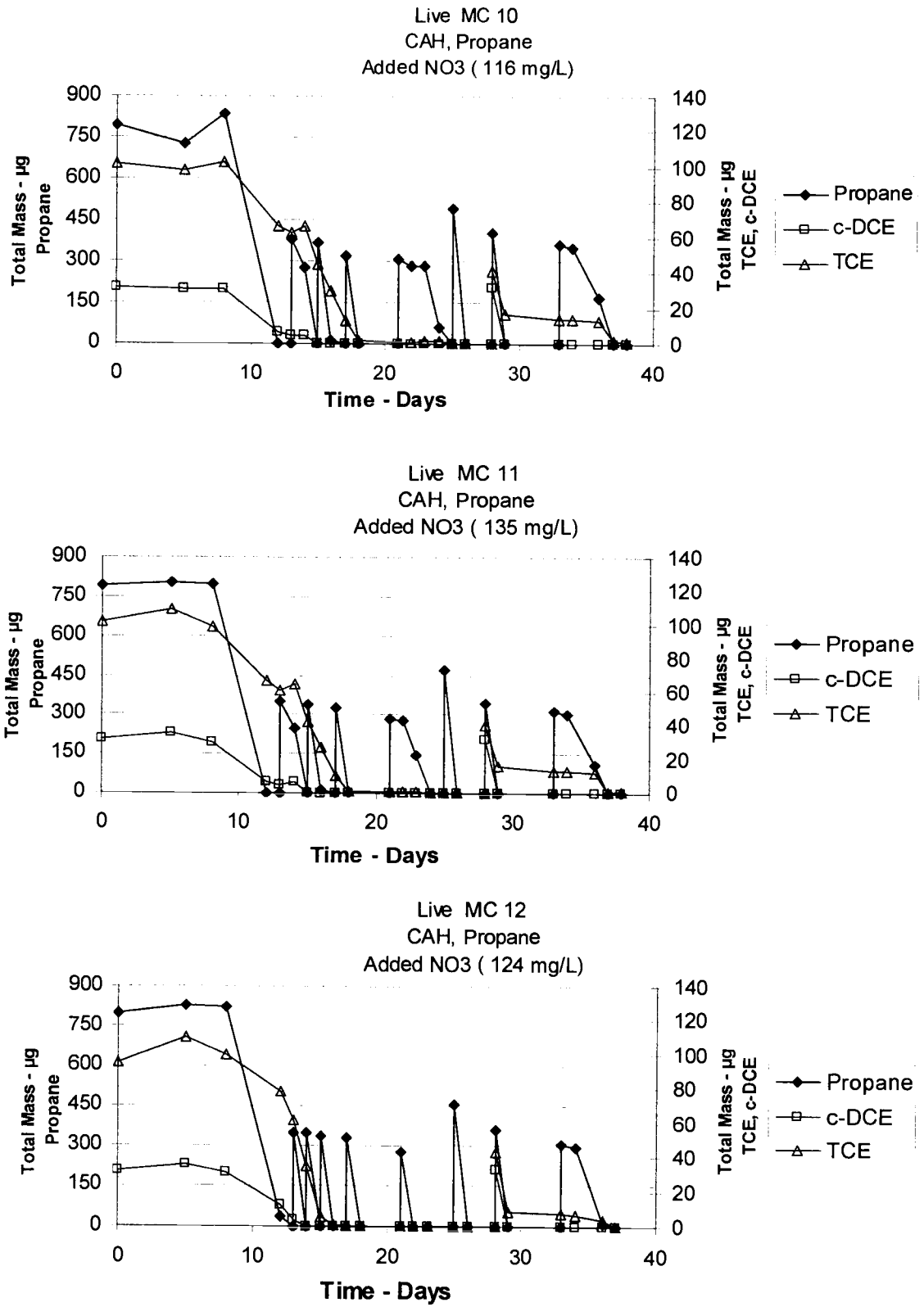

Figure 3.4 Live microcosms MC 10-12 with nitrogen amendments.

After day 15, propane utilization and CAH transformation rates did not decrease with successive additions of propane, as they did in microcosm MC 4-6. Table 3.6 presents microcosm set MC 10-12 (nitrate amended) after the third addition of propane, which can be compared to Table 3.3 (non-nitrate amended).

Table 3.6 Lag times, propane utilization, and CAH transformation in microcosms MC 10-12 with nitrate amendments after the third addition of propane

Microcosm	Lag Time (Days)	Propane Utilized (μg)	c-DCE Transformed μg (% Transformed)	TCE Transformed μg (% Transformed)
10	9-12	1530	32 (100%)	102 (98%)
11	9-12	1480	32 (100%)	102 (99%)
12	9-12	1490	32 (100%)	95 (100%)

Transformation yield for microcosms MC 10-12 (0.066 μg TCE/μg propane) was slightly greater than the 0.052 μg TCE/μg propane calculated for microcosms 4-6. The amounts of propane utilized were similar, but utilization rates in the unamended microcosms began to decrease after the third addition of propane (Table 3.3). Propane utilization in microcosms MC 10-12 did not decrease with successive additions of propane, but lag periods up to three days were observed when rest periods occurred between propane additions. These lag periods occurred when TCE was transformed in the absence of propane. By the third addition of propane, 99% of the TCE was removed in the nitrate-amended microcosms, while 74% was removed in the unamended microcosms.

On day 28, propane and CAHs were again added to the nitrate-amended microcosms and a detailed analysis was completed over a 32-hour period (Figure 3.5). Propane was completely utilized within 14 hours of operation, yielding a utilization rate of 26 μg propane/hr. Very reproducible transformation was observed in the triplicate microcosms. It was observed that c-DCE was more rapidly transformed than TCE, and transformation of c-DCE occurred while propane was being utilized. The maximum transformation rates observed for TCE and c-DCE were 2.5 μg/hr and 3.3μg/hr, respectively. Most of the TCE was transformed after propane was reduced to a low concentration, and continued for only a few hours after propane was consumed.

In these nitrate-amended microcosms, the average transformation yield was 0.077 μg TCE/μg propane. These are likely inflated values due to prior additions of propane in the absence of any CAHs permitting biomass accumulation (Figure 3.4). Additions of propane without any CAHs present increased the available cell mass in the microcosms for transformation, around day 25, and resulted in the rapid transformation of propane, c-DCE, and TCE when added on day 28.

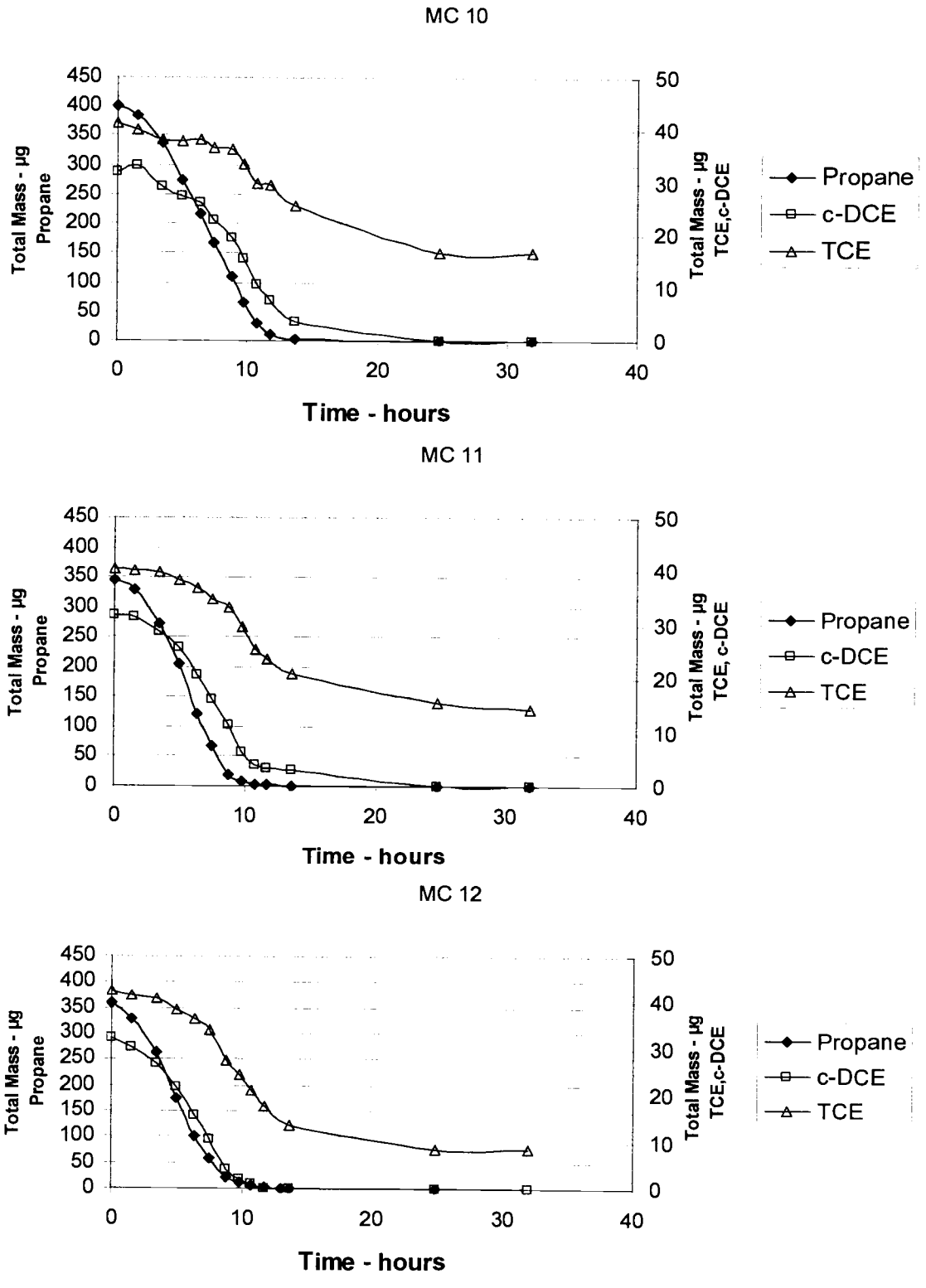

Figure 3.5 Propane utilization and CAH transformation in microcosms MC 10-12 over a 32-hour period (days 28-29).

TCE remained in all three microcosms for four days after the experiment. Over the four day period TCE was still being transformed, but at a very slow rate. A total of 1.3-2.7 μg TCE was transformed in the three microcosms without the presence of propane. Long-term transformation of up to 23 days occurred in previous propane fed microcosms (Tovanabootr and Semprini, 1998). Propane was again added to the nitrate-amended microcosms on day 33. Two to three days were required for propane utilization and TCE concentrations remained constant until propane concentrations were substantially reduced (Figure 3.4). This delay in utilization of the primary substrate suggests a decrease in the microorganisms' ability to consume propane. It appears that TCE transformation had a negative effect on the propane-utilizing microbial population.

3.3.5 McClellan Live Microcosms MC 13-15

Microcosms MC 13-15 were amended with nitrate (122 mg/L as NO_3^-), and were fed propane but were not challenged with CAHs. These microcosms were used to determine: 1) if the lag time to stimulate the indigenous microorganisms would be different when nitrate was added but with CAHs absent; 2) propane utilization rates; 3) and if any other nutrients may be limiting other than nitrate. Table 3.1 shows the amount of propane added and the associated aqueous concentrations.

Figure 3.6 presents the propane utilization occurring with repeated additions over 38 days. As with previous microcosms, a lag period of 9-12 days occurred before propane utilization began. This set of microcosms can be compared with microcosms MC 7-9 to show the effects of nitrogen limitations. No decrease in propane utilization

rates was observed in microcosms MC 13-15, as was observed in microcosms MC 7-9 (Table 3.4). A maximum propane utilization rate of 34 μg/hr was observed (day 37-38). The results indicate that other nutrients, such as phosphorous, were not limiting, and bioavailable nitrogen was the main limiting factor, which could be overcome through nitrate addition.

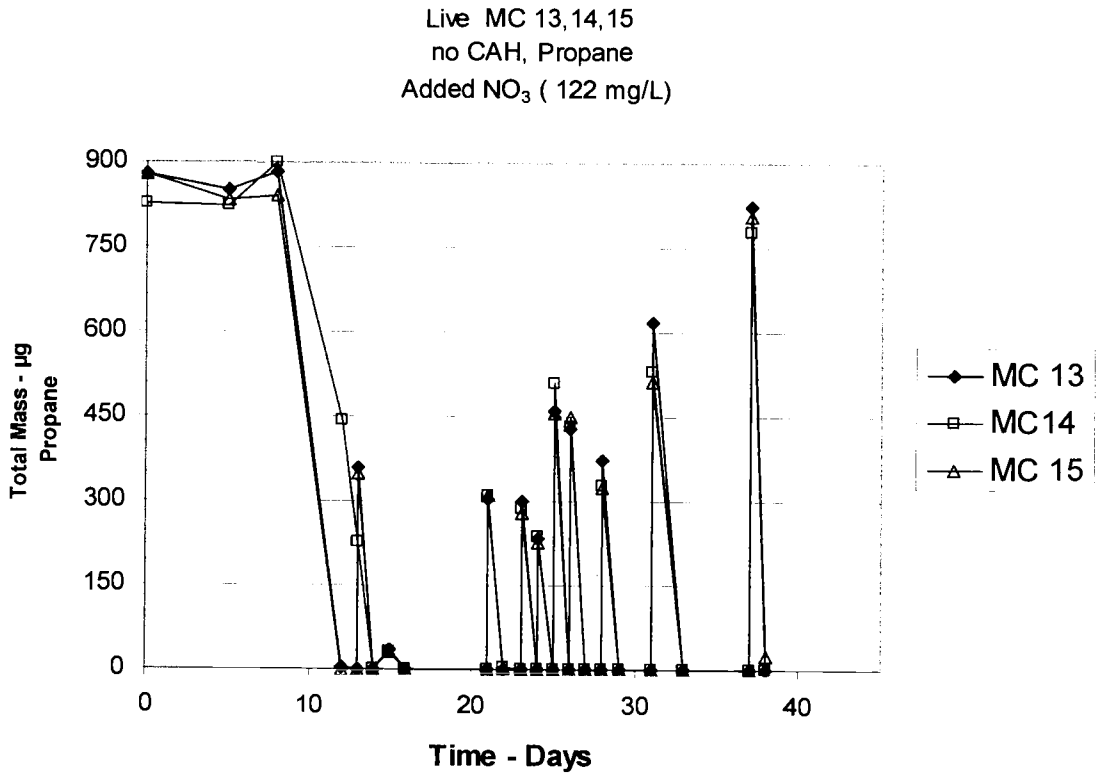

Figure 3.6 Propane utilization over a 38-day period in microcosms MC 13-15 that were amended with nitrate.

3.3.6 Ethylene Test

Ethylene was a component in the propane used at the McClellan field test at a mole fraction of approximately 1%. The impact of ethylene on the transformation of

CAHs at the site is unknown. Ethylene has been found in some cases to inhibit the transformation of some CAHs, and the intermediate ethylene oxide can also be toxic to microorganisms (Freedman and Herz, 1996). A microcosm experiment was conducted to test for any inhibitory effects from the 1% ethylene contained in the propane. Propane gas samples were shipped from the field site for this study. Microcosms MC 10-12 (Figure 3.4 and 3.5) that had nitrate added were used for this test. The ethylene experiment was conducted as those shown in Figure 3.5, except the contaminated propane gas from the field was used. The two tests were separated by a 40-day interval. To create a similar experiment propane was added twice the week before the experiment. This was similar to the propane injections that occurred in the absence of CAHs before the experiment in Figure 3.5.

Results are presented in Figure 3.7. Very reproducible results were achieved in all three microcosms, and were essentially the same as those shown in Figure 3.5. The presence of ethylene in the propane at the field site did not negatively effect CAH transformation. Ethylene was effectively transformed at a rate of 1.5 μg/hr during the period when propane was being utilized. Similar maximum rates and extents of TCE and c-DCE transformation were achieved with ethylene present (Table 3.7).

Table 3.7 Comparison between propane and CAH rates with and without ethylene present in microcosms MC 10-12.

