STABLE CARBON ISOTOPE FRACTIONATION OF TRANS-1,2-DICHLOROETHYLENE BY METHANOTROPHIC BIODEGRADATION

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A thesis submitted to the Faculty and the Board of Trustees of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Master of Science Geochemistry.

Golden, Colorado

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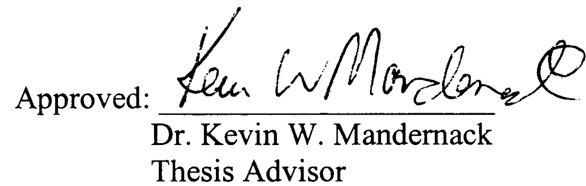
Approved: 
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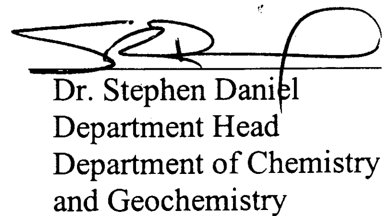
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ABSTRACT

Changes in the carbon isotope value ($\delta^{13}C$) of *trans*-1,2-dichloroethylene (t-DCE) were measured during its cometabolic degradation by *Methylomonas methanica*, a type I methanotroph, and *Methylosinus trichosporium* OB 3b, a type II methanotroph. In closed-vessel incubation experiments with each bacterium, the residual t-DCE became progressively enriched in ^{13}C, indicating isotopic fractionation. From these experiments the biological fractionation during t-DCE cometabolism, expressed as ε, was measured to be –3.3‰ and –3.7‰ for duplicate experiments with the type I culture and –6.7‰ for the type II culture. This fractionation effect and subsequent enrichment in the $\delta^{13}C$ of the residual t-DCE suggests that measured changes in the $\delta^{13}C$ values of chlorinated solvents may be used to monitor the extent of biodegradation in laboratory or field settings where cometabolism by methanotrophs occurs.

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Section 1.0

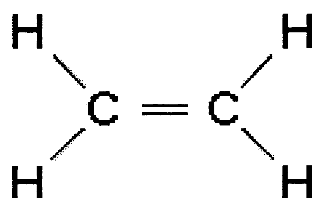
INTRODUCTION

Experiments were conducted to investigate the possibility of measuring the changes in the stable carbon isotopic composition ($\delta^{13}C$) of trans-1,2-dichloroethylene (t-DCE) as a result of isotopic fractionation by methanotrophic bacteria during cometabolic degradation. This section provides a background review of the chlorinated ethenes, natural attenuation as a remediation technique for chlorinated ethenes, methanotrophic (methane-oxidizing) bacteria and their role in degrading chlorinated ethenes, and microbial stable isotope fractionation and its potential application for monitoring in-situ bioremediation.

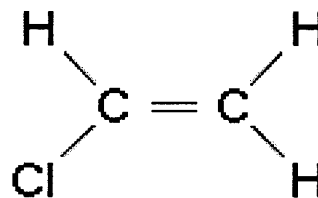
1.1 Chlorinated Ethenes

Of the 25 most commonly encountered groundwater contaminants at hazardous waste sites, 10 are chlorinated volatile organic compounds (VOCs) (1), including chlorinated aliphatic hydrocarbons (CAHs) such as the chlorinated ethenes trichloroethylene (TCE), dichloroethylene (DCE), and vinyl chloride (VC). The molecular structure of ethene is shown in Figure 1-1. Replacing a hydrogen atom in an ethene molecule with one to four chlorine atoms results in a chlorinated ethene (Figure 1-1). Past research has demonstrated that the more highly chlorinated ethenes, such as perchloroethylene (PCE)

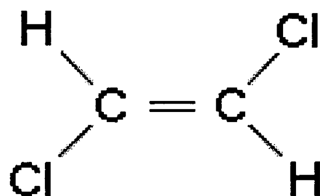
and TCE can undergo dehalogenation in the field, resulting in relatively high concentrations of the DCE isomers (t-DCE, *cis*-1,2-DCE, and 1,1-DCE) and VC (see Section 1.3) (2-12). Therefore, concentrations of the contaminants typically observed at field sites may be the result of disposal practices in addition to subsequent degradation of the more highly chlorinated parent compounds.



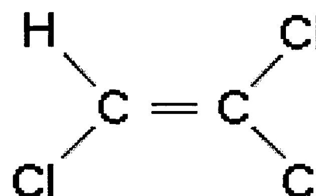
ethene



vinyl chloride



trans-1,2-dichloroethylene



trichloroethylene

Figure 1-1. The structure of ethene and some of the chlorinated ethenes

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1.1.1 Industrial Uses

Some CAHs possess chemical properties that make them effective industrial solvents. For example, TCE was used extensively as a cold cleaning industrial solvent for degreasing metal parts over a period of decades. TCE was also used in dry cleaning agents, fungicides, insecticides, oils, resins, paints, adhesives, and disinfectants (13).

1.1.2 Health Effects

Chlorinated ethenes are known or suspected carcinogens (14-18). After the use of TCE was already widespread, it was discovered that malignant tumors were produced in laboratory animals exposed to TCE (19-21). As a result, The Food and Drug Administration banned much of its use (13); however, some risk of exposure still exists due to historical disposal practices. During the 1970's, high concentrations of PCE, TCE, DCE, and VC were found in aquifers near industrial waste sites. Some of these aquifers were thought to supply drinking water to residential areas. Many industrial workers and neighborhood residents potentially exposed to elevated concentrations of chlorinated ethenes experienced negative health effects such as skin rashes, leukemia, cancers of the lung, colon, kidney, pancreas, bladder, and liver (22-24). Although a direct correlation cannot be made between these health effects and chemical exposures, the results of animal studies have led researchers to believe that chlorinated ethenes represent a significant health hazard to a large portion of the human population, and therefore require some form of remediation.

1.1.3 Difficulty in Remediating

In addition to the proposed dangerous health effects, CAHs possess other unfavorable characteristics. They are highly recalcitrant in most groundwater environments, and drinking water limits are very low due to their potential as carcinogens (14-18). Most of these organic compounds belong to a class of fluids referred to as dense non-aqueous phase liquids (DNAPL), which possess a very complex physical behavior in subsurface environments. Some compound-specific and environment-specific characteristics responsible for the complex behavior of DNAPL are as follows:

- DNAPL are immiscible with water;
- DNAPL are more dense than water and can therefore penetrate to considerable depths below the water table;
- DNAPL with low water solubilities can persist in the subsurface for many years or decades;
- DNAPL can serve as a long-term source of dissolved contaminants which move with groundwater to substantial distances away from the contaminant source;
- Small amounts of DNAPL can contaminate large volumes of water;
- Materials through which DNAPL has moved retain a portion of the DNAPL fluid;
- Geologic heterogeneity can result in highly complex distributions of DNAPL in saturated and unsaturated zones; and

- DNAPL cannot be completely removed by pumping and is immobile at residual saturation (material is said to have residual saturation if ~5% of the pore space is occupied by DNAPL) (1).

1.1.4 Remediation Techniques

As a consequence of the rapid emergence of the need for groundwater remediation spurred by the U.S. Congress in the early 1980's, the fastest and most obvious methods of remediation were developed. One of these methods, based on aquifer hydraulics, is the pump-and-treat system where water is pumped from the aquifer, then treated in a separate above-ground facility. Due to their complex subsurface behavior, as discussed in Section 1.1.3, the chlorinated ethenes and other CAHs can be difficult to remove from contaminated soils and aquifers using conventional pump-and-treat methods. Consequently, the results of using these treatment systems for remediation have not been favorable (1). From 1982 to 1994, 73% of selected hazardous waste sites established under the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) were using pump-and-treat systems, and approximately 90% of those sites had not yet achieved their clean-up objectives (1).

General industry dissatisfaction with pump-and-treat systems has led researchers to develop more effective methods of remediating groundwater contaminated with CAHs. Alternative methods such as soil vapor extraction, excavation and thermal treatment, groundwater recirculation wells, and passive barrier treatment have recently shown

favorable results in field settings. Another approach to remediation that is relatively inexpensive and requires lower levels of engineering is natural attenuation, whereby the CAHs are degraded through natural processes in the field.

1.2 Natural Attenuation

Natural attenuation as a method of in situ bioremediation has emerged as a successful and cost-effective method for remediating soil and groundwater contaminated with chlorinated solvents (25). The U.S. Environmental Protection Agency defines monitored natural attenuation as the following (26):

> *The term "monitored natural attenuation," as used in this Directive, refers to the reliance on natural attenuation processes (within the context of a carefully controlled and monitored clean-up approach) to achieve site-specific remedial objectives within a time frame that is reasonable compared to other methods. The "natural attenuation processes" that are at work in such a remediation approach include a variety of physical, chemical, or biological processes that, under favorable conditions, act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in soil and ground water. These in-situ processes include biodegradation, dispersion, dilution, sorption, volatilization, and chemical or biological stabilization, transformation, or destruction of contaminants.*

1.2.1 Bioremediation

Natural attenuation can include bioremediation, whereby microorganisms remove or detoxify unwanted chemicals in subsurface environments. Bioremediation can be enhanced by adding nutrients to the subsurface to stimulate the background population of microorganisms responsible for degradation of the contaminants. This method has

become an attractive alternative for remediating contaminated sites that cannot be accessed with equipment necessary for heavily engineered remediation, or for areas containing widespread plumes of low level contamination (25). However, in order to determine its effectiveness, a means of accurately tracking and quantifying the biotransformation process must be developed (25).

1.3 Anaerobic Biodegradation

Anaerobic bacteria degrade chlorinated ethenes through reductive dehalogenation as shown in Figure 1-2 (2-12). Through reductive dehalogenation, chlorine atoms in the highly chlorinated ethenes (PCE, TCE, and DCE) are replaced by hydrogen atoms. As depicted in Figure 1-2, an end product of this reductive process is vinyl chloride, a very volatile compound and a known carcinogen. Freedman and Gossett (11) showed that under anaerobic and methanogenic conditions, vinyl chloride can further be degraded to ethene; however, the step is rate limiting with significant levels of vinyl chloride persisting. In the absence of methanogenesis, DiStefano, et al. demonstrated that only near millimolar concentrations of PCE could be reduced to ethene with little residual vinyl chloride (12). In contrast to these findings, the end products of aerobic degradation of chlorinated ethenes appear to be carbon dioxide and water (27-44).