Microcosm	Propane Rate (μg/hr)	c-DCE Rate (μg/hr)	TCE Rate (μg/hr)
MC 10	43	3.5	1.8
MC 10 w/ Ethylene	47	3.4	2.4
MC 11	42	3.3	2.2
MC 11 w/ Ethylene	38	2.7	1.9
MC 12	39	3.0	3.3
MC 12 w/ Ethylene	46	3.6	3.0

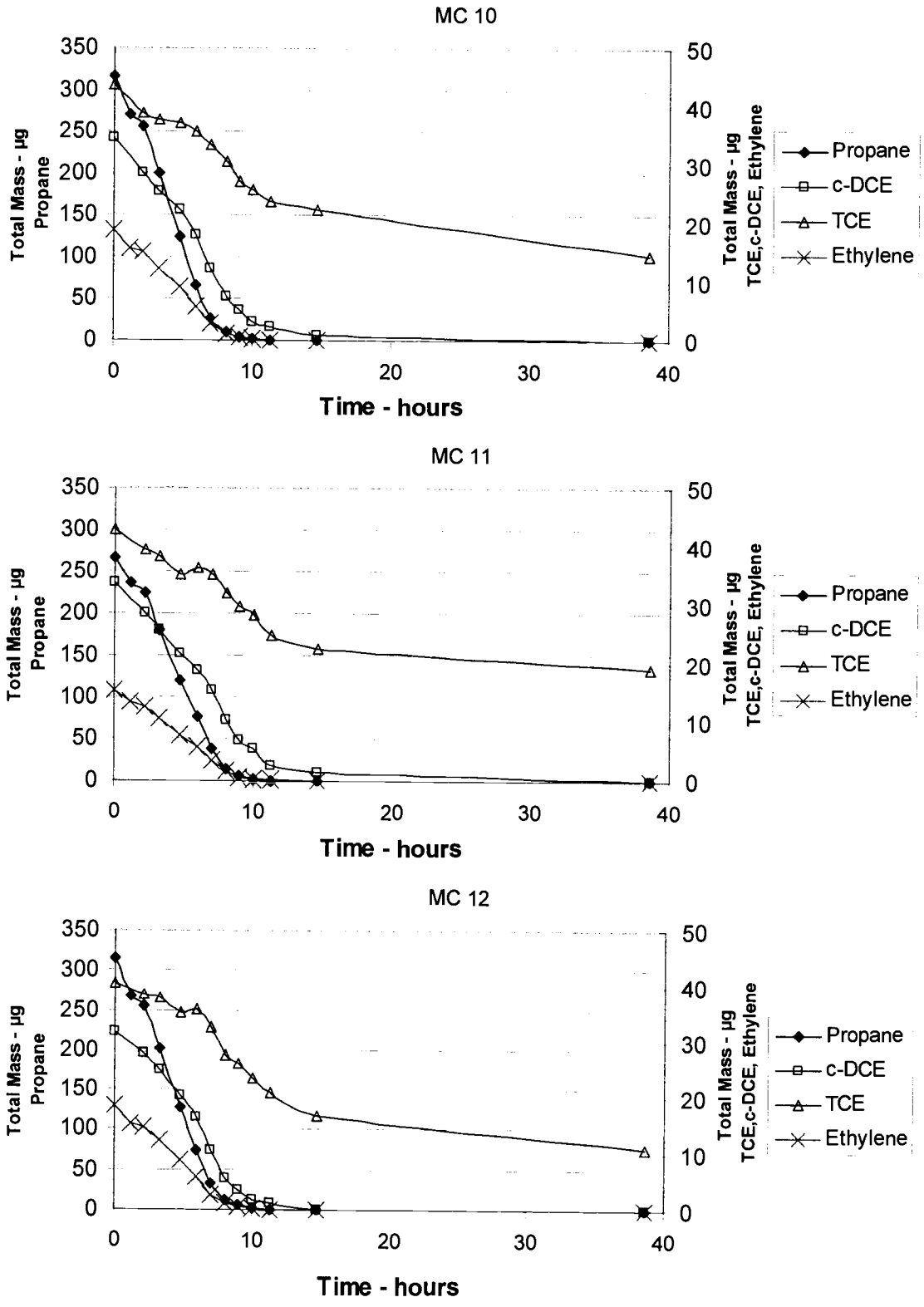

Figure 3.7 Results of the propane and ethylene experiment using microcosms MC 10-12.

The transformation yield for this experiment was 0.092 μg TCE/μg propane, which is higher than the 0.077 μg TCE/μg propane achieved without the presence of ethylene. Lag times for the transformation of c-DCE and TCE were also similar. The rate of c-DCE transformation in both experiments was faster than TCE. Transformation of TCE was slow throughout the propane utilization phase, and significantly increased once the total mass of propane dropped below 100 μg. In both cases the rate of TCE transformation increased significantly after the concentration of propane and c-DCE decreased, and continued for several hours after propane was utilized.

3.3.7 Repeated Additions of CAHs

An experiment was conducted to test the propane-utilizing microorganisms ability to degrade successive additions of CAHs. Three additions of TCE and c-DCE were added to nitrogen-amended microcosms MC 10-12 over a 65-day period. The last addition tested the ability of the propane-utilizers to transform higher concentrations of CAHs. Aqueous concentrations of 5.5 mg c-DCE/L and 3.5 mg TCE/L were achieved for the experiment. The objectives for this experiment were to determine: 1) if any other macro/micro nutrients may be limiting; 2) the propane-utilizers ability to transform multiple additions of CAHs; 3) transformation yields for each CAH addition; and 4) if propane-utilizers can utilize propane at higher concentrations of CAHs.

All three microcosms had residual TCE (1.3 μg TCE) left from the previous experiments. An average of 825 μg propane was initially added to each microcosm. A lag period of 4-6 days was observed in all three microcosms. TCE concentrations were

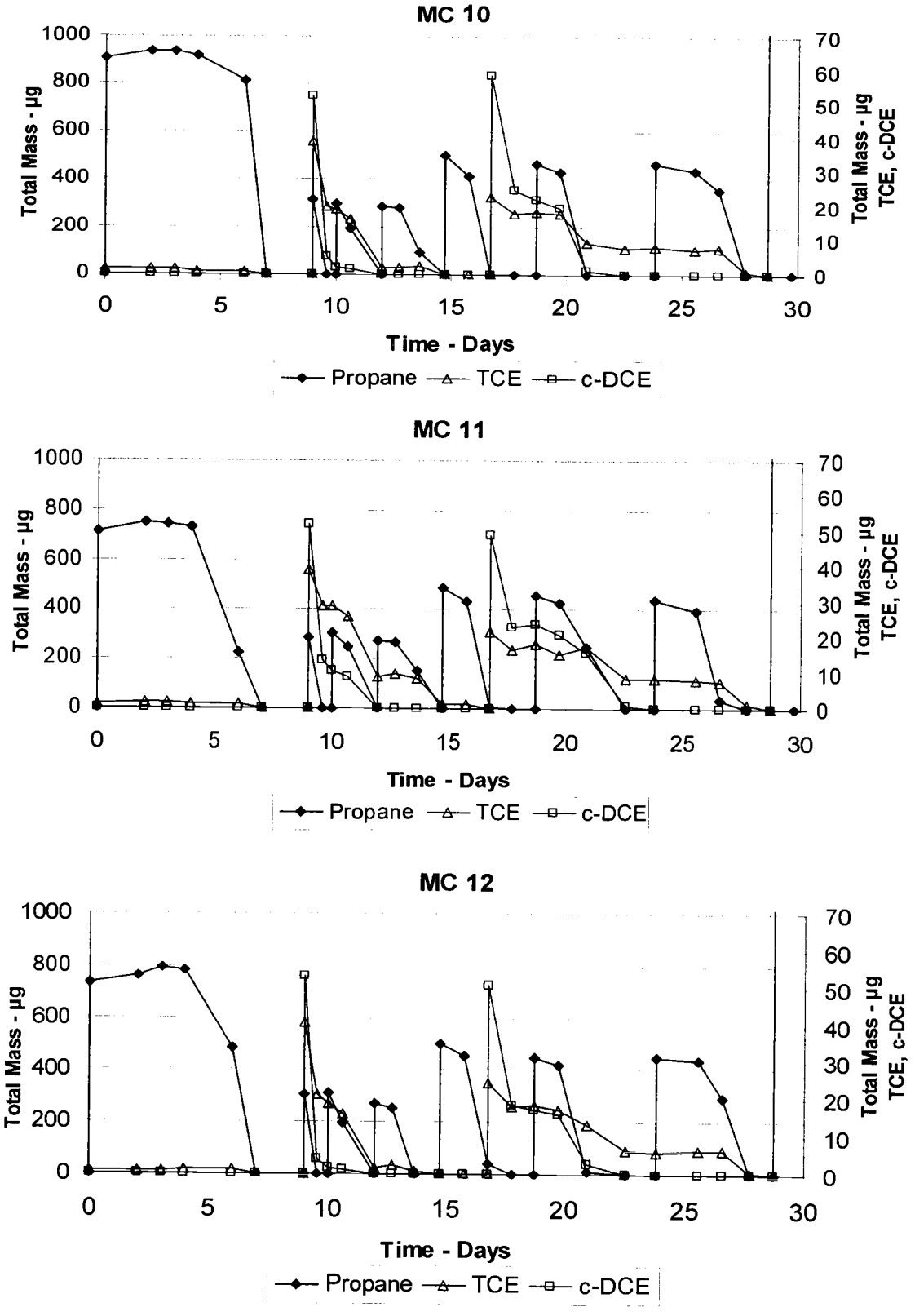

Figure 3.8 First two successive additions of CAHs at lower concentrations.

below detection limits in all three after the first incubation with propane. Figure 3.8 shows the first two additions of CAHs with an average of 53 μg c-DCE and 39 μg TCE added on day 9, and an average of 52 μg c-DCE and 23 μg TCE added on day 17. Two different transformation yields were calculated for the third and sixth addition of propane. For the third addition of propane the average transformation yield was 0.075 μg CAH/μg propane, and the sixth addition was 0.069 μg CAH/μg propane. Only TCE was left after the sixth addition of propane. One more addition of propane was added to transform the remaining TCE before the high concentration test.

The third addition of CAHs was added on day 29 with an average of 542 μg c-DCE (5.5 mg/L aqueous concentration) and 386 μg TCE (3.5 mg/L aqueous concentration). For two days the CAHs were allowed to equilibrate before adding 455 μg propane on day 31. A lag period of six days was observed in all three microcosms before propane began to be utilized (Figure 3.9). It took twelve days to utilize the initial addition of propane after the lag period. Propane was never fully utilized in these microcosms, and an average of 15 μg propane remained in each one. Transformation yields for this first propane addition ranged from 0.12-0.14 μg c-DCE/μg propane, and 0.040-0.064 μg TCE/μg propane. This initial propane addition had a higher transformation yield for c-DCE than previously observed (0.036 μg c-DCE/μg propane), but a similar transformation yield for TCE. The total CAH transformation yield was 0.19 μg CAH/μg propane, which is 2.5 times greater than the transformation yield obtained at the lower CAH concentration level (0.075 μg CAH/μg propane). The majority of CAH transformation occurred from day 41 to 45 when the propane concentration significantly decreased.

Nitrate concentrations were measured during this experiment. Before the experiment began the nitrate concentrations in MC 10-12 were 99 mg/L, 110 mg/L, and 102 mg/L, respectively. After the first two CAH additions the nitrate concentrations were 83 mg/L, 99 mg/L, and 87 mg/L, respectively. The total volume of groundwater in the microcosms is 75 mL. From this the total mass of nitrate consumed over these first two CAH additions was 1.2 mg NO_3^-, 0.85 mg NO_3^-, and 1.2 mg NO_3^-, respectively.

Two more additions of propane were added to these microcosms from day 49 to day 58. These additions (1585 μg propane) were over 3 times higher than the initial propane addition (455μg propane). From day 49 to day 51 the rate of propane utilization with the first large addition of propane was slow, but increased significantly from day 51 to 53. Transformation of CAHs occurred during day 51-53 when propane utilization rates increased, and propane concentrations decreased. Transformation of CAHs was not observed from day 53-54 when propane was not available in the microcosms, indicating no long-term transformation ability of the propane utilizers without the presence of propane. On day 54, the second large addition of propane was added. There was no observed decrease in propane utilization rate as was previously noticed with the first large addition of propane. By day 59 all propane had been utilized and transformation of CAHs had ceased. No significant loss of CAHs was measured from day 59-65 when propane was absent, indicating no long-term transformation occurred. The transformation yields for the two large additions of propane ranged from 0.022-0.051 μg c-DCE/μg propane, and 0.005-0.01 μg TCE/μg propane. The total CAH transformation yield was 0.042 μg CAH/μg propane, which is

4.5 times less than the transformation yield obtained with the smaller addition of propane and slightly less than the yield obtained during previous experiments (0.075 μg CAH/μg propane). It is unknown why the transformation yield was so much greater for the first addition of propane in the presence of high concentrations of CAHs. Some studies have found that the positive effects of high concentrations of growth substrate can be overwhelmed by inhibition between the CAHs and growth substrate (Chang and Alvarez-Cohen, 1995).

Nitrate concentrations were measured during this high concentration experiment. Before the experiment began the nitrate concentrations in MC 10-12 were 83 mg/L, 99 mg/L, and 87 mg/L, respectively. After the three additions of propane to MC 10-12 the nitrate concentrations were 68 mg/L, 83 mg/L, and 74 mg/L, respectively. The total volume of groundwater in the microcosms is 75 mL. From this the total mass of nitrate consumed over these first two CAH additions was 1.1 mg NO_3^-, 1.2 mg NO_3^-, and 0.9 mg NO_3^-, respectively. Comparing these values to the previous observations with the lower concentration successive additions of CAHs there are no significant differences. The average total mass of nitrate used at the successive lower concentrations of CAHs was 1.1 mg NO_3^-, and the total mass of nitrate used at the higher concentrations was also 1.1 mg NO_3^-. Table 3.8 shows the comparison of propane consumed, CAHs transformed, and mol nitrogen/mol propane utilized between the low and high concentration tests.

Table 3.8 Comparison of propane utilized, CAHs transformed, and molar ratio of nitrogen to propane between low and high concentration exposure.

CAH Conc.	MC	Propane Utilized (μg)	c-DCE Transformed (μg)	TCE Transformed (μg)	mol Nitrogen / mol Propane
Low	10	3260	111	64	0.26
	11	2990	101	62	0.20
	12	3078	104	66	0.28
High	10	3803	139	47	0.25
	11	3407	216	58	0.30
	12	3703	149	41	0.22

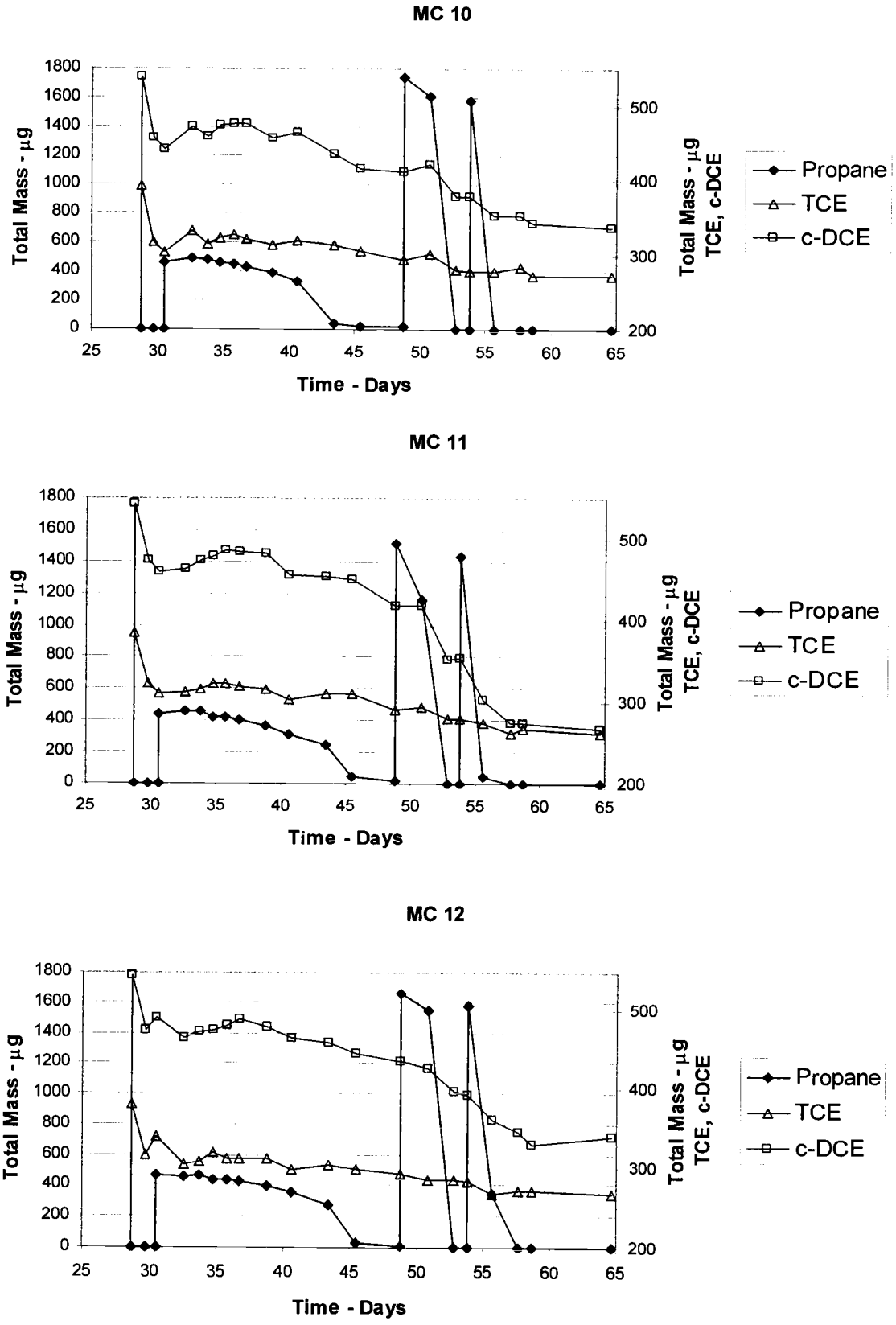

Figure 3.9 Three additions of propane at high CAH concentrations (3.5-5.5 mg/L).

3.4 Summary

The ability to stimulate microbes on propane was demonstrated in microcosms constructed with McClellan subsurface solids and groundwater. Concentrations of propane and CAHs obtained in the microcosms were similar to those during the CAS demonstration. Background data collected during the initial 40 days of the CAS demonstration showed groundwater concentrations of TCE and c-DCE ranging from 100-1000 μg/L (Tovanabootr et al., 2000). Average concentrations in the microcosms were 350 μg c-DCE/L and 950 μg TCE/L. After sparging at the field site, propane concentrations ranged from 0.3 to 1.5 mg/L. Microcosm concentrations for propane ranged from 0.75 to 0.88 mg/L.

The first significant result of the microcosm study was that the lag times required to stimulate the indigenous microorganisms around 10 days. It was unknown whether the presence of CAHs or the lack of nutrient amendments would have negative effects on the amount of time required to obtain a viable population of propane utilizers. These experiments showed no negative effect on the lag time required to stimulate the indigenous microorganisms since lag times were very similar in all the microcosms. The addition of high concentrations of nitrate had no beneficial response on lag time. This is consistent with the background nitrate present in the groundwater being sufficient for the initial utilization of the propane present. During the CAS demonstration a fairly long lag period of 22 to 59 days occurred before propane utilization was evident in the saturated zone (Tovanabootr et al., 2000). This corresponded well to the previous microcosm study where the lag time before utilization occurred was 24-50 days (Tovanabootr, 1997). It is unknown why the

microcosms in this study had a significantly shorter lag period than observed in previous lab and field experiments. Contamination is unlikely due to no activity in control microcosm MC 2 (Autoclaved). The control was treated and sampled the same way as the other live microcosms. It is likely that the aquifer material used in these experiments had a larger initial population of microorganisms than the previous materials used, which may have caused the decrease in lag time.

Another finding of the microcosm tests was the effects of nitrogen limitations on propane utilization and CAH transformation. Background nitrate levels at the site could only maintain propane utilization and CAH transformation for a limited time (1.9 mg propane) before showing the negative effects of nitrogen limitations. However, the initial rates of propane utilization and CAH transformation were similar in the nitrate-amended and non-nitrate-amended microcosms. Having a larger initial concentration of nitrate did not increase the transformation rates. Propane utilization rates gradually decreased with each addition, and CAH transformation eventually ceased in the non-amended microcosms. Nitrogen was shown to be the limiting nutrient as the nitrate amended microcosms never showed a decrease in propane utilization rate or CAH transformation rate. Nitrate levels were also found to be below analytical detection level soon after the decreases in rates were observed. Microcosms with ample amounts of nitrate had multiple additions of substrate and did not show any decrease in utilization rates indicating sufficient nutrients, such as phosphorous, were available in the groundwater and aquifer material.

The microcosms without any nutrient amendments could only support propane utilization and CAH transformation for a limited time. During the initial propane

additions at the field site effective removal of CAHs was observed in the saturated zone where effective propane and DO delivery occurred (Tovanabootr et al., 2000). It was also noticed at the field demonstration that the rates of propane uptake decreased and CAH transformation ceased at several locations that received high propane doses (Tovanabootr et al., 2000). Nitrogen was found to be the limiting nutrient and ammonia gas was added to the sparge gas as a nitrogen source. Upon the addition of ammonia gas the propane rates increased and CAH removal resumed (Tovanabootr et al., 2000).

By comparing different sets of microcosms under different conditions, with and without the presence of CAHs, estimates could be made to the amount of nitrogen required for the indigenous microorganisms to transform CAHs. A comparison was made between the two sets that had only background levels of nitrate. The set without the CAHs present was able to utilize an average of 3780 μg propane before showing a significant loss in propane utilization. The set with CAHs present was only able to utilize an average of 1890 μg propane before showing a significant loss in propane utilization. This greater consumption of nitrogen is likely due to the toxic effects the propane-utilizers encounter when they transform TCE. Several studies have observed toxic effects from highly reactive intermediates that caused covalent modification of cellular components during the oxidation of TCE by other organisms (Chu and Alvarez-Cohen, 1999; Fox et al., 1990; Oldenhuis et al., 1991; Wackett and Householder, 1989). Chu and Alvarez-Cohen (1999) speculated that TCE oxidation exerts a broad range of toxic effects that damage both specific and non-specific cellular functions. Propane-utilizers are able to survive in the presence of TCE, but when they

begin to transform it, toxicity occurs. TCE transformation significantly decreased soon after propane utilization, so the microorganisms were not able to use it as a sole carbon and energy source. Propane-utilizers were left in the presence of TCE for several days without any propane, but still were stimulated within a few days after propane addition. It was observed that the transformation of CAHs resulted in transformation product toxicity, which was evident in the lag periods after propane addition (Figure 3.4). This toxicity manifested itself by causing an increased demand of the indigenous microorganisms for nitrogen. It is suggested that significantly more nitrogen was needed for growth and maintenance by these microorganisms when CAHs were being transformed.

Evaluating the transformation yield was also of interest. Initial transformation yields between the microcosms with (0.056 μg TCE/μg propane) and without (0.049 μg TCE/μg propane) nitrogen amendments were similar, but the rate of transformation in the non-amended microcosms decreased, while the nitrate amended microcosms maintained CAH transformation ability. This result indicates that nitrogen requirements must be met at the site in order to maintain transformation rates. Transformation of c-DCE was observed to be faster than TCE, which is what was observed in the CAS demonstration (Tovanabootr et al., 2000). TCE transformation occurred after propane concentrations were reduced to low levels, while c-DCE transformation occurred during propane utilization. Both CAHs were completely removed below detection limits from the microcosm with nitrate amendments, which corresponds to what was observed at the field site.

Transformation yields in the initial experiments ranged from 0.049 – 0.077 μg TCE/μg propane. Previous microcosm experiments using propane have achieved transformation yields ranging from 0.0056 – 0.048 μg TCE/μg propane (Chu and Alvarez-Cohen, 1995; Tovanabootr and Semprini, 1998). A possible reason for the higher transformation yields in this experiment is because the other two experiments either used high concentrations of substrate or grew up a culture without TCE and then exposed it to TCE. Chu and Alvarez-Cohen (1995) found that the addition of low concentrations of growth substrate enhanced TCE transformation capacities and rates, presumably due to the regeneration of reducing energy (NADH). Also in their experiment they grew up a culture in pure propane and nitrate medium salts containing copper. The microbial consortia in the microcosms used in this study were indigenous and possibly consisted of many species working together. Also, they were exposed to TCE from the beginning, which may have led to a selection of organisms capable of surviving and co-metabolizing in the presence of CAHs.

Long-term transformation was noticed in the previous propane fed microcosm experiment for up to 20 days (Tovanabootr and Semprini, 1998). These microcosms were not tested for their long-term transformation abilities, but transformation of TCE was not observed after 2-3 days in the absence of propane. Neither c-DCE nor TCE was found to significantly decrease after a few days without propane present during the high concentration experiment.

The indigenous propane-utilizers in this experiment were able to survive and grow in the presence of 5.5 mg c-DCE/L and 3.5 mg TCE/L. A transformation yield of 0.042 μg CAH/μg propane was observed during the two large propane additions, and

0.19 μg CAH/μg propane was observed during the initial smaller propane addition. The yield obtained during the two large additions of propane was lower than the range or yields obtained in the previous experiments (0.069-0.075 μg CAH/μg propane). However, the yield obtained from the initial lower addition of propane was significantly higher than all the rest. From this experiment it was noticed that feeding patterns might play an important role in maximizing transformation yields. It has been reported that cometabolic activity depends upon microbial community structure and that the community structure can be manipulated by altering the growth substrate-feeding pattern (Shih et al., 1996). It has also been suggested that the positive effects of growth substrates can be overwhelmed by inhibition of CAH transformation at higher concentrations (Chang and Alvarez-Cohen, 1995). It was observed in a previous study that two out of the three propane-utilizing cultures were not able to maintain TCE transformation abilities when aqueous TCE concentrations exceeded 2.0 mg TCE/L (Tovanabootr, 1997). The propane-utilizers stimulated in these experiments could survive and effectively transform CAHs at concentrations of 3.5-5.5 mg/L.

The ability to transform ethylene and its effects on CAH transformation was also tested. The concentration of ethylene present in the propane used at the field site did not negatively effect CAH transformation. Instead, the transformation of CAHs was slightly greater for the microcosms that had ethylene present. Ethylene has been used as a primary substrate for the transformation of VC in microcosms (Freedman and Herz, 1996; Hartmans and de Bont, 1992). The broad specificity of the enzyme produced by the propane-utilizers was probably able to transform the ethylene as it transformed the CAHs. The microcosm test indicated that the concentration of the

ethylene in the sparge gas used for the CAS demonstration likely did not negatively effect CAH transformation.

CHAPTER 4
USING AMMONIA AS A NITROGEN SOURCE FOR AEROBIC TRANSFORMATION OF TCE, C-DCE BY PROPANE-UTILIZING MICROORGANISMS STIMULATED FROM THE MCCLELLAN SUBSURFACE

4.1 Introduction and Objectives

Nutrients are needed to maintain the growth of subsurface microorganisms that cometabolize TCE and other CAHs. Nitrogen is one of the most essential macronutrients that can be limiting in groundwater, with nitrate being the most common available nitrogen source. The addition of a nitrogen source such as ammonia or nitrate to the nitrogen-limited subsurface may be required to enhance CAH cometabolism.

Most nitrogen in the environment is in inorganic forms, either as NH_3 or NO_3^-. In the subsurface environment nitrate concentrations are generally much higher than ammonium concentrations, although ammonium is invariably the preferred nitrogen source when it is available. Information concerning the mechanisms of uptake of the two forms of nitrogen is limited, and most data have been derived from measurements of uptake in higher plants rather than measurements of uptake in algae and bacteria (Reay et al., 1999). Bacteria assimilate nitrate and ammonia by two different pathways. Nitrate assimilation involves three pathway specific steps: uptake, reduction to nitrite, and further reduction to ammonium. Assimilatory enzymes are cytoplasmic so the nitrate has to be transported into the cell and then reduced to ammonium. Energy is required to reduce nitrate to ammonium ion within the cell. Ammonia assimilation is a more efficient pathway in microorganisms. Ammonia likely is transported by diffusion

across the cell membrane. Studies, however, have indicated possible transport systems for ammonia into cells (Brown, 1980). Invariably ammonia is the preferred source of nitrogen for bacterial growth, however nitrate can be converted to ammonium ion for biosynthesis. Without available nitrogen microorganisms will not be able to grow efficiently or produce the enzymes required for CAH cometabolism.

The goal of this study was to determine the effects of ammonia addition on propane-utilizers stimulated in McClellan microcosms. Ammonia was added to microcosms that were previously shown to be nitrogen limited. This limitation was manifested by decreases in propane utilization and CAH transformation rates. Another set of tests were conducted in microcosms that were never exposed to CAHs, to determine nitrogen needs in the absence of CAH transformation.

The objectives of these tests were used to determine: 1) if ammonia could be used as a possible source of nitrogen for the propane-utilizers; 2) the lag period before propane-utilization rates increased after ammonia addition; 3) the transformation yields of c-DCE and TCE using ammonia as the primary nitrogen source; and 4) the nitrogen requirements in the presence and absence of CAH transformation.

4.2 Materials and Methods

4.2.1 Microcosm Construction and Set-up

The microcosms used in this study had been previously used to obtain the results reported in Chapter 3. Two sets of microcosms were chosen. Microcosm MC 4-6 were used because nitrate levels were found to be below detection limits (< 25

μg/L), and propane utilization had significantly decreased and TCE transformation had ceased (Figure 3.2). Monitoring of propane and TCE concentration was continued on day 62, during this time they were left in a 20 °C room after propane was added to re-stimulate activity in the microcosms, and ammonia was later added to two of the three microcosms. Microcosm set MC 7-9 was the second set of microcosms used. These microcosms had never been exposed to CAHs, and had exhibited a decrease in propane utilization due to nitrogen limitations. For six months these microcosms were not fed propane. They were then incubated with propane for the ammonia addition tests.

In the first ammonia experiment, propane from the field site that contained ethylene (1%) was added. Two microcosms, MC 4 and MC 5, had 0.2 mL ammonia gas added, while microcosm MC 6 did not receive any ammonia.

The second ammonia experiment used only propane gas (no ethylene) to compare with first ammonia experiment with ethylene. Two sequential additions (0.2 mL) of ammonia gas were added to microcosms MC 4-6. Microcosms MC 7-9 were also stimulated on propane during this experiment. These microcosms only received one addition of 0.2 mL ammonia gas, and did not have any CAHs present. Propane was added several times until utilization rates decreased significantly.

4.2.2 Chemical Sources, Saturated and Standard Solutions

Anhydrous ammonia (99.99%) was purchased from Scott Specialty Gases in Plumsteadville, PA. Trichloroethylene (TCE;>99%) and c-DCE (>97%) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Propane (98%) and ethylene (99%) were also purchased from Aldrich Chemical Co. (Milwaukee, WI). Saturated

stock solutions of CAHs were prepared at room temperature by adding specific amounts of the liquid to 125-mL serum bottles containing autoclaved deionized water. This procedure eliminated the use of carrier solvents, such as methanol. The bottles were shaken for 6 hours to ensure saturation, and then were allowed to settle for at least 24 hours before use.

Standard solutions were made for creating standard calibration curves for CAH GC analysis. A given mass of the compound was dissolved in a known volume of methanol. A given volume of methanol solution was added to a capped 28 mL vial that contained 10 mL of deionized water. The solution equilibrated on a shaker table at 20 °C. Headspace samples were taken and analyzed using GC methods. Mass balances using Henry's Law were made to determine the corresponding liquid concentration, and to construct standard curves.

4.2.3 Sampling and Analytical Methods

The microcosm tests required the measurement of headspace concentrations of the gaseous substrate and CAHs of interest. Headspace samples were obtained using a 100-μL gas-tight syringe. Samples were manually injected onto the appropriate GC immediately after sampling. During the course of the microcosm incubation, headspace sampling and propane and oxygen consumption created a vacuum inside the microcosms. After propane was consumed the headspace was equilibrated to atmospheric pressure under a laminar flow hood by adding pure oxygen. This maintained oxygen concentrations in the microcosms so that aerobic conditions were maintained throughout the experiment.

CAH and gaseous substrate concentrations were quantified by GC analysis. The HP 6890 GC was equipped with a photo ionization detector (PID) in conjunction with a flame ionization detector (FID). The CAHs with unsaturated bonds were detected with a PID, while the FID detected the gaseous cometabolic substrate (propane). The advantage of using PID/FID detectors in series was that the gaseous substrate and chlorinated ethenes could be determined in the same analysis. Separation was obtained by using a GS-Q capillary column (VWR Scientific Inc.) operated with a temperature gradient for better separation of the gaseous substrate and chlorinated ethenes. High-grade helium, at a head pressure of 60 psi, was used as the carrier gas. The method was calibrated by using the external standards. The total mass of compounds in the microcosms was determined by mass balance assuming Henry's law equilibrium partitioning.

Nitrate was measured by ion chromatography (IC) using a Dionex 40001 ion chromatograph equipped with a conductivity detector. A Dionex Ionpac AS4A column was used for the chromatographic separation. The column uses a regenerate containing and H_2SO_4, Na_2CO_3, and $NaHCO_3$. A 0.7 mL aqueous microcosm sample was centrifuged to remove any suspended particulates. After being centrifuged 0.5 mL was used in the analysis. External standards of $NaNO_3$ were used for the calibration of this method.

4.3 Results and Discussion

4.3.1 Transformation of CAHs and Ethylene After the First Addition of Ammonia Gas to Nitrogen-Limited Microcosms MC 4-6.

The previous study showed that nitrogen was a limiting nutrient effecting substrate utilization and CAH transformation. The background nitrate levels at the field site (4.8 mg/L; 0.36 mg) were able to maintain propane-utilization and CAH transformation for a period of six days after initial stimulation in microcosms MC 4-6 with 1.9 mg of propane. After three additions of propane the utilization rates began to decrease with each addition (Figure 3.2). From day 21 to 41 no measurable TCE was transformed in microcosms MC 4-6.

Propane was not added to microcosms MC 4-6 from day 41 to day 61. These microcosms all had residual TCE (5-9 μg) during this time period. On day 62, propane (370 μg) was added and concentrations were monitored. A three-day lag period occurred before propane was utilized, at slow rates in all three microcosms. Ammonia (0.2 ml of 99.99%) gas was then injected into the headspace of microcosms MC 4 and 5 on day 72, while microcosm 6 served as a control with no NH_3 addition. Figure 4.1a and 4.1b show the results from microcosms MC 4 and 5. The rate of propane utilization increased within 1-2 days after ammonia was added to MC 4 and 5. By day 73 the propane was completely utilized and all the residual TCE was transformed.