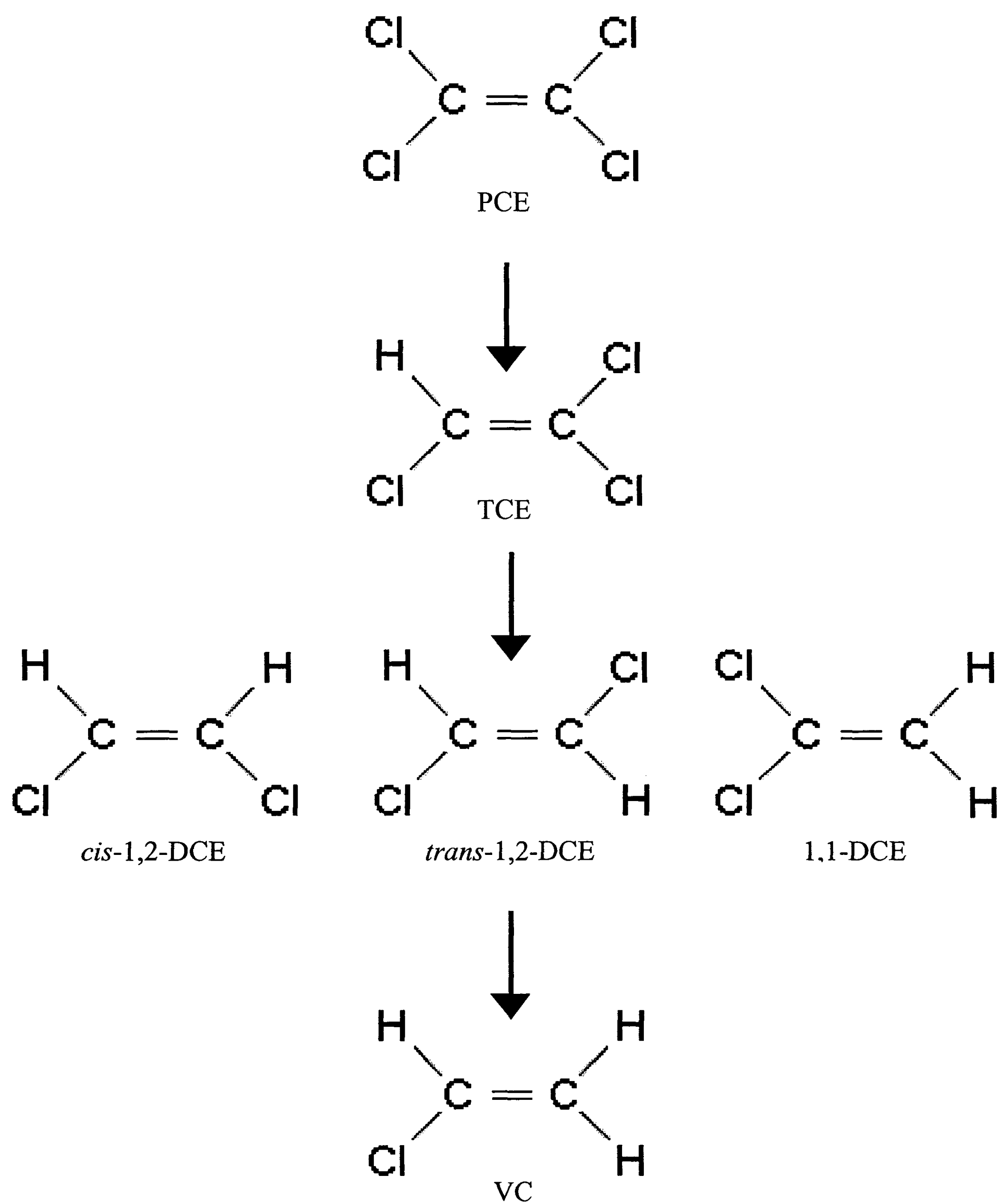


Figure 1-2. Chlorinated ethene reaction series for reductive dehalogenation

1.4 Aerobic Biodegradation

Methanotrophic (methane-oxidizing) bacteria, through aerobic cometabolism, degrade chlorinated ethenes without producing harmful degradation products such as vinyl chloride. Numerous studies involving the use of methanotrophic bacteria to degrade CAHs have emerged over the past two decades. Wilson and Wilson were the first to demonstrate that a chlorinated ethene could be degraded aerobically to carbon dioxide in the presence of air and methane (27). Presumably, addition of the methane and air stimulated a background population of methanotrophic bacteria that, as subsequent studies have confirmed, degrade various chlorinated organics (28-44).

1.5 Physiology and Ecology of Methanotrophs

Methanotrophs are a specialized group of gram negative, obligately aerobic bacteria, capable of oxidizing methane and other one-carbon compounds (45-46). Methanotrophs are classified as type I or type II, depending on their internal cell membrane structures, methane monooxygenase enzymes, and carbon assimilation pathways (45-46). Methane is produced by methanogenic bacteria in anaerobic environments such as sediments, marshes, wetlands, rice paddies, and landfills (45). Methane is a major constituent of natural gas and can also be found in coal formations. Methanotrophs use methane as an electron donor for energy generation and as a source of carbon (45-46). They are widespread in both terrestrial and aquatic environments, especially in soils, and fresh water and marine sediments where oxygen and a stable source of methane exist. Their

ubiquity in natural soils and water combined with the ability to degrade various contaminants suggests that methanotrophs could be an important component of natural attenuation or bioremediation.