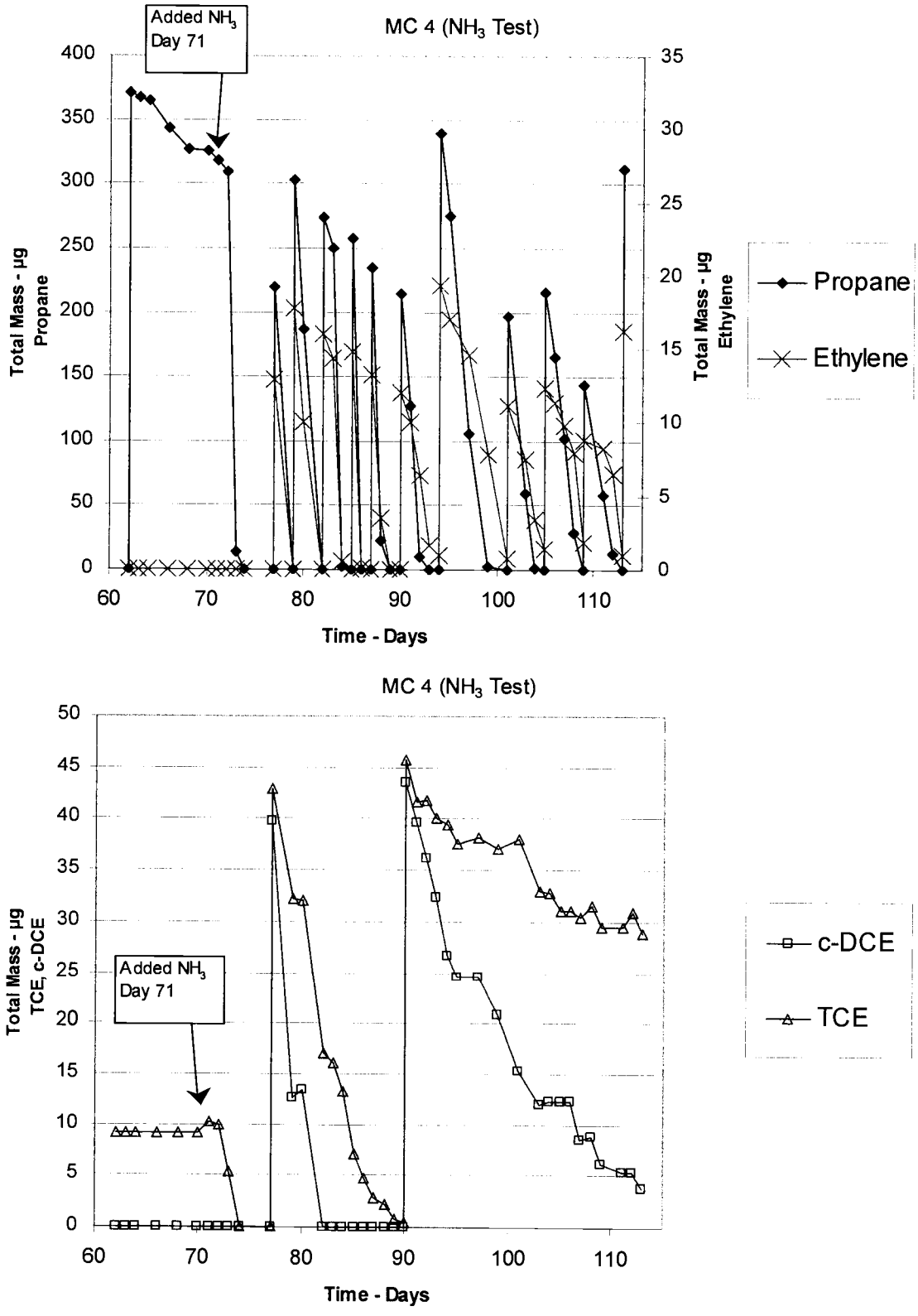

Figure 4.1a Effects of ammonia addition in microcosm MC 4.

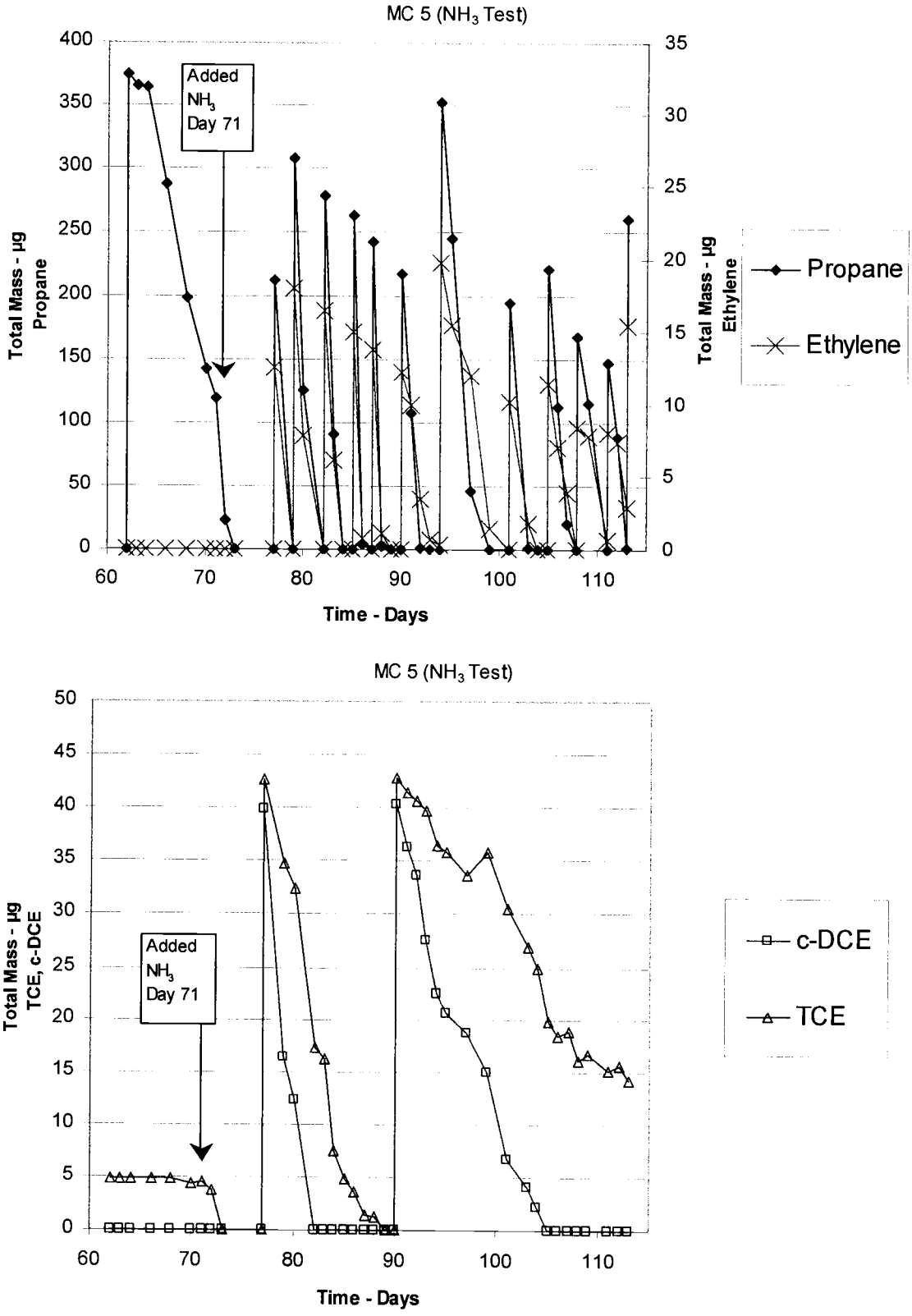

Figure 4.1b Effects of ammonia addition in microcosm MC 5.

On day 77, propane (216 μg), ethylene (13 μg), c-DCE (40 μg), and TCE (43 μg) were added to microcosms MC 4 and 5. The propane/ethylene mixture was obtained from the propane tank that was used for the CAS demonstration. Ethylene was present in the propane gas at 1% by volume. The propane/ethylene gas was rapidly consumed in successive additions over the period from 75 to 90 days. Both c-DCE and TCE also were also transformed. The successive additions of propane, however, likely inhibited c-DCE transformation, as can be seen between days 79-80 and days 94-97 in both MC 4 and 5. The change in the rate of c-DCE transformation is apparent upon these two propane additions. The similar decrease in rate can be seen with TCE transformation in the presence of a high concentration of propane. All of the c-DCE was transformed by day 82, while TCE was fully transformed by day 90. Both c-DCE and TCE had similar initial masses in both microcosms, but it took five days to fully transform c-DCE and 15 days to fully transform TCE. This indicates c-DCE has a higher transformation rate than TCE. One non-biological reason is that c-DCE has significantly lower Henry's law constant than TCE, so more c-DCE is present in the aqueous phase than TCE. Since the transformation of CAHs occurs in the aqueous phase c-DCE has a concentration advantage over TCE for the propane monooxygenase enzyme. Inhibition of its transformation by propane may be less than the inhibition of TCE transformation due to the higher aqueous concentration.

Transformation yields were calculated for the second and third propane/ethylene additions. After the second addition of propane/ethylene the TCE transformation yield for microcosms MC 4 and MC 5 was 0.049 and 0.037 μg TCE/μg propane, and the total CAH transformation yields were 0.092 and 0.11 μg CAH/μg propane,

respectively. This transformation yield does not consider ethylene as a potential substrate that is also being cometabolized. Only TCE was present for the third incubation with propane/ethylene. Microcosms MC 4 and 5 transformation yields for this addition increased to 0.051 and 0.057 μg TCE/μg propane, respectively.

On day 90, c-DCE (42 μg) and TCE (45 μg) were again added along with propane (216 μg) and ethylene (12 μg). The propane utilization rate and ethylene, c-DCE, and TCE rates slowed. The re-addition of propane on day 95 again shows the inhibitory effect of a high concentration of propane on c-DCE and TCE transformation.

Propane utilization began to slow after 90 days. By day 105, approximately 38-67% of the TCE and from 92-100% of the c-DCE had been transformed in microcosms MC 4 and 5. After day 105, TCE transformation ceased in MC 4, while limited transformation occurred in MC 5. The transformation yield for MC 5 during this period was 0.01 μg TCE/μg propane. There was no c-DCE present in MC 5 over this time period. During this same time period some c-DCE, but no TCE was transformed from day 105-113 in microcosm MC 4. A transformation yield of 0.024 μg c-DCE/μg propane was achieved. It is an interesting observation that TCE transformation ceased, but c-DCE still occurred at a decreased rate. This suggests that c-DCE transformation is less susceptible to nitrogen-limiting conditions than TCE transformation is.

The amount of nitrogen consumed during this study was estimated based on the amount of ammonia added. The 0.2 mL ammonia added is equal to 0.14 mg ammonia (0.12 mg nitrogen). The microcosms were considered to be experiencing a nitrogen-limitation when there was a 50% decrease in propane utilization rates, which for microcosm MC 4 and MC 5 occurred on day 90 and 94, respectively (Table 4.1). Even

when the propane utilization rates decreased to less than half their rate, CAH transformation still continued in both microcosms until day 105, but at reduced rates.

Table 4.1 Decreases in propane utilization rates for MC 4 and MC 5.

Microcosm	Day (87-88) Propane Utilization Rate (μg/hr)	Day (90-91) Propane Utilization Rate (μg/hr)	Day (106-107) Propane Utilization Rate (μg/hr)
MC 4	8.8	3.6	2.6
MC 5	10.0	4.1*	3.8

* Day 95-97

Similar amounts of propane and CAHs were transformed in microcosms MC 4 and MC 5 after the addition of ammonia gas. Day 105 was chosen as the time at which propane utilization rates decreased to the point where TCE transformation stopped. Propane utilization rates were below 4 μg/hr for both microcosms at this time. Total mass of propane consumed and CAHs transformed through day 105 are presented in Table 4.2. Similar amounts of propane were consumed and CAHs transformed in the microcosms. The molar ratio of nitrogen to propane consumed was calculated to be 0.17 mol nitrogen/mol propane. This ratio is used to compare with the next ammonia experiments in the absence of CAHs, as will be discussed.

Table 4.2 Total mass of propane utilized and CAHs transformed by the addition of ammonia to MC 4 and MC 5.

Microcosm	Propane Utilized (μg)	c-DCE Transformed (μg)	TCE Transformed (μg)	mol Nitrogen / mol Propane
MC 4	2360	71	58	0.16
MC 5	2190	80	66	0.17

Microcosm 6 did not receive any ammonia gas as a source of nitrogen. A three-day lag period was observed for the initial utilization of propane, which was the same as microcosms MC 4 and 5 (Figure 4.2). It took six days to fully utilize the initial propane added. After the initial utilization a limited amount of TCE was transformed (4 μg). Propane was added three more times to this microcosm, with the last two additions containing ethylene. A continuous decrease in the rate of propane utilization occurred in contrast to those that received ammonia. No TCE transformation occurred after the first utilization of propane in this microcosm. The initial rate of propane and TCE transformation was possibly due to some organic nitrogen that might have been in the microcosm subsurface material.

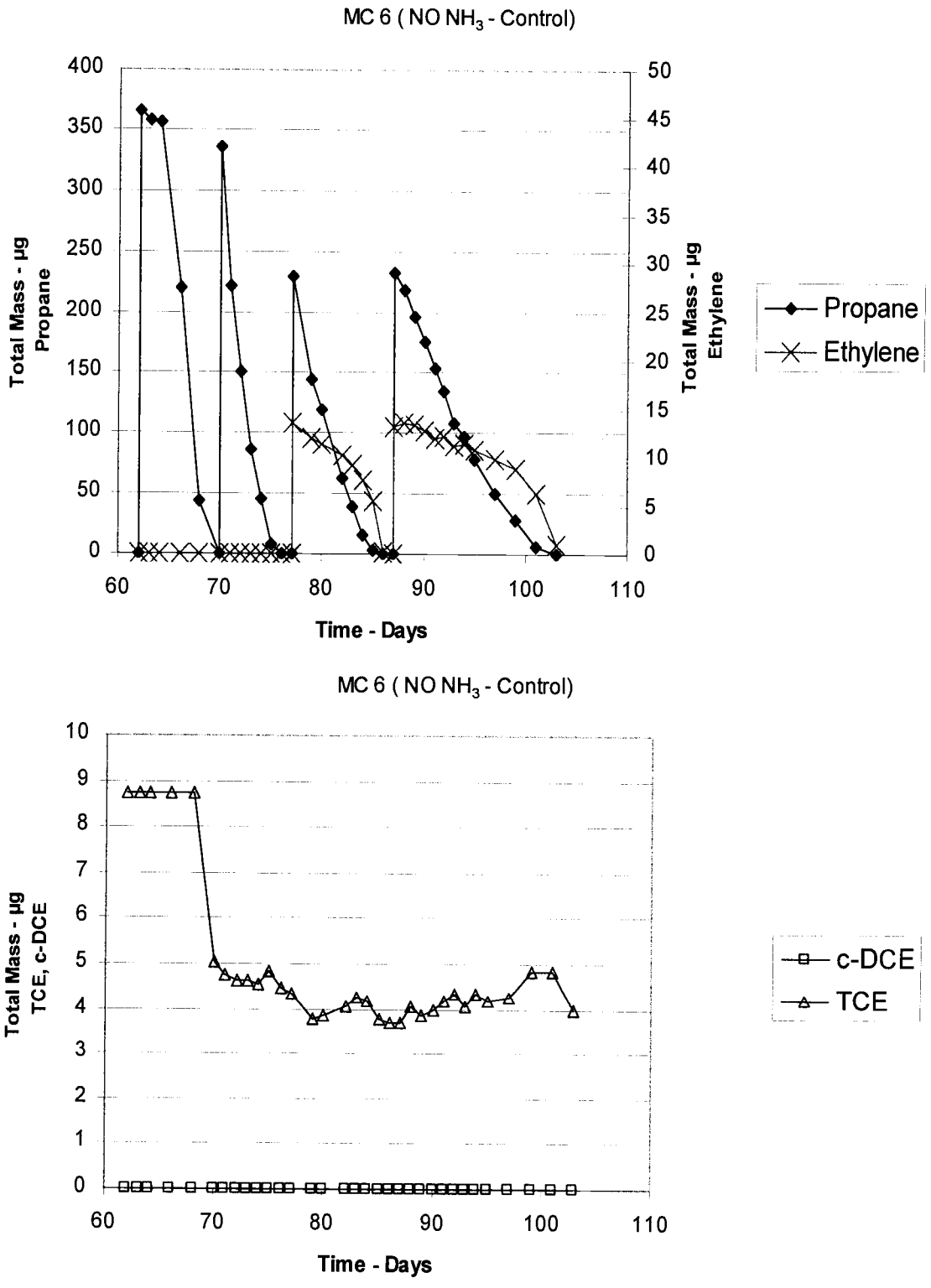

Figure 4.2 Microcosm MC 6 without any ammonia addition.

Ethylene transformation occurred after propane concentrations decreased to low levels (<100 μg), indicating propane was inhibiting ethylene transformation. In all three microcosms the rate of ethylene transformation was closely tied to the rates of propane utilization (Table 4.3).

Table 4.3 Propane utilization, ethylene transformation, and ratio of ethylene to propane rates for microcosms MC 4-6.