1.6 Methane Oxidation by Methanotrophs

The overall pathway for methane oxidation by both types of methanotrophs is as follows:

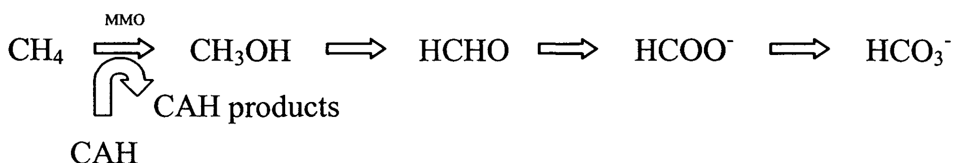
$$CH_4 \xrightarrow{MMO} CH_3OH \Rightarrow HCHO \Rightarrow HCOO^- \Rightarrow HCO_3^-$$

The first step, where methane is oxidized to methanol, involves an enzyme called the methane monooxygenase enzyme (MMO), which is common to all methanotrophs. Monooxygenases catalyze the transfer of one of the two oxygen atoms in O_2 to an organic compound as a hydroxyl group (OH^-), with the second atom being reduced to water (47). Two forms of the MMO enzyme have been found in methanotrophic bacteria, the soluble MMO (sMMO) and the particulate MMO (pMMO) (45). Type I methanotrophs typically utilize only a pMMO, whereas type II methanotrophs use both a pMMO and an sMMO (45). The latter is generally expressed when available copper is limiting.

1.7 Methanotrophic Degradation of CAHs

Methanotrophic bacteria degrade chlorinated organics through cometabolic processes that use the MMO enzyme (39-44). Besides facilitating the oxidation of methane to

methanol, the MMO enzyme has a low substrate specificity and is therefore able to co-oxidize or dehalogenate a variety of organic contaminants (43,48-51). During methanotrophic biodegradation, CAHs are fed into the methane oxidation pathway at the first step where the MMO enzyme acts as indicated below (29,44).



Historical research has shown that mixed methanotrophic and pure *M. trichosporium* OB3b cultures degrade TCE when sMMO is expressed (39-41). However, more recently, Lontoh et al. (1997) observed that *M. trichosporium* OB3b cells expressing pMMO grown with an abundance of copper can degrade larger quantities of TCE than cells grown under lower concentrations of copper, though not low enough to allow for sMMO expression (42). In addition, Anderson et al. (1998) reported that for a mixed methanotrophic culture expressing pMMO, transformation yields for t-DCE and VC were 20 times greater than the yields reported by others for cells expressing sMMO, although transformation yields for TCE, c-DCE, and 1,1-DCE were similar or less than for cultures expressing sMMO (43). In summary, these results demonstrate that both the sMMO and the pMMO enzyme contribute to the degradation of CAHs.

1.8 Stable Isotopes

Isotopes of specific elements have the same number of protons, but different numbers of neutrons in the atomic nucleus. Isotopes are referred to as "stable" if they do not undergo natural radioactive decay. Carbon has two stable isotopes, ^{12}C and ^{13}C, which have relative natural abundances of 98.89% and 1.11%, respectively. Stable carbon isotope measurements are expressed using the standard δ notation, which relates the $^{13}C/^{12}C$ ratio of a sample to the $^{13}C/^{12}C$ ratio of a standard that is assigned an arbitrary value of 0 ‰. For carbon, the standard is the Peedee Belemnite carbonate. The $\delta^{13}C$ value of a given sample is defined as follows:

$$\delta\ (in\ per\ mil,\ ‰) = \left(\frac{R_x}{R_{std}} - 1\right) \times 1000 \quad (1)$$

where R_x is the $^{13}C/^{12}C$ ratio in the sample, and R_{std} is the $^{13}C/^{12}C$ ratio in the standard.

1.8.1 Stable Isotope Fractionation

The chemical behavior of isotopes of the same element is typically identical; however, isotope property differences exist due to mass differences between the isotopes (52). The vibrational frequency of atoms in a molecule with respect to one another causes isotope effects (52). Isotopes with a lower mass have a higher vibrational frequency. Consequently, the lighter isotopes in a molecule will have a lower disassociation energy than the heavy isotope, thus weaker bonds are generally formed by

the light isotope. Therefore, if two phases are in an equilibrium mixture, the heavier isotope will tend to accumulate more in the phase with the stronger bonds, resulting in equilibrium isotopic fractionation between the two phases. An example of this fractionation is the equilibrium between dissolved CO_2 and HCO_3^-, whereby ^{13}C tends to accumulate more in the HCO_3^- phase.

Molecules containing the light isotope will generally react more readily than those with the heavy isotope (52). Using methane as an example, because the ^{12}C isotope has a lower disassociation energy then the ^{13}C isotope, the ^{12}C-H bond is weaker than the ^{13}C-H bond. As a result, the activation energy required to convert CH_4 to CH_3OH is lower for ^{12}C-H bonds than for ^{13}C-H bonds (53). Thus, molecules with ^{12}C-H bonds will generally react more rapidly than molecules with ^{13}C-H bonds (52). This is an example of a kinetic isotope effect (KIE), which can also be influenced by enzymes that mediate biological reactions. In general, biologically mediated reactions will favor the lighter isotope. The KIE for a given reaction can be defined in terms of the individual rate constants for the heavy and light isotope as follows:

$$^{13}R \xrightarrow{k_{13}} {}^{13}P \qquad\qquad {}^{12}R \xrightarrow{k_{12}} {}^{12}P$$

where R and P are reactants and products, respectively, containing the ^{13}C and ^{12}C isotopes as designated by the superscript, and KIE is equal to the ratio of the isotopic rate constants, k_{12}/k_{13} (54).