Microcosm	Time (Days)	Propane Rate (μg/hr)	Ethylene Rate (μg/hr)	Rate Ethylene / Rate Propane
4	91-93	2.7	0.18	0.07
	97-99	2.2	0.14	0.07
5	91-92	4.5	0.27	0.06
	95-99	2.6	0.15	0.06
6	80-85	0.97	0.05	0.05
	95-101	0.51	0.03	0.06

Figure 3.7 shows the results of the first experiment using the propane/ethylene mixture in MC 10-12. The propane utilization rates, ethylene transformation rates, and the ratio of ethylene rates to propane rates are presented in Table 4.5. The ratio of the rate of ethylene transformation to propane utilization was the same for microcosms 10-12, amended with nitrate, as it was for the ammonia amended microcosms 4-6 (Table 4.3 and 4.4). The propane utilization rates used in Table 4.4 were much higher than the rates used in Table 4.3 to calculate the ratios.

Since the ratio is the same over a broad range of utilization rates (0.5-36 μg/hr) it supports the idea that the microbial mass and the enzyme activity of these microorganisms are proportional to ethylene transformation. With greater amounts of microbial mass there is an increased level of enzyme activity, which would transform the ethylene at an increased rate.

Table 4.4 Propane utilization, ethylene transformation, and ratio of ethylene to propane rates for microcosms MC 10-12.

Microcosm	Time (Hours)	Propane Rate (μg/hr)	Ethylene Rate (μg/hr)	Rate Ethylene / Rate Propane
10	2-9	36	2.1	0.06
11	2-9	31	1.7	0.05
12	2-9	36	2.1	0.06

4.3.2 Transformation of CAHs After the Second and Third Addition of Ammonia Gas to Nitrogen-Limited Microcosms MC 4-6 without Ethylene Present

A second and third ammonia addition was added to the microcosm set MC 4-6 110 days after the first ammonia addition experiment. On day 215 this ammonia addition experiment was begun and is represented in subsequent figures and discussion as day 1. Two successive ammonia additions were conducted in microcosms MC 4-6. Ammonia was added to all three microcosms and no ethylene was included in the propane feed gas.

Propane (1060 μg) was added to microcosms MC 4-6 to determine if any residual nitrogen was present that might support propane utilization. Figure 4.3 shows the propane concentrations were stable for 19 days, indicating insufficient nitrogen was present to support microbial growth. On day 10, c-DCE was added (41 μg) to all three microcosms. c-DCE concentrations were stable from day 10-19, and TCE concentrations were stable from day 0-19. Ammonia gas (0.2 mL) was added on day 19. A 2-3 day lag period was observed before propane utilization rates increased and CAHs were transformed (Day 22). Prior to the stimulation of the propane-utilizers the total mass of TCE ranged from 2-9 μg TCE and c-DCE ranged from 39-41 μg c-DCE in the microcosms. After propane was consumed, 1-6 μg TCE and 6-7 μg c-DCE remained. The c-DCE was more rapidly transformed than TCE. The transformations were similar in all three microcosms.

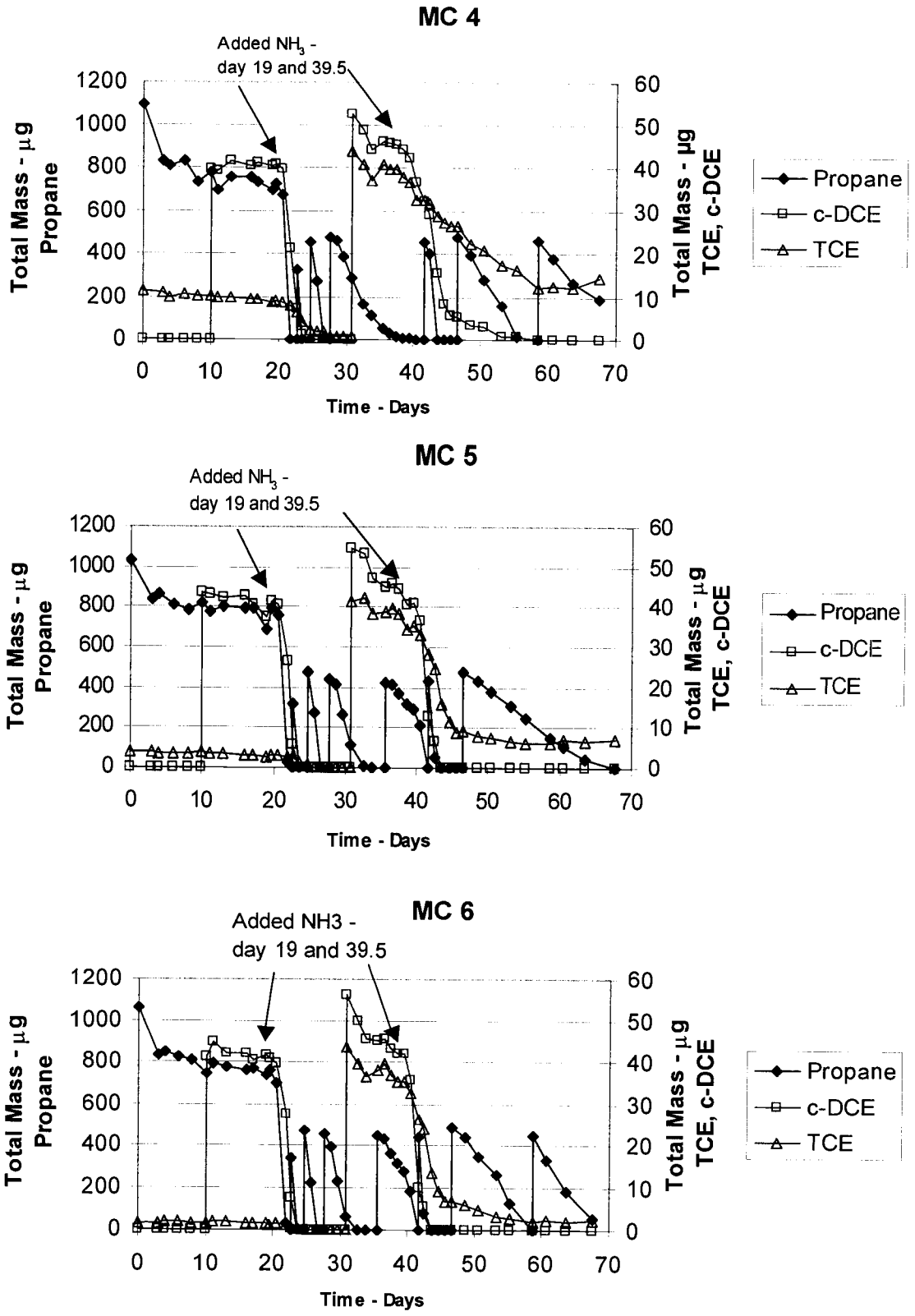

Figure 4.3 Second and third addition of ammonia to microcosms MC 4-6.

The second addition of propane was rapidly utilized and c-DCE was fully transformed, and the transformation of TCE increased. TCE was fully transformed in MC 5 and 6 after the second addition of propane. By the fourth addition of propane (day 27) the propane utilization rates had significantly decreased, indicating the microcosms were becoming nitrogen limited. Table 4.5 shows the decrease in propane utilization rates with each addition.

TCE (43 μg) and c-DCE (54 μg) were added again on day 31, while the fourth propane addition was being utilized at a slow rate. From day 31 to 34 limited transformation occurred after propane concentrations decreased. Another addition of propane on day 35.5 for MC 5 and 6 resulted in limited CAH transformation. Transformation of CAH was observed to be significantly limited when the propane utilization rate was reduced to around 4 μg/hr (Table 4.5).

Table 4.5 Propane utilization rates for microcosms MC 4-6 after second addition of ammonia gas.

Microcosm	Propane Utilization Rates (μg/hr)			
	Day 20.6-21.9	Day 24.6-26.6	Day 28.6-32.5	Day 36.5-39.5
4	> 28	9.4	3.0	0.35
5	> 30	10	4.2	1.8
6	> 28	9.7	4.1	2.2

The 0.2 mL ammonia added is equal to 0.14 mg ammonia (0.12 mg nitrogen). Table 4.6 shows the amount of propane utilized, CAHs transformed, and the molar ratio of nitrogen to propane consumed after the first addition of ammonia from day 20 to day 40. The average ratio of mol nitrogen to mol propane consumed was 0.21 (0.037 std. dev.), which was slightly higher than the ratio calculated with the previous addition of ammonia to these microcosms (Table 4.3).

Table 4.6 Total mass of propane utilized and CAHs transformed after the first addition of ammonia to MC 4-6 from day 20 to day 40.

Microcosm	Propane Utilized (μg)	c-DCE Transformed (μg)	TCE Transformed (μg)	mol Nitrogen / mol Propane
MC 4	1500	47	12	0.25
MC 5	2040	51	6	0.18
MC 6	2020	51	6	0.20

A second addition of ammonia gas (0.2 mL) was added on day 39.5. Prior to this addition, propane utilization rates were below 2.2 μg/hr and CAH transformation had ceased. MC 4-6 showed a 1-2 day lag period before propane utilization increased and CAH transformation resumed. All three microcosms were fed propane again on day 41.5, and MC 5 and 6 began to utilize the propane immediately, while MC 4 had a one-day lag period before significant utilization occurred. c-DCE transformation increased significantly in MC 4, while TCE was transformed at a much slower rate. MC 5 and 6 had transformed most of the c-DCE with the previous addition of propane, which resulted in TCE transformation increasing upon utilization of propane and c-

DCE was fully transformed. TCE transformation ceased two days after propane was utilized.

Two more additions of propane occurred on day 46 and day 58, propane was rapidly utilized in MC 4 and a significant amount of c-DCE and TCE was transformed. Propane utilization decreased in MC 4, and transformation of TCE ceased after the propane addition on day 58 (Table 4.7). MC 5 and 6 transformed very little TCE with the last two propane additions, as propane utilization rates had decreased. The TCE transformation yield for these microcosms was 0.034 μg TCE/μg propane, and the total CAH transformation yield was 0.088 μg CAH/μg propane.

Table 4.7 Propane utilization rates for microcosms MC 4-6 after addition of ammonia gas on day 40.

Microcosm	Propane Utilization Rates (μg/hr)			
	Day 40.3-41.6	Day 41.5-42.4	Day 46.5-55.2	Day 58.6-63.6
4	*	17 **	2.2	1.6
5	> 8.9	16	1.1	*
6	> 6.3	15	1.7	2.2

* Rate not measured
** Day 42.4-43.5

The total amount of CAHs transformed, propane utilized, and molar ratio of nitrogen to propane consumed during this test is shown in Table 4.8. The ratio of nitrogen to propane for the second ammonia addition was significantly higher than the ratio calculated for the first ammonia addition. The main difference between the two additions was the initial concentrations of CAHs prior to the ammonia addition. The

second addition had much higher concentrations of TCE than the first addition. The amount of TCE transformed during the second addition of ammonia was three times higher than the amount of TCE transformed in the first addition. This difference in the amount of TCE transformed appeared to cause an increase in the demand for nitrogen.

Table 4.8 Total amount of propane utilized, CAHs transformed, and molar ratio of nitrogen to propane after the second addition of ammonia to MC 4-6 from day 40 to day 60.

Microcosm	Propane Utilized (μg)	c-DCE Transformed (μg)	TCE Transformed (μg)	mol Nitrogen / mol Propane
4	930	42	23	0.39
5	720	41	28	0.51
6	710	42	33	0.51

4.3.3 Propane Utilization After Addition of Ammonia Gas to Nitrogen-Limited Microcosms MC 7-9

Microcosms MC 7-9 were used to determine the amount of propane utilized after ammonia addition in the absence of CAHs. These microcosms were used for this experiment because they had previously shown nitrogen limitations, and had never been exposed to CAHs (Figure 3.3).

Before adding propane to these microcosms liquid samples were taken to determine nitrate concentrations. MC 7 and 9 had nitrate levels below the detection limit, while MC 8 had a nitrate concentration of 0.3 mg/L NO_3^-. Propane (880 μg) was added to all three microcosms, but no CAHs were added (Figure 4.4). The residual nitrate in microcosm MC 8 was enough to stimulate the propane-utilizers, and a lag

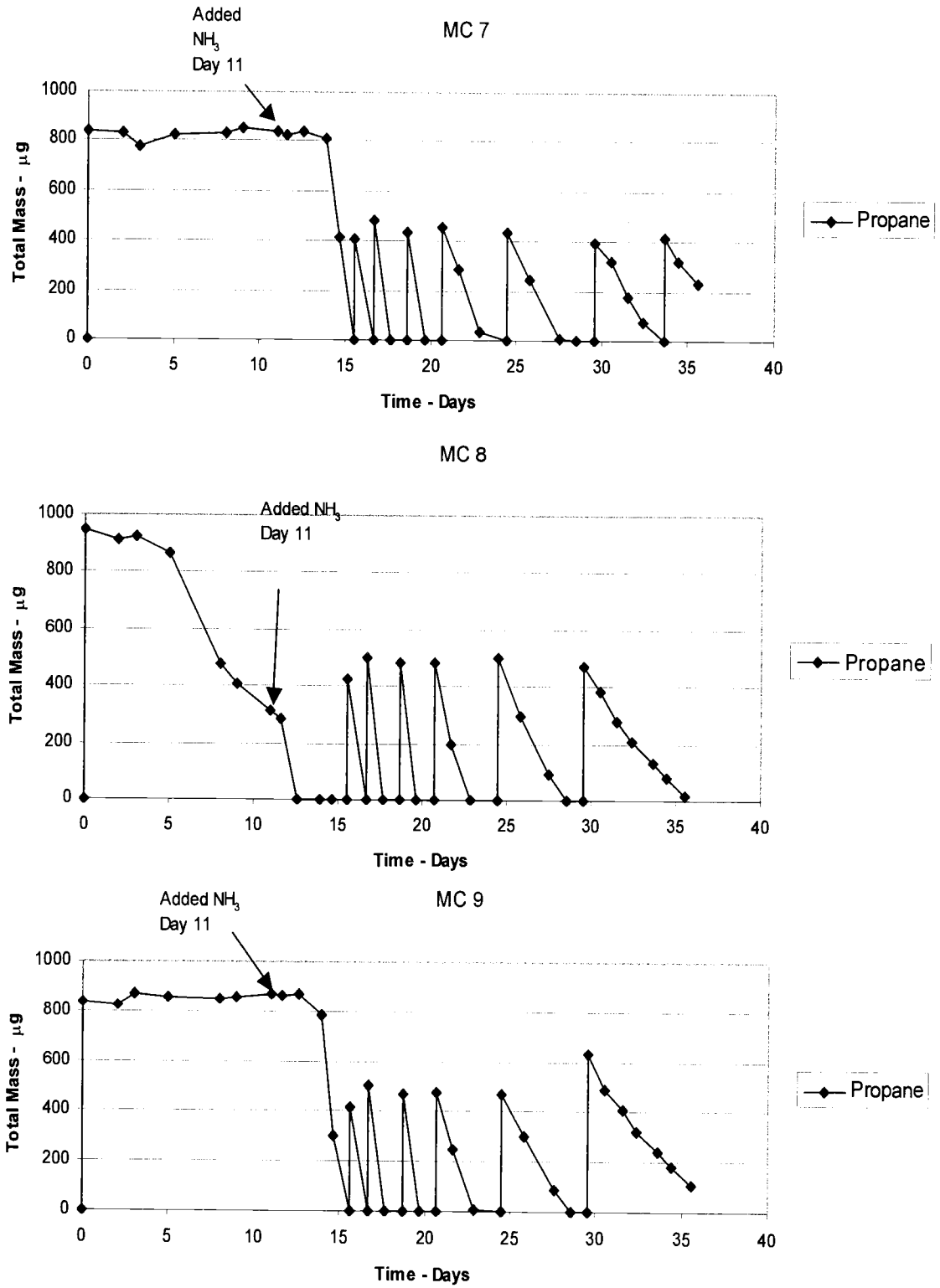

Figure 4.4 Propane utilization before and after the addition of ammonia gas to microcosms MC 7-9.

period of 5-8 days was observed. Propane utilization decreased significantly from day 5 through day 11, indicating the microcosm had become nitrogen limited. Nitrate concentrations in MC 8 were found to be below detection limit on day 11. Microcosms MC 7 and 9 showed no signs of propane utilization through day 11.

On day 11, 0.2 mL of ammonia gas was added to all three microcosms. Slightly different lag times were observed between the microcosms. MC 7 and 9 had a lag period of 3-4 days before utilization occurred. MC 8 had a shorter lag period of 1.5 days before the remaining propane was consumed, presumably due to microbial stimulation provided by the residual nitrate. After the initial addition, several successive additions of propane (400-500 μg) were made to each microcosm. Propane was rapidly utilized for the first three additions. Utilization rates began to decrease on the fourth addition (day 20.7-22.8), when rates decreased by half. Table 4.9 shows the decrease of propane utilization rates with each addition. In the previous studies where CAHs were present, when the propane utilization rate was 4 μg/hr CAH transformation essentially ceased. When CAHs were not present this rate was observed after the six addition of propane on day 29.5.

Table 4.9 Propane utilization rates for microcosms MC 7-9.

Microcosm	Propane Utilization Rates (μg/hr)			
	Day 18.7-19.7	Day 20.7-22.8	Day 24.5-27.5	Day 29.5-33.6
7	> 18	8.8	6.0	4.1
8	> 20	10	5.6	3.5
9	> 20	9.8	5.3	4.0

The total mass of propane utilized before the decreased utilization rate occurred was 2990 μg propane (Table 4.10). In the absence of CAHs the propane-utilizers were able to consume a significantly larger amount of propane than in the presence of CAHs (790-1850 μg) for the same nitrogen addition. The reduced nitrogen demand without CAHs present is presumably due to the lack of a transformation product toxicity effect.

Table 4.10 Total amount of propane utilized and molar ratio of nitrogen to propane for microcosms MC 7-9.

Microcosm	Propane Utilized (μg)	mol Nitrogen / mol Propane
7	3060	0.12
8	2710	0.14
9	3210	0.11

4.3.4 Comparison of Nitrate and Ammonia (Nitrogen) Requirements During Various CAH Exposures.

The amount of nitrogen required by the propane-utilizing microorganisms to utilize propane and effectively transform CAHs was measured under a variety of CAH concentration exposures. Nitrogen requirements were calculated and compared at various CAH concentrations using either nitrate or ammonia as the primary nitrogen source. The effects of CAH transformation on the nitrogen requirements of propane-utilizers were compared.

The molar ratio of nitrogen to propane consumed was calculated for four ammonia experiments. The first and second additions of ammonia in the presence of

CAHs (Tables 4.6 and 4.8), and the addition of ammonia in the absence of CAHs were compared (Table 4.10).

Figure 4.5 shows a bar graph comparing the molar ratios obtained in the three experiments. It is apparent that the propane-utilizers in the absence of CAH transformation were able to utilize significantly more propane than when CAHs were being transformed. The transformation products of the CAHs were potentially causing negative effects on cell function and maintenance, increasing the demand for nitrogen.

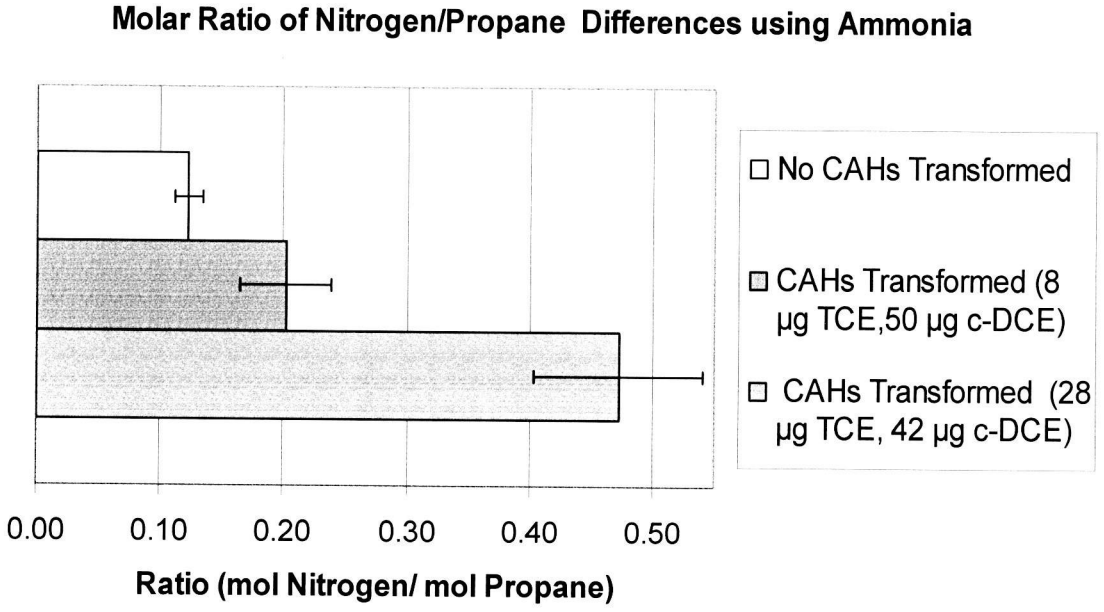

Figure 4.5 Comparison between the molar ratios of nitrogen to propane when ammonia was the primary nitrogen source.

Figure 4.6 shows the comparison of the molar ratios of nitrogen to propane consumed by the propane-utilizing microorganisms when using nitrate as the primary

nitrogen source. Values were obtained from the first experiments in Chapter 3 conducted with microcosms MC 4-9 with only background levels of nitrate available, and from microcosms MC 10-12 during the high concentration test. The amount of nitrogen required when CAHs are not being transformed is significantly less that the nitrogen required when CAHs are being transformed. From these observations it appears that as the CAH transformation increases the amount of nitrogen required for growth and maintenance by the propane-utilizers increases.

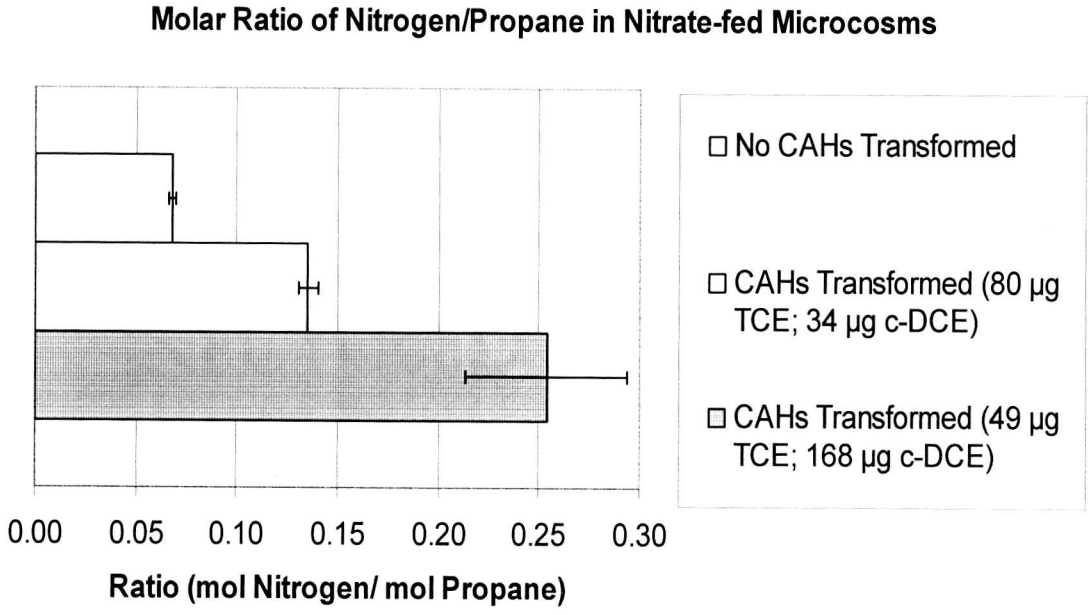

Figure 4.6 Comparison between the molar ratios of nitrogen to propane when nitrate was the primary nitrogen source.

4.4 Summary

Ammonia gas was a good nitrogen source for the propane-utilizing microorganisms stimulated from the McClellan aquifer material. Upon addition of ammonia a 1-3 day lag time was observed before propane utilization increased and CAH transformation resumed. This correlates well to the increase in propane utilization and CAH transformation observed in the field after the addition of ammonia gas to the sparge gas (Tovanabootr et al., 2000). This short lag time indicates that propane-utilizers were able to quickly adapt to the presence of ammonia, and use it for growth and maintenance.

Transformation of CAHs using ammonia was not significantly different than the transformations observed when nitrate was the primary nitrogen source. Total CAH transformation yields obtained in the ammonia experiments were 0.088-0.10 μg CAH/μg propane, which are similar to the average total transformation yields obtained when nitrate was the primary nitrogen source (0.083-0.092 μg CAH/μg propane). Even though microorganisms are thought to prefer ammonia as a nitrogen source it did not seem to have a significant effect on transformation yields.

Microcosms MC 7-9, which did not have any CAHs present, were able to utilize 2990 μg propane after the addition of 0.2 mL ammonia gas (0.12 mg nitrogen). These same microcosms had previously utilized 3780 μg propane using only the background nitrate concentration found at the field site (0.08 mg nitrogen). The molar ratio of nitrogen to propane utilized was 0.068 when nitrate was the primary nitrogen source, and 0.123 when ammonia was the primary nitrogen source. The amount of propane utilized when nitrate was the nitrogen source was 1.8 times greater than the amount of

propane utilized when ammonia was the nitrogen source. Analysis of the aquifer solids for nitrogen sources was not made, thus we do not know if nitrate in the groundwater was the only source of nitrogen present. However, the fate of the ammonia gas in the microcosms is also unknown. The microcosms were never analyzed for ammonia content and it may be that the amount of ammonia added was over-estimated. It is also possible that some of the ammonia was lost due to sorption onto the cap or aquifer solids, or that all of the ammonia was not available to the microorganisms.

The major observation of these experiments was that the amount of nitrogen required was greater when CAHs were being transformed. The demand for nitrogen increased by 1.6-3.8 times when CAHs were present and ammonia was the nitrogen source (Figure 4.5). A similar 2.0-3.8-fold increase in nitrogen consumption was observed with nitrate as the primary nitrogen source. As the CAH concentrations increased, and CAH transformation increased, the nitrogen demand also increased. The effects of transformation product toxicity may result in increased biosynthesis of nitrogen-containing cellular macromolecules such as nucleic acid bases and amino acids.

From this experiment it is difficult to say whether ammonia or nitrate is a better source of nitrogen for the propane-utilizers stimulated from the McClellan subsurface. Little is known regarding the assimilatory pathways of these two potential sources of nitrogen. What was observed was that ammonia gas was as efficient as nitrate in facilitating the transformation of the CAHs of interest in this study. Ammonia gas would be a good source of nitrogen to use at the site since it could be included in the sparge gas. A gaseous nitrogen source such as ammonia may also be appropriate as a

potential source of nitrogen in the vadose zone. However, further investigations into the pH effects caused by using ammonia gas are needed.

CHAPTER 5
VADOSE ZONE MICROCOSMS

5.1 Introduction and Objectives

One of the goals of the CAS demonstration at the McClellan field site was to remediate TCE and c-DCE contamination from the vadose zone. A fairly thick vadose zone exists at the site, with contaminated groundwater being approximately 108 ft below the surface. Propane was effectively delivered to the vadose zone, and was very well distributed spatially indicating sparging was a good way of delivering propane. Decreases in propane were observed early on and at the end of the test after ammonia gas was added to the sparge gas. Results from the test indicated some cometabolic removal of TCE and c-DCE by 20% and 35%, respectively (Tovanabootr, 2000). However, it is likely that nitrogen limitations may have stopped propane utilization and effective CAH removal. It was unknown why propane utilization was less effective in the vadose zone compared to the saturated zone. This limited study attempted to gain insight into the processes occurring in the vadose zone.

This microcosm study uses a combination of different nutrient and contaminant conditions to test the ability of the indigenous propane-utilizers to transform CAHs under different initial conditions. These microcosms were used to determine: 1) if indigenous propane-utilizing microorganisms could be stimulated under vadose conditions at the site; 2) the lag time required to stimulate these microorganisms; 3) how effectively these microorganisms transform TCE and c-DCE at the concentration levels found at the site; 4) the transformation yields; 5) the nitrogen requirements of the

indigenous microorganisms; 6) and if ammonia could be used as a possible nitrogen source.

5.2 Materials and Methods

5.2.1 Subsurface Material

Subsurface material was collected at the field site to create the matrix of vadose zone microcosms for the McClellan tests. The material was obtained from three cores taken March 19-24, 1999 during the construction of the monitoring wells for the CAS demonstration. Since cores from the vadose zone were not available cores from monitoring wells C2, C3, and C4, at depths ranging from 112-113 ft-bgs, below the water table, were used. The cores were aseptically removed from the brass sleeves under a laminar flow hood, wet sieved through a No. 4 sieve to remove the large particles, and thoroughly mixed together before using. The core material was light brown in color and had a sandy-clay texture. The moisture content of the subsurface material out of the brass sleeves was measured to be approximately 2 g water per 10 g of aquifer material. This material was packed directly into the microcosms and was not dried. The bulk density (g/cm^3) was measured to be 1.69.

5.2.2 Microcosm Construction and Matrix

All materials used in microcosm construction were autoclaved (140 °C for 60 min), including batch media bottles, caps with septa, and all implements used during the construction. Aseptic construction methods help to ensure that the microorganisms

that are stimulated come from the core sample. Microcosm bottles were 150 mL media bottles with gray butyl rubber septa and screw cap (Wheaton Glass Co., Millville, NJ). The bottles are called 125 mL serum bottles, but they have a total volume of 150 mL. The subsurface solids were lightly packed into the microcosms and occupied a total volume of approximately 75 mL. An average of 113 g of solids was added to each microcosm bottle, which had a average 23 g water (0.2 g water/g solids). This left a headspace volume of 75 mL in each bottle. Using the bulk density and the density of quartz (2.65 g/cm^3) the porosity for the volume of subsurface solids in the microcosms was calculated to be 27 mL. This resulted in a total headspace volume of 102 mL. The headspace permitted sampling of the gaseous substrate and CAHs.

The microcosm experimental matrix was as follows: Control microcosm VZ 1 (no propane, only TCE and c-DCE), Live microcosm VZ 2 (propane, ammonia gas), Live microcosm VZ 3 (propane only), Live microcosm VZ 4 (propane, TCE, c-DCE), and Live microcosm VZ 5 (propane, TCE, c-DCE, ammonia gas).

Gaseous propane (98%) was added (1 mL) to all the microcosms, except control microcosm VZ 1. A 2% mixture of propane to air (vol/vol) was used for the CAS demonstration. The initial concentration used in the microcosms was 1% propane by volume. This volume of propane converts to approximately 1830 μg propane total mass using the gas law constant of 24.03 L/mol. The initial total mass calculated for the gaseous phase in each microcosm was by headspace analysis using a gas chromatograph. The average total mass of propane was 1550 μg, which is 85% of the expected total mass added. Approximately 1000 μL TCE and 200 μL c-DCE saturated water solutions (20 °C) were added to microcosms VZ 1 and VZ 4 and 5. The

measured concentration of water saturated with TCE at 20 °C is 1100 mg/L, and 6000 mg/L for c-DCE. Using these saturation values the expected total mass of TCE and c-DCE added to each microcosm was calculated to be 1100 μg and 1200 μg, respectively. The average initial mass analyzed in the headspace by gas chromatography for TCE and c-DCE was 580 μg and 290 μg, respectively. This represents 53% of the total mass for TCE and a 24% for c-DCE. The remaining mass of CAHs was present in the liquid fraction or sorbed onto the aquifer material in the microcosms, as will be discussed in the results section.

5.2.3 Chemical Sources, Saturated and Standard Solutions

Trichloroethylene (TCE;>99%) and c-DCE (>97%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Propane (98%) was also purchased from Aldrich Chemical Co. (Milwaukee, WI). Saturated stock solutions of CAHs were prepared at room temperature by adding specific amounts of the liquid to 125-mL serum bottles containing autoclaved deionized water. This procedure eliminated the use of carrier solvents, such as methanol. The bottles were shaken for 6 hours to ensure saturation, and then were allowed to settle for at least 24 hours before use.

Standard solutions were made for creating standard calibration curves for CAH GC analysis. A given mass of the compound was dissolved in a known volume of methanol. A given volume of methanol solution was added to a capped 28 mL vial that contained 10 mL of deionized water. The solution equilibrated on a shaker table at 20°C. Headspace samples were taken and analyzed using GC methods. Mass balances

using Henry's Law were made to determine the corresponding liquid concentration, and to construct standard curves.

4.2.3 Sampling and Analytical Methods

The microcosm tests required the measurement of headspace concentrations of the gaseous substrate and CAHs of interest. Headspace samples were obtained using a 100-μL gas-tight syringe. Samples were manually injected onto the appropriate GC immediately after sampling. During the course of the microcosm incubation, headspace sampling and propane and oxygen consumption created a vacuum inside the microcosms. After propane was consumed the headspace was equilibrated to atmospheric pressure under a laminar flow hood by adding pure oxygen. This maintained oxygen concentrations in the microcosms so that aerobic conditions were maintained throughout the experiment.

CAH and gaseous substrate concentrations were quantified by GC analysis. The HP 6890 GC was equipped with a photo ionization detector (PID) in conjunction with a flame ionization detector (FID). The CAHs with unsaturated bonds were detected with a PID, while the FID detected the gaseous cometabolic substrate (propane). The advantage of using PID/FID detectors in series was that the gaseous substrate and chlorinated ethenes could be determined in the same analysis. Separation was obtained by using a GS-Q capillary column (VWR Scientific Inc.) operated with a temperature gradient for better separation of the gaseous substrate and chlorinated ethenes. High-grade helium, at a head pressure of 60 psi, was used as the carrier gas. The method was calibrated by using the external standards. The total mass of compounds in the

microcosms was determined by mass balance assuming Henry's law equilibrium partitioning.