1.8.2 Stable Isotope Fractionation by Methanotrophs

As a result of a strong kinetic isotope effect during the oxidation of methane to carbon dioxide by methanotrophs, the carbon dioxide formed instantaneously at any point in time can be depleted in the ^{13}C isotope relative to the methane source (53,55-57). As the reaction proceeds in a closed-vessel batch culture, the fractionation effect in the reactant is compounded over time, and the difference in the $\delta^{13}C$ values of the reactant methane and product carbon dioxide becomes greater due to the Rayleigh distillation effect (58). The Rayleigh model, as shown in Equation 2, calculates a kinetic isotopic fractionation factor, epsilon (ε), of the product relative to the substrate, which can be used to calculate F, the fraction of the reactant remaining in a system. The Rayleigh model as derived by Mariotti et al. is as follows (58):

$$10^3 \ln \frac{10^{-3}\delta^{13}C_{T1}+1}{10^{-3}\delta^{13}C_{T0}+1} = \varepsilon \ln \frac{X_{T1}}{X_{TO}} \quad (2)$$

or in simplified form,

$$\delta^{13}C_{T1} - \delta^{13}C_{T0} = \Delta^{13}C = \varepsilon \ln \frac{X_{T1}}{X_{T0}} \quad (3)$$

where $\delta^{13}C_{T0}$ and $\delta^{13}C_{T1}$ are the isotopic composition of the reactant at Time (T) = 0 and T_{final} of the reaction, respectively, and X_{T0} and X_{T1} are the concentrations of the reactant at T_0 and T_{final}. The ratio X_{T1}/X_{T0} represents F, the fraction of reactant remaining, and is also a measure of the extent of the reaction. Epsilon (ε) is the isotopic enrichment factor of

the product relative to the substrate ($\varepsilon = 1000*(\alpha-1)$, where α equals the kinetic isotopic fractionation). As indicated in Equation 3, ε is equal to the slope of the line when the change in $\delta^{13}C$ from T_0 to T_{final} ($\Delta^{13}C$) is plotted against ln F.

Studies have reported that the oxidation of methane by methanotrophs has imparted fractionations of carbon isotopes by as much as ~30 per mil (‰) with the residual methane enriched in ^{13}C (53,55-57,59-60). In addition, the ability of microorganisms to isotopically fractionate a variety of substrates has been observed at contaminated sites experiencing petroleum hydrocarbon degradation (61-63), and carbon isotope fractionation of halogenated hydrocarbons during biotic or abiotic degradation has been observed in laboratory experiments (64-68) and in the field (68). These studies suggest that isotopic measurements may be used as a means for monitoring chlorinated solvent biodegradation. By measuring the changes in $\delta^{13}C$ values of contaminants as a result of degradation, and applying the appropriate values of ε as determined empirically from Equation 3, the value of F, the extent of contaminant biodegradation, could be estimated in the field. Therefore, $\delta^{13}C$ measurements could provide an alternative method for quantifying natural attenuation in field settings.

1.9 Research Objectives

This study consists of an investigation of whether stable carbon isotope measurements may eventually be used as a tool for quantifying chlorinated solvent biodegradation in field settings. Specific investigations included whether carbon isotope

fractionation occurs during cometabolism of *trans*-1,2-DCE by pure cultures of type I and type II methanotrophs, and whether this fractionation effect could potentially be used to predict the extent of biodegradation by methanotrophs.

Section 2.0

ISOTOPE FRACTIONATION OF *trans*-1,2-DCE DURING BIODEGRADATION

Experiments were conducted to measure the change in $\delta^{13}C$ associated with the degradation of *trans*-1,2-DCE by methanotrophs over time. The following section describes: 1) the methods and materials that were used to culture the type I and type II methanotrophs, and for measuring t-DCE concentrations and stable isotope compositions; 2) the results of experimentation; 3) a discussion of the results; and 4) a hypothetical example using the results in a field application.

2.1 Culture of Organisms

Pure cultures of *Methylomonas methanica*, Oak Ridge (type I) and *Methylosinus trichosporium* OB 3b (type II) were obtained from Dr. Richard Hanson of the University of Minnesota. With sterile techniques, bacterial inocula were mixed with 80-ml of methanotrophic nutrient media, as described in Table 2-1 (28), and sealed with gray butyl rubber septa in 160-ml serum bottles under an air atmosphere. Using a sterile syringe, 10-ml of methane was injected into the headspace of each bottle. The cultures were shaken at 95 rpm on an orbital shaker in a 20°C environmental chamber. These serum

bottles contained the stock cultures for all subsequent experiments. Methane and oxygen were periodically injected to maintain the live cultures.

Table 2-1. Composition of methanotrophic nutrient media

Stock Label (amount used)	Compound	Stock Conc. (mg/L)
F1 (5ml/L)	$MgSO_4 \cdot 7H_2O$	40,000
	KCl	3,000
F2 (5ml/L)	$FeSO_4 \cdot 7H_2O$	600
	$CaCl_2 \cdot 2H_2O$	4,000
F3 (0.1ml/L)	$CoCl_2 \cdot 6H_2O$	1,000
	$NiCl_2 \cdot 6H_2O$	200
	$MnCl_2 \cdot 4H_2O$	200
	H_3Bo_3	200
	$CuCl_2$	100
	$Na_2MoO_4 \cdot 2H_2O$	300
	$ZnSO_4 \cdot 7H_2O$	700
F4 (10ml/L)	KH_2PO_4	16,000
	Na_2HPO_4	18,400
	$NaNO_3$	100,000

The cultures used in the isotope experiments were prepared by mixing 40-ml of common stock culture with 360-ml of methanotrophic media. Ten of these cultures were mixed then sealed in 1-L serum bottles with 60-ml methane overpressure and placed on the orbital shaker at 20°C for 10 days. Every three days, oxygen was added to remove the vacuum created by gas consumption, and 60-ml additional methane was injected.

Cell numbers were determined by direct counting using microscopy and a petrof-hauser counting chamber.

2.2 t-DCE Degradation Measurements

Degradation experiments with *M. methanica* were performed twice with separate stocks of bacterial cultures, while experiments with *M. trichosporium* were performed once. In order to eliminate growth discrepancies between bottles, for each experiment, the 400-ml cultures grown in the 1-L bottles were mixed together in a sterile 5-L beaker after 10 days of growth. After mixing, a 400-ml aliquot of the culture was poured into each of the ten 1-L bottles for duplicate sampling at the following times: 0 hour, 6 hour, 12 hour, 24 hour, and 48 hour. Control bottles with methanotrophic media and no culture, as well as sodium hydroxide-killed cultures were also prepared. All bottles were sealed with Teflon-lined gray butyl rubber septa, and injected with 60-ml of methane using a sterile syringe. Bacterial culture bottles and control bottles were injected with 20 and 100-µl of t-DCE, respectively. Time 0 samples were taken immediately for concentration measurements while the other bottles were incubated while shaken at 95 rpm in a 20°C environmental chamber.

Trans-DCE concentration in each bottle was measured using a Hewlett Packard Model 5890II gas chromatograph equipped with a 55-m J&W DBVRX capillary column and an electron capture detector. Helium was used as the carrier gas. The instrument was calibrated using a five-point calibration curve. Each time point sample was run with

a control sample, and all samples were run in triplicate. After concentration measurements were taken, 10-ml of 10M sodium hydroxide was injected into each control and culture bottle. Sodium hydroxide served three purposes in this experiment: 1) to kill the bacteria and therefore stop the degradation reaction; 2) to remove excess carbon dioxide in the headspace of the sample bottle; and 3) to remove a degradation product whose concentration increased with time during the degradation experiment. The bottles were then inverted and stored in the 20°C chamber to prevent leakage of t-DCE until $\delta^{13}C$ analysis. Control experiments indicated that storage for one week resulted in no change in the concentration or $\delta^{13}C$ value of the t-DCE.

2.3 t-DCE Stable Carbon Isotope Measurements

Within three days of measuring t-DCE concentrations, and after sodium hydroxide additions, the stored culture and control bottles were sampled for isotopic measurements. Using a 100-ml gas tight syringe, 86-ml of headspace was removed from a sample bottle and injected onto a vacuum line for cryogenic distillation and separation of the t-DCE from the remaining gases. All bottles were overpressured with air or methane (a non-condensable gas) before the headspace sample was removed in order to prevent a vacuum from occurring inside the syringe, which would result in sample loss. Duplicate samples were collected from each of the ten culture bottles. Condensable gases (t-DCE and trace carbon dioxide remaining after NaOH addition) were first collected in a liquid nitrogen trap, and non-condensable gases (methane, oxygen, and nitrogen) were pumped away.

An ethanol/dry ice trap was then used to separate the t-DCE from any remaining carbon dioxide collected in the sample. Using a liquid nitrogen bath, the t-DCE was collected and vacuum sealed in a 20-cm x 6-mm Pyrex sample tube containing 2-g cupric oxide and 0.25-g pure silver wool. To remove trace organics the Pyrex tubes had previously been heated to 550°C for 1 hour and stored in a 105°C oven. The copper oxide served as the oxidant for combustion of t-DCE to form carbon dioxide and water, and the silver wool scavenged chlorine resulting from the combustion (69). The sealed tubes were heated to 550°C for 4 hours in a muffle furnace to convert the t-DCE to carbon dioxide. After combustion, the sample tubes were cracked on a vacuum line and gases were collected in a liquid nitrogen bath. An isopropanol/dry ice bath was used to remove any water in the samples before they were collected in o-ring glass stopcock vials for transfer to the mass spectrometer. Stable isotope measurements were performed using either a Finnigan MAT 251 or 252 isotope ratio mass spectrometer in the U.S. Geological Survey laboratory in Denver.

2.4 t-DCE Degradation Results

During 48 hour incubation, *M. methanica* reduced t-DCE concentrations from 18 mg/L to 4 mg/L, while *M. trichosporium* OB3b reduced concentrations from 15 mg/L to 7 mg/L (data listed in Tables 2-2 and 2-3). Degradation by both organisms ended at ~48 hours of incubation due to cell death, as evidenced by the termination of gas consumption in the sample bottles and by lack of cell movement. First-order reaction rate constants for

Table 2-2. Concentrations and $\delta^{13}C$ values of t-DCE during its degradation by *Methylomonas methanica*, a type I methanotroph

Time (hours)	X (mg/L)	ln[X]	$\delta^{13}C$
0, experiment 1	17.8	2.9	-22.07
0, experiment 1	17.8	2.9	-21.97
0 control, experiment 1	64.8	3.1	-
6, experiment 1	13.4	2.6	-21.19
6, experiment 1	12.7	2.5	-21.02
6 control, experiment 1	65.7	3.1	-
12, experiment 1	11.1	2.4	-20.86
12, experiment 1	11.2	2.4	-20.57
12 control, experiment 1	66.9	3.1	-
24, experiment 1	8.5	2.1	-19.67
24, experiment 1	8.7	2.2	-19.57
24 control, experiment 1	62.7	3.0	-
48, experiment 1	4.2	1.4	-18.81
48, experiment 1	4.0	1.4	-17.22
48 control, experiment 1	66.1	3.1	-
0, experiment 2	15.4	2.7	-21.79
0, experiment 2	15.4	2.7	-21.83
0 control, experiment 2	66.7	3.1	-
24, experiment 2	6.3	1.8	-18.63
24, experiment 2	7.4	2.0	-19.39
24 control, experiment 2	70.2	3.2	-
48, experiment 2	4.4	1.5	-17.09
48, experiment 2	4.6	1.5	-15.61
48 control, experiment 2	68.5	3.1	-