5.3 Results and Discussion

5.3.1 Control Microcosm

The initial total mass of c-DCE and TCE added to microcosm VZ1 was 1200 μg and 1100 μg, respectively. After the addition of c-DCE and TCE the total mass in the headspace of the microcosm were 310 μg and 560 μg, respectively (Figure 5.1). The remaining mass of CAHs was either present in the liquid phase or sorbed onto the aquifer material and rubber septa cap. The volume of water in the microcosm was calculated by using the moisture content of the aquifer material plus the amount of water added with the CAH water saturated solutions. The total volume of water in microcosm VZ 1 was 22.3 mL. Using this volume of water and the corresponding Henry's coefficients the aqueous phase concentration was calculated. The total mass of CAHs sorbed onto subsurface solids and septa was calculated by subtracting the total mass of CAHs in the gaseous and liquid phases from the initial total mass added. Figure 5.2 shows the calculated amount of CAHs sorbed onto the subsurface solids and septa over the first 44 days of operation. For the first ten days a significant amount of CAHs were sorbed, and after that the concentrations became steady with only a small amount of sorption occurring through day 44. Partition coefficients (K_d), C_i solids/C_i aqueous, were calculated from this data.

The formula used to calculate the partition coefficient values was:

$$K_d = 0.63\ K_{ow}\,(f_{oc})(\varepsilon\ /\rho_b) \qquad [L^3/M] \qquad \text{(McCarty and Rittmann, 2001)}$$

The amount of dry subsurface material was 84.3 grams in microcosm VZ 1. An organic fraction value, f_{oc}, of 0.015 was used in the estimates of expected sorption coefficient. The subsurface material was obtained from a depth of 112 ft and had a sandy-clay texture, which should not contain a high organic fraction. The constant, 0.63, is an empirical conversion factor (mass solid-volume octanol/mass-volume water); ρ_b is the bulk density of the subsurface material; and ε is the porosity.

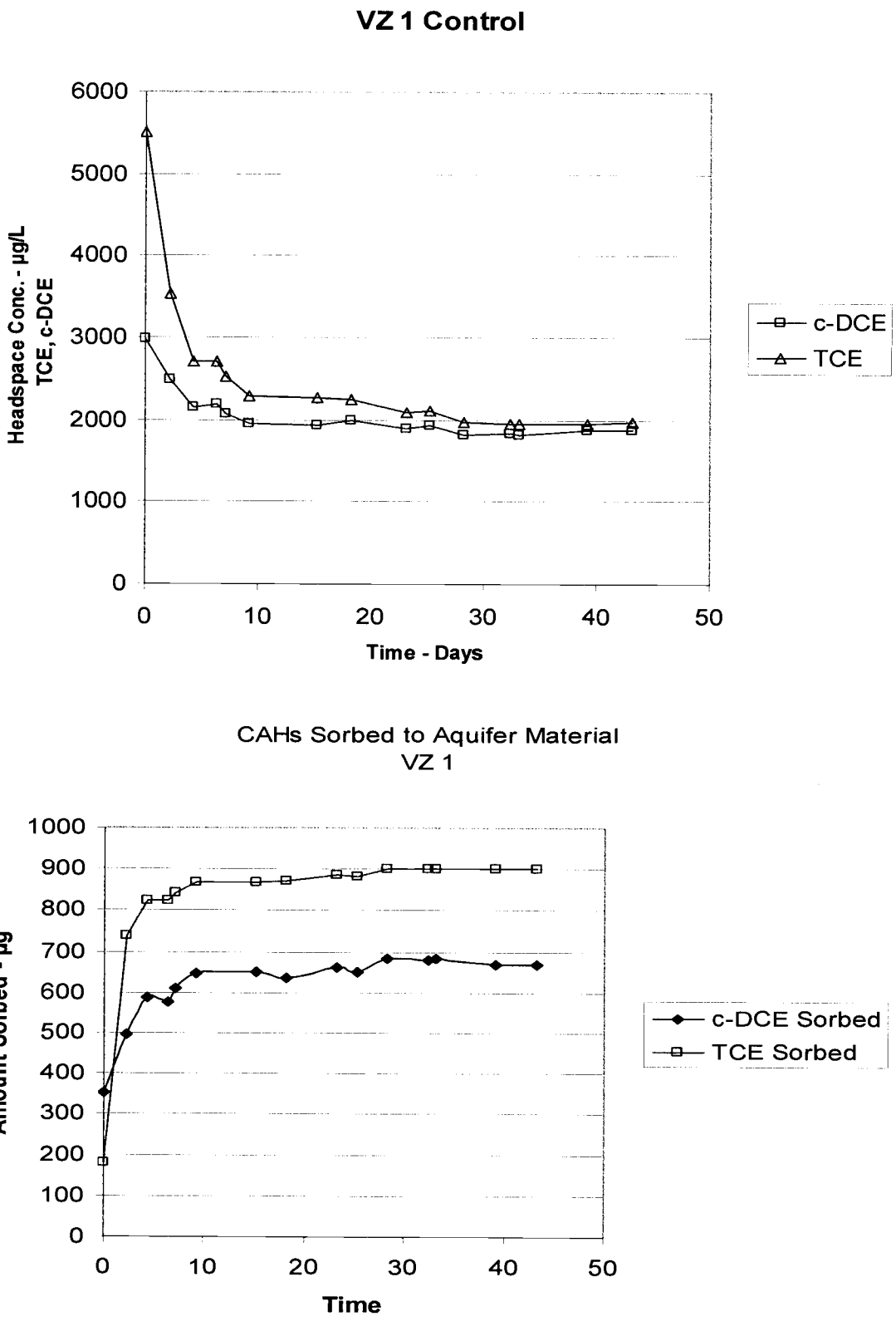

Figure 5.1 Headspace concentrations of CAHs in control microcosm VZ 1 and total amount CAHs sorbed on to subsurface material from day 0 to day 44.

Table 5.1 Calculated partition coefficients for TCE and c-DCE on to the subsurface material in microcosm VZ 1.

Microcosm VZ	Calculated Kd (m^3/g) c-DCE	Calculated Kd (m^3/g) TCE
1	5.21E-07	1.86E-06

These results show that 92% of the initial total mass of TCE and 57% of the initial mass of c-DCE were sorbed onto the subsurface solids over a 44-day time period. The resulting gaseous concentration of c-DCE and TCE by day 44 was 1.87 mg/L and 1.97 mg/L, respectively. Assuming equilibrium partitioning the resulting aqueous concentration of c-DCE and TCE by day 44 was 15.2 mg/L and 5.8 mg/L, respectively.

These concentrations were higher than the gaseous phase concentrations found at the field site (0.5-0.6 mg/L). To lower the concentration of CAHs in microcosm VZ 1, 200 mL of air was purged into the microcosm. Figure 5.2 shows the headspace concentrations initially decreased, but increased over the next five days due to partitioning into the gas phase from the solids. Two more purges with air were required before headspace concentrations were lowered to concentrations that represented those in the field. The final headspace concentration achieved on day 81 for c-DCE and TCE were 0.49 mg/L and 0.69 mg/L, respectively. Headspace concentrations of microcosm VZ 1 remained essentially constant after day 81 indicating steady-state conditions were obtained.

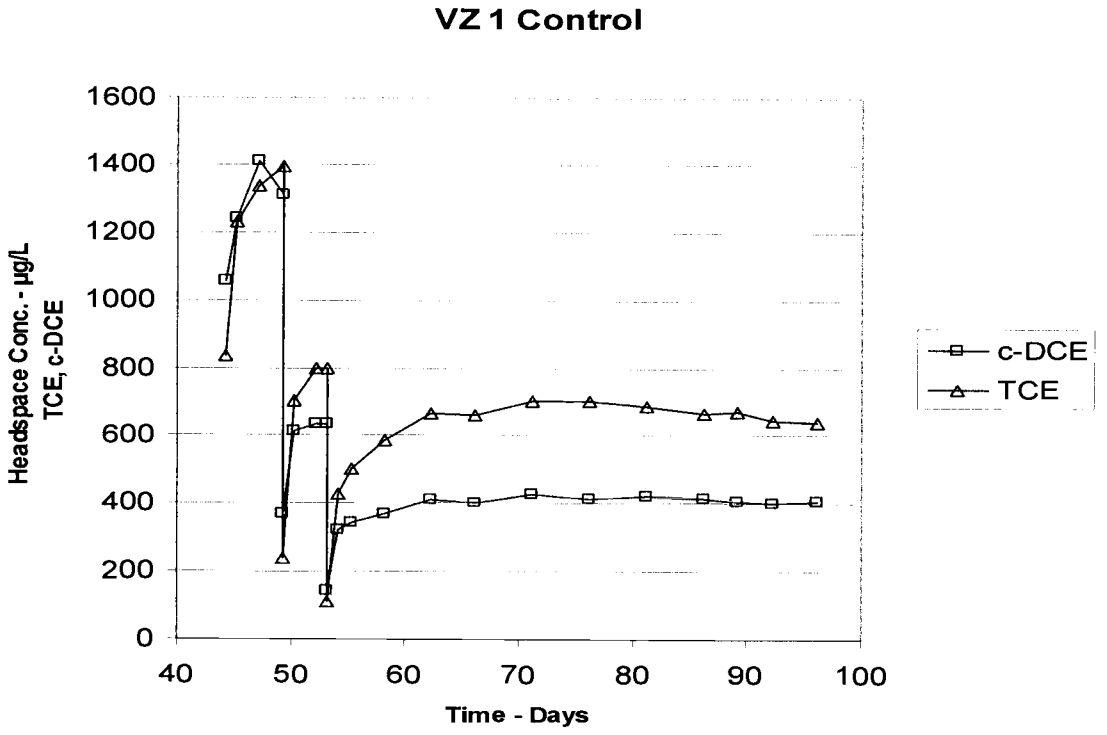

Figure 5.2 Headspace concentrations after purging microcosm VZ 1 three times with air.

5.3.2 McClellan Vadose Zone Microcosms VZ 2 and 3

Microcosms VZ 2 and 3 were used to investigate propane utilization in the absence of CAHs, with and without the presence of ammonia gas. Both VZ 2 and 3 had 1 mL of propane gas added, but only VZ 2 had a 0.2 mL of ammonia gas added. The initial headspace concentration of propane was 15.2 mg/L.

Figure 5.3 shows the headspace concentrations of propane over a 100-day time period. An initial lag period of 13-15 days was observed in the two microcosms before propane utilization began. Table 5.2 shows the propane utilization rates for VZ 2 and 3 over several time periods. There was a significant difference in propane utilization

rates as the experiment progressed. Microcosm VZ 2, with the ammonia addition, had a propane utilization rate that was 1.5 times higher than VZ 3. Microcosm VZ 2 maintained a higher rate of propane utilization than VZ 3 until propane concentrations in the headspace fell below 1000 μg/L (Day 39) in VZ 2.

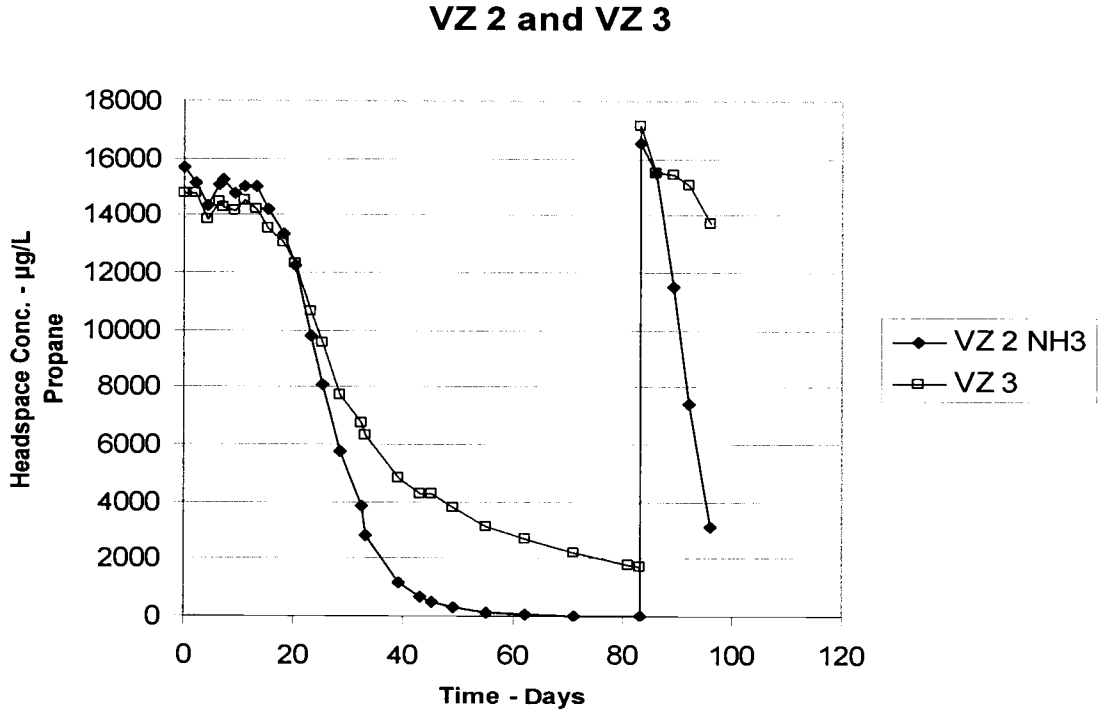

Figure 5.3 Propane utilization in microcosms VZ 2 and 3.

Table 5.2 Propane utilization rates over various times in microcosms VZ 2 and 3.

	Propane Utilization Rates			
Microcosm	**Day 18-23 (μg/hr)**	**Day 28-33 (μg/hr)**	**Day 39-49 (μg/hr)**	**Day 86-96 (μg/hr)**
VZ 2	3.0	2.5	0.37	5.3
VZ 3	2.0	1.2	0.43	0.75

On day 83, propane gas (1 mL) was added again to both microcosms. Microcosm VZ 2 received another addition of ammonia gas (0.2 mL), while VZ 3 did not receive any nitrogen amendments. Table 5.2 shows the difference in propane utilization rates upon this addition became even more apparent (Day 86-96). VZ 2 maintained a high propane utilization rate compared to VZ 3. The propane utilization rate of VZ 2 did not significantly decrease throughout the experiment except between days 39-49 when propane concentrations were below 1000 μg/L, indicating that nitrogen was the limiting nutrient.

Comparisons were made between the propane utilization rates of these vadose microcosms and the saturated microcosms in Chapters 3 and 4, in the absence of CAHs. A significant difference in the rate of propane utilization was observed. Propane utilization rates observed in the saturated microcosms ranged from 20-34 μg/hr, when nitrate or ammonia was used as the primary nitrogen source (Table 3.4 and 4.7, Figure 3.6). The utilization rates in the vadose microcosms ranged from <1-5.3 μg/hr, when ammonia was the nitrogen source. Rates of propane utilization were a factor of five lower in the vadose zone.

5.3.3 McClellan Vadose Zone Microcosms VZ 4 and 5

Microcosms VZ 4 and 5 were used to determine the ability of the propane-utilizers to transform CAHs at varying concentrations with and without the presence of ammonia gas. These microcosms were operated for 100 days.

The headspace concentrations for these microcosms are shown in Figure 5.4 for the first 44 days. Both microcosms initially had a total mass of 1200 μg c-DCE and

1100 μg TCE added. Propane (1 mL) was added to both microcosms, but only VZ 5 had a 0.2 mL addition of ammonia gas. These microcosms behaved similarly to the control microcosm VZ 1 that showed a large amount of the CAHs partitioning to the aquifer solids over the first 10 days of incubation (Figures 5.5). Headspace concentrations of 2.0 mg c-DCE/L and 2.4 mg TCE/L were achieved from the initial addition of water-saturated solutions. These concentrations were a factor of four times greater than the vapor phase concentrations at the field site. The associated c-DCE and TCE liquid phase concentrations were calculated to be 16 mg/L and 7 mg/L, respectively. No utilization or sorption of propane was observed in either microcosm during this time period. It was assumed that the high concentrations of c-DCE and TCE present in the aqueous phase and sorbed onto the subsurface material were potentially inhibiting the stimulation of propane-utilizers.