Table 2-3. Concentrations and $\delta^{13}C$ values of t-DCE during its degradation by *Methylosinus trichosporium* OB3b, a type II methanotroph

Time (hours)	X (mg/L)	ln[X]	$\delta^{13}C$
0	15.2	2.7	-21.98
0	15.2	2.7	-21.91
0 control	59.1	3.0	-
6	13.8	2.6	-21.06
6	13.6	2.6	-21.01
6 control	62.2	3.0	-
12	11.2	2.4	-20.09
12	11.2	2.4	-20.22
12 control	63.3	3.0	-
24	9.2	2.2	-19.04
24	9.4	2.2	-18.98
24 control	63.9	3.1	-
48	7.0	1.9	-16.30
48	6.5	1.9	-16.54
48 control	62.7	3.0	-

t-DCE degradation were calculated for both the type I and type II methanotrophs (Figures 2-1 and 2-2, respectively). The corresponding first-order kinetic degradation rate constants are 0.029/hr, 0.025/hr, and 0.016/hr for the type I, experiment 1; type I, experiment 2; and type II cultures, respectively. The respective R^2 values of 0.98, 0.95, and 0.97 for the type 1 experiment 1, type I experiment 2, and type II experiments indicate that t-DCE degradation behaved as first-order kinetics.

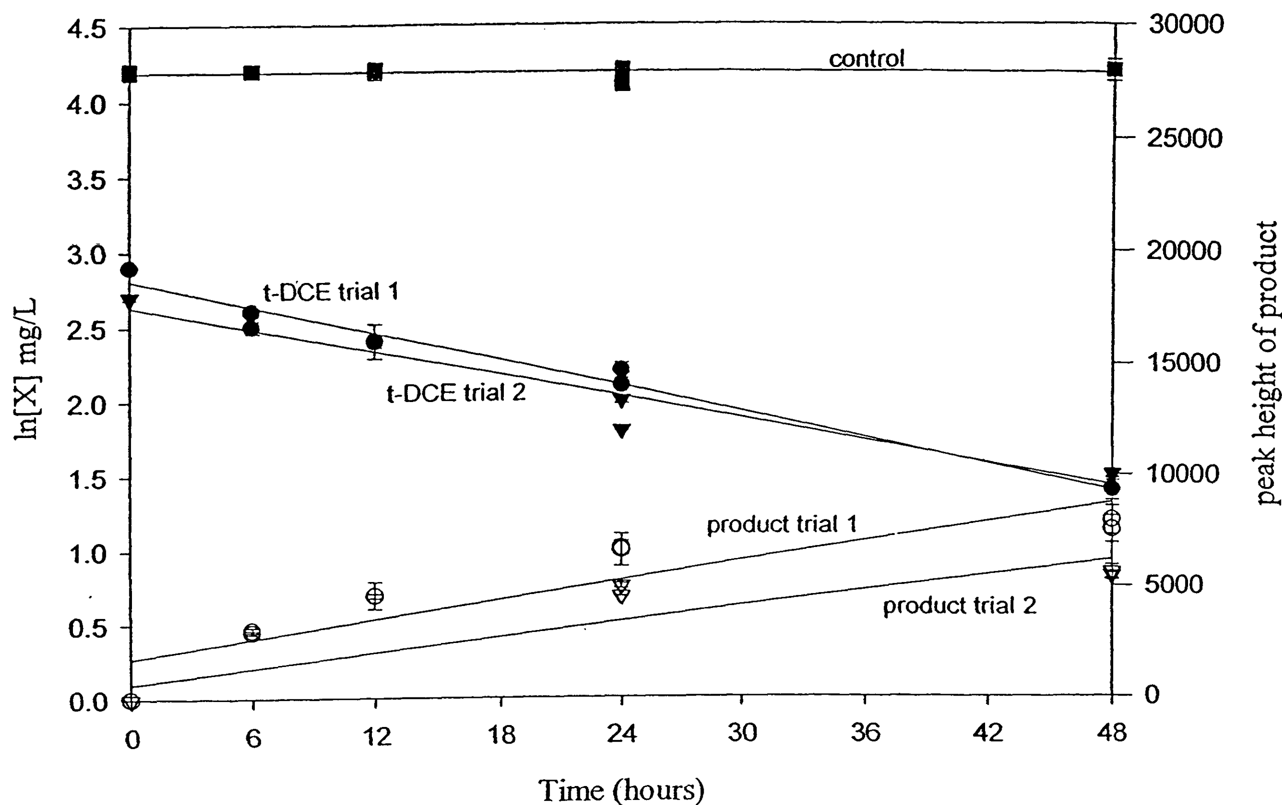


Figure 2-1: First-order reaction rate constants for the degradation of t-DCE by *Methylomonas methanica*, a type I methanotroph, over a 48 hour incubation period, combined with the production of a degradation product. Error bars represent standard deviations of triplicate measurements. Data are represented by the following symbols: ■ control; ● t-DCE trial 1; ▼ t-DCE trial 2; O product trial 1; ∇ product trial 2. The slopes of the lines indicate the rate constants. Linear regressions are as follows:
control: $y = -0.0001x + 4.19$, $R^2 = 0.006$;
t-DCE trial 1: $y = -0.029x + 2.808$, $R^2 = 0.9799$;
t-DCE trial 2: $y = -0.025x + 2.633$, $R^2 = 0.9515$;
product trial 1: $y = 144.87x + 1800.6$, $R^2 = 0.8020$; and
product trial 2: $y = 115x + 667.67$, $R^2 = 0.8478$.