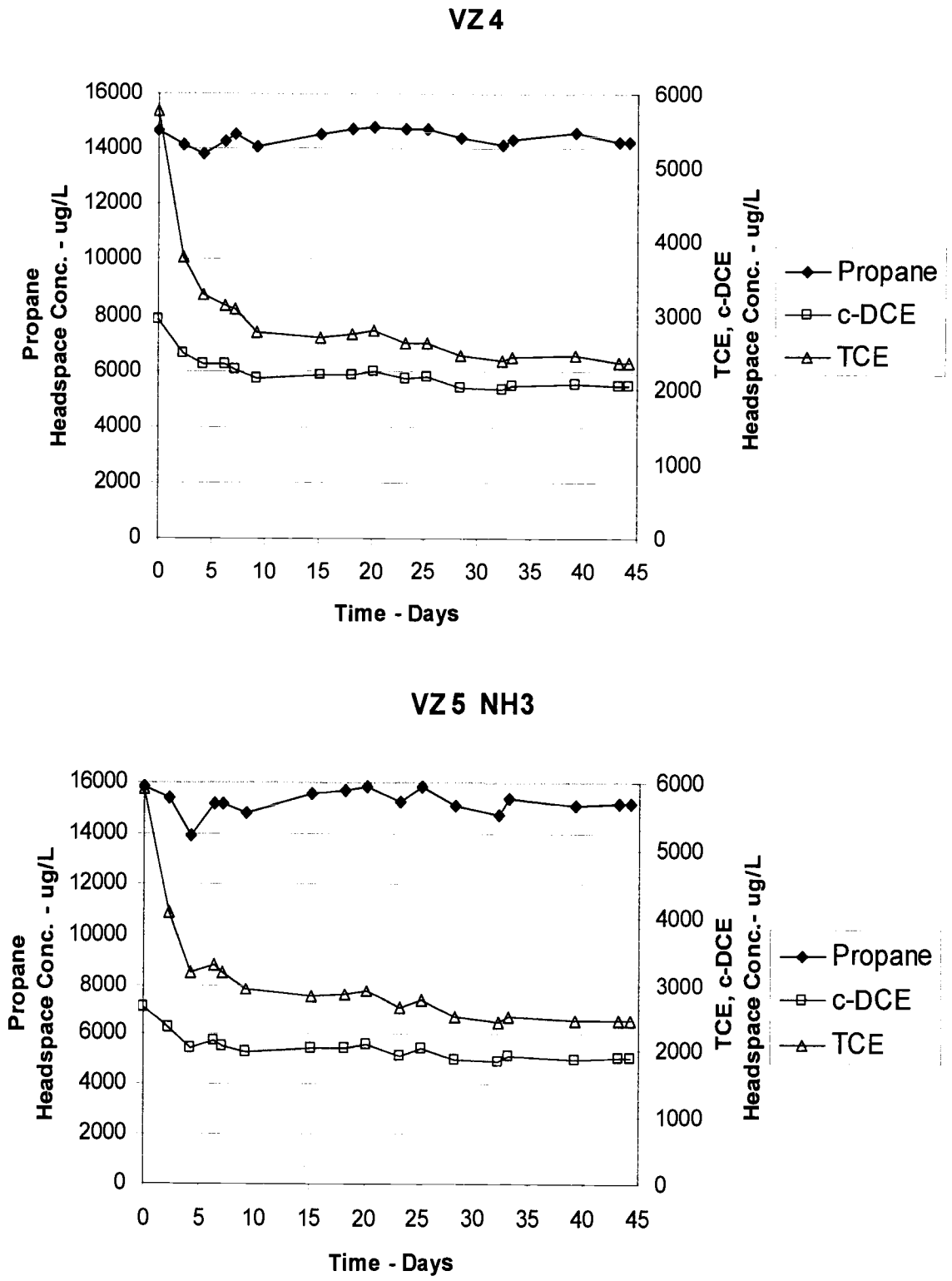

Figure 5.4 First 44 days of operation for microcosm VZ 4 and 5. Microcosm VZ 5 had NH_3 added.

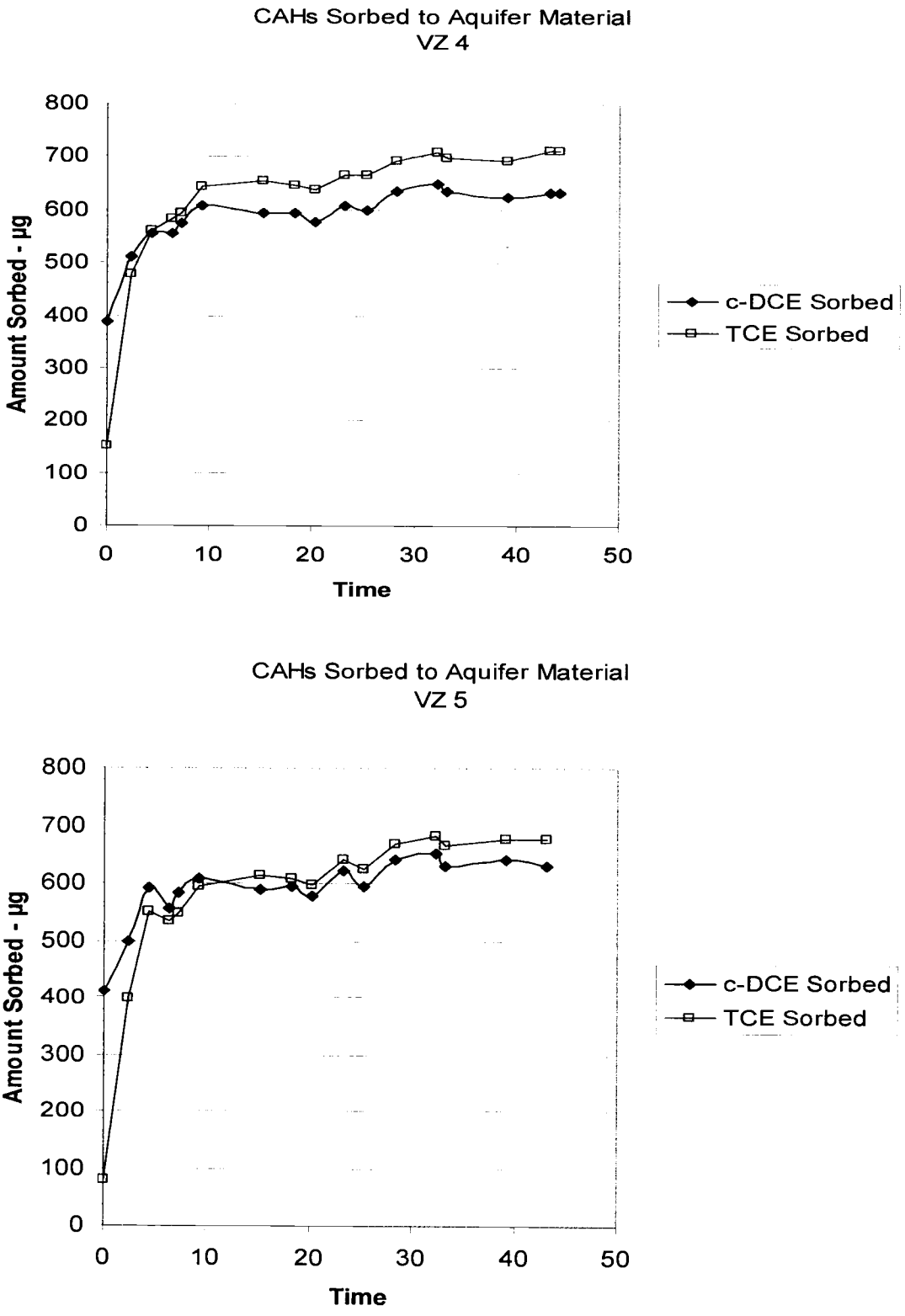

Figure 5.5 Amount of CAHs sorbed to subsurface material in VZ 4 and 5.

In order to achieve more representative concentrations of c-DCE and TCE found in the vadose zone of the field site, microcosms VZ 4 and 5 were purged with air three times to decrease the mass in the microcosms (Figure 5.6). These microcosms were purged in the same fashion as the control microcosm VZ 1. To lower the concentration of CAHs, 200 mL of air was purged into the microcosms. Headspace concentrations initially decreased, but increased over the next five days due to partitioning into the gas phase from the subsurface solids. Two more purges with air were required before headspace concentrations were lowered to concentrations that represented those in the field. The final headspace concentration achieved on day 66 for c-DCE and TCE were 0.41 mg/L and 0.71 mg/L, respectively.

Propane was completely removed from the microcosms after the three successive purges with air. On day 55, propane (1 mL) was added to microcosms VZ 4 and 5. This yielded a headspace concentration of 16.2 mg/L. Ammonia (0.2 mL) was also added to VZ 5. Propane utilization was observed after three days in VZ 5, with a rate of 0.87 μg/hr from day 55-96. To date, after 40 days of incubation, microcosm VZ 4 has not shown significant evidence of propane utilization. The rates in VZ 5 were over three times slower than the propane utilization rates observed in microcosms VZ 2 and 3. The presence of CAHs negatively effected propane utilization.

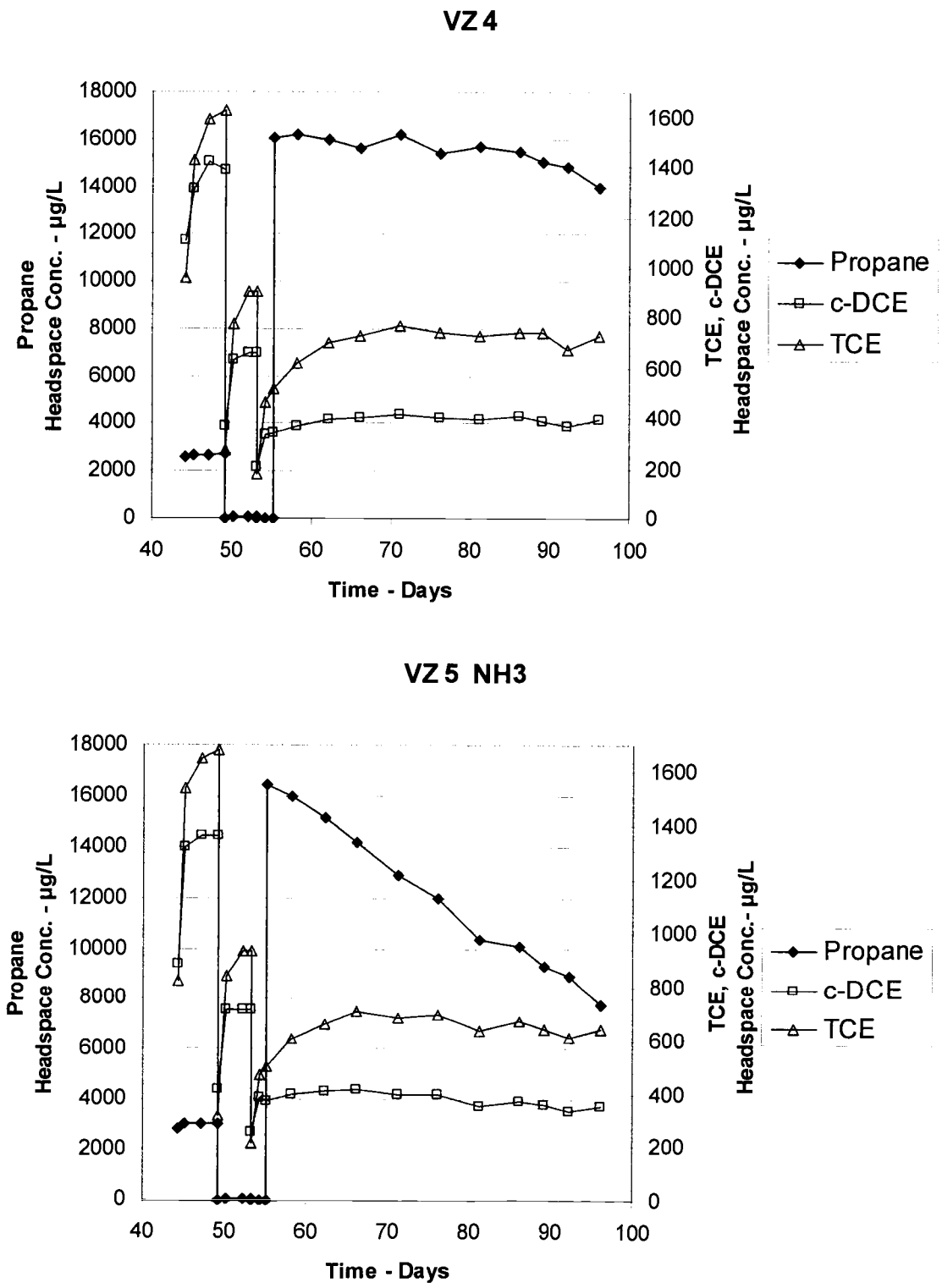

Figure 5.6 Microcosms VZ 4 and 5 after purging with air to reduce CAH concentrations. Microcosm VZ 5 had ammonia added.

Transformation of CAHs in VZ 4 and 5 were not observed during this experiment. Headspace concentrations of c-DCE and TCE were stable from day 66-96 in both microcosms. However, propane concentrations had not decreased to low enough concentrations for effective CAH transformation based on observations in saturated microcosms. Also a large amount of the CAHs were sorbed onto the aquifer material, which may have re-partitioned into the headspace effectively masking small amounts of CAH transformation. Further monitoring of propane utilization and CAH concentrations is needed before any conclusions can be made regarding CAH transformation.

5.4 Summary

This experiment provided some preliminary observations that may be used to explain observations during the CAS demonstration at McClellan AFB. Headspace concentrations, after purging, were approximately 350 μg/L c-DCE and 650 μg/L TCE. These were similar to the TCE and c-DCE concentrations (300-600 μg/L) surveyed during the first 40 days of testing at the CAS field site (Tovanabootr et al., 2000). Propane concentrations in the microcosms were 0.9% in air, while the field site concentrations were 2%.

One observation of the experiment was the lag times required to stimulate the propane-utilizers in the absence and presence of CAHs. A lag time of 13 days was observed before propane utilization occurred in the microcosms without any CAHs present. The addition of ammonia gas did not have any effect on the lag time in these microcosms. The initial incubation of propane in the microcosm with the higher

concentration of CAHs did not show any propane utilization for 44 days. After the headspace concentrations were reduced a three-day lag period was observed in the microcosm with ammonia added. In comparison, the microcosm without ammonia did not show significant propane utilization over 40 days of incubation. These lag times were shorter than the 40-day lag period observed during the CAS demonstration.

A major observation of this experiment was the propane utilization rates observed in the presence and absence of CAHs, and the effects of adding ammonia gas on these rates. Microcosms without CAHs present had propane utilization rates a factor of three times greater than the microcosms with CAHs. From this observation the presence of CAHs negatively affected the ability of the propane-utilizers to consume propane. Another major factor affecting the propane utilization rates was the presence of ammonia gas. The microcosms with the nitrogen amendment were able to utilize propane a factor of 1.5 times faster than the microcosms without the nitrogen amendment. Also the microcosms with the ammonia added were able to maintain propane utilization rates, while rates in microcosms without the nitrogen amendment gradually decreased. These observations correlate well to what was observed in the CAS demonstration at McClellan AFB. Little propane utilization or CAH transformation was observed in the vadose zone during the first 188 days of operation (Tovanabootr et al., 2000). Upon addition of ammonia to the sparge gas (0.01%), propane and CAH concentrations began to decrease. The ammonia may have provided a source of bioavailable nitrogen for the propane-utilizers. These results also correlate well to the results in Chapter 4 where it was observed that the presence of bioavailable nitrogen was required for effective propane utilization and CAH transformation.

However, rates of propane utilization in the vadose zone microcosms were consistently lower than in saturated microcosms.

This experiment did not show transformation of CAHs by the propane-utilizers. Concentrations of propane were still high at the end of this experiment, and had not yet decreased enough to observe any changes in the CAH concentrations in the headspace of the microcosms. During the CAH demonstration, CAH concentrations in the vadose zone slowly decreased after ammonia was added to the sparge gas indicating some cometabolic removal (Tovanabootr et al., 2000). Further monitoring of these microcosms is required before any conclusions can be made regarding CAH transformation ability.

CHAPTER 6
CONCLUSIONS AND ENGINEERING SIGNIFICANCE

The primary objective of this study was to use microcosms to better understand the cometabolic processes that occurred during the CAS demonstration at McClellan AFB. The CAS demonstration experienced effective removal of c-DCE and TCE in the saturated zone, but only limited removal in the vadose zone. Background nitrate concentrations in the groundwater were only able to support propane utilization and CAH transformation for a limited time (Tovanabootr et al., 2000). Ammonia gas was added as a source of bioavailable nitrogen that caused propane utilization and CAH transformation to resume.

The results of this study showed results similar to those observed in the field, and demonstrated that microcosms can be used to better understand the processes that may be occurring at a field site. Concentrations of c-DCE decreased more rapidly than TCE, as was observed in the field test. Propane utilization slowed when nitrate became limiting, causing CAH transformation to decrease. The addition of ammonia gas provided bioavailable nitrogen to the propane-utilizers causing an increase in propane utilization and CAH transformation. A significant difference between the microcosms and the field demonstration were the lag times in the microcosms being shorter than the lag time observed in the field.

Another major observation of this study was the nitrogen requirements of the propane-utilizers in the presence of CAHs. Both nitrate and ammonia were found to be good sources of bioavailable nitrogen for the microorganisms. As the amount of CAHs transformed increased the microorganisms demand for nitrogen also increased. If

nitrogen was not available the microorganisms' ability to utilize propane and transform CAHs gradually decreased. Maintaining a sufficient source of bioavailable nitrogen should be a consideration of any treatment system in the field in order to achieve effective removal of CAHs.

Preliminary results from the vadose microcosms indicated that the utilization of propane occurs at an order of magnitude lower than the rate in the saturated zone. Also bioavailable nitrogen is required to maintain propane utilization rates. However, the higher CAH concentrations were found to inhibit the stimulation of the propane-utilizers.

These results show potential incompatibility of the combined treatment through sparging of the vadose zone and saturated zone. Two separate systems for substrate addition may be required for effective CAH removal. The high sparge concentrations of propane to achieve groundwater concentrations are difficult to remove once they are in the vadose zone. One possibility was that the moisture content was too low in the vadose zone to achieve enhanced rates of propane utilization. Further microcosm studies are needed in order to gain insight into these slower processes that are occurring so field applications can be modified to create a more efficient remediation system in the vadose zone. The effect of moisture content in the vadose zone needs to be closely examined.

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