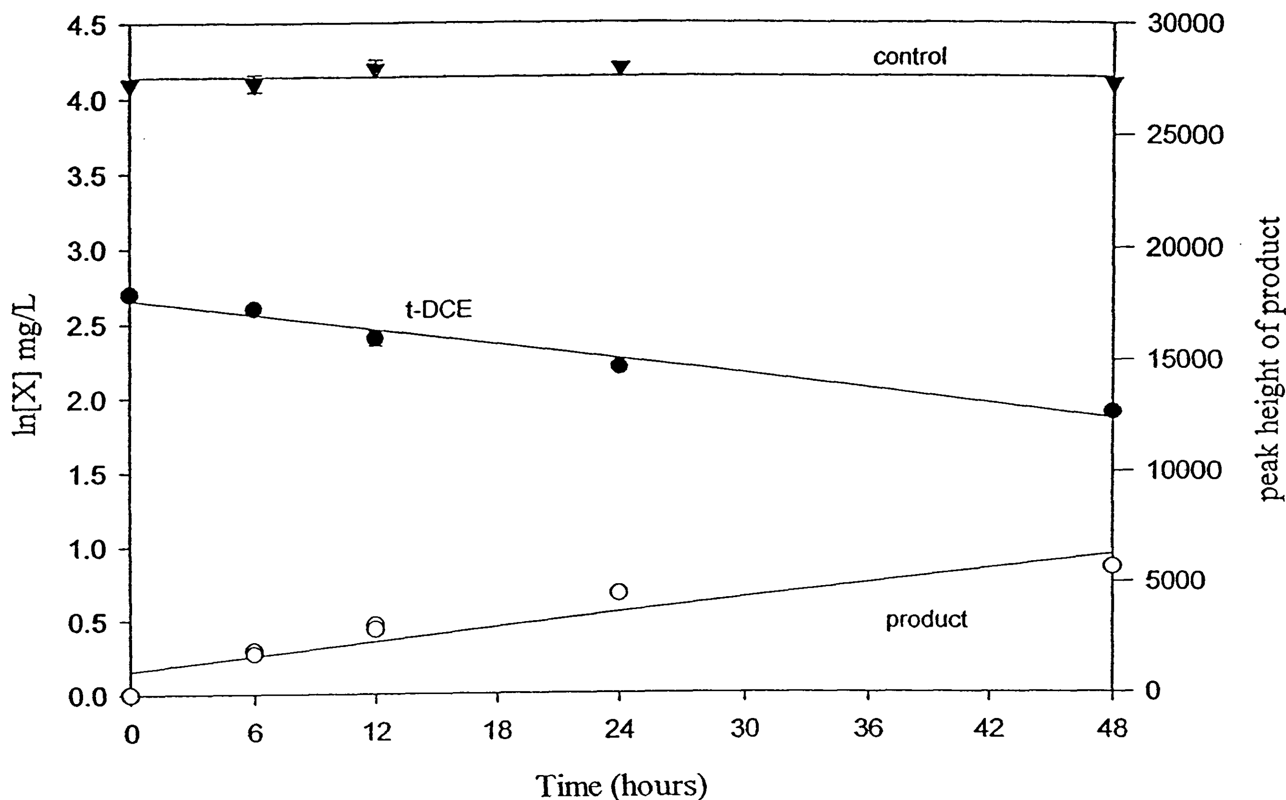


Figure 2-2: First-order reaction rate constants for the degradation of t-DCE by *Methylosinus trichosporium* OB3b, a type II methanotroph, over a 48 hour incubation period, combined with the production of a degradation product. Error bars represent standard deviations of triplicate measurements. Data are represented by the following symbols: ▼ control; ● t-DCE; O product. The slopes of the lines indicate the rate constants. Linear regressions are as follows:
control: $y = 6E\text{-}17x + 4.14$, $R^2 = 5E\text{-}28$;
t-DCE: $y = -0.016x + 2.66$, $R^2 = 0.9709$; and
product: $y = 108.21x + 1061.7$, $R^2 = 0.8651$.

2.4.1 t-DCE Degradation Product

Trans-DCE degradation by both types of methanotrophs produced a compound that could not be identified with certainty, although past research suggests that the compound may be t-DCE epoxide (30-31,48,70,71). The concentration of this compound increased at a fairly constant rate as t-DCE was degraded (Figures 2-1 and 2-2), and stopped when t-DCE degradation ceased. As noted earlier, addition of sodium hydroxide completely removed this compound from the gaseous phase. Further details about this degradation product, and experiments conducted in order to identify and suppress the compound are described in Section 3.0.

2.5 t-DCE Stable Carbon Isotope Composition Results

Replicate analysis (n = 4) of the t-DCE stock solution provided a $\delta^{13}C$ value of -22.0 (± 0.1‰ standard deviation). The $\delta^{13}C$ values and concentrations of the residual t-DCE during degradation by the type I and type II cultures are listed in Tables 2-2 and 2-3 and shown in Figures 2-3 and 2-4. Degradation by both types resulted in an increase in $\delta^{13}C$ over time, indicating that bacterial fractionation of the carbon isotopes occurred.

As indicated in Equation 3, ε is equal to the slope of the line when the change in $\delta^{13}C$ from T_0 to T_{final} ($\Delta^{13}C$) is plotted against ln F. It follows from Equation 3 that linear regression of the experimental data on the plot shown in Figure 2-5 gives the value of the kinetic isotope fractionation (ε) corresponding to each series of experiments (the calculated data used for the plots in Figure 2-5 are provided in Table 2-4). The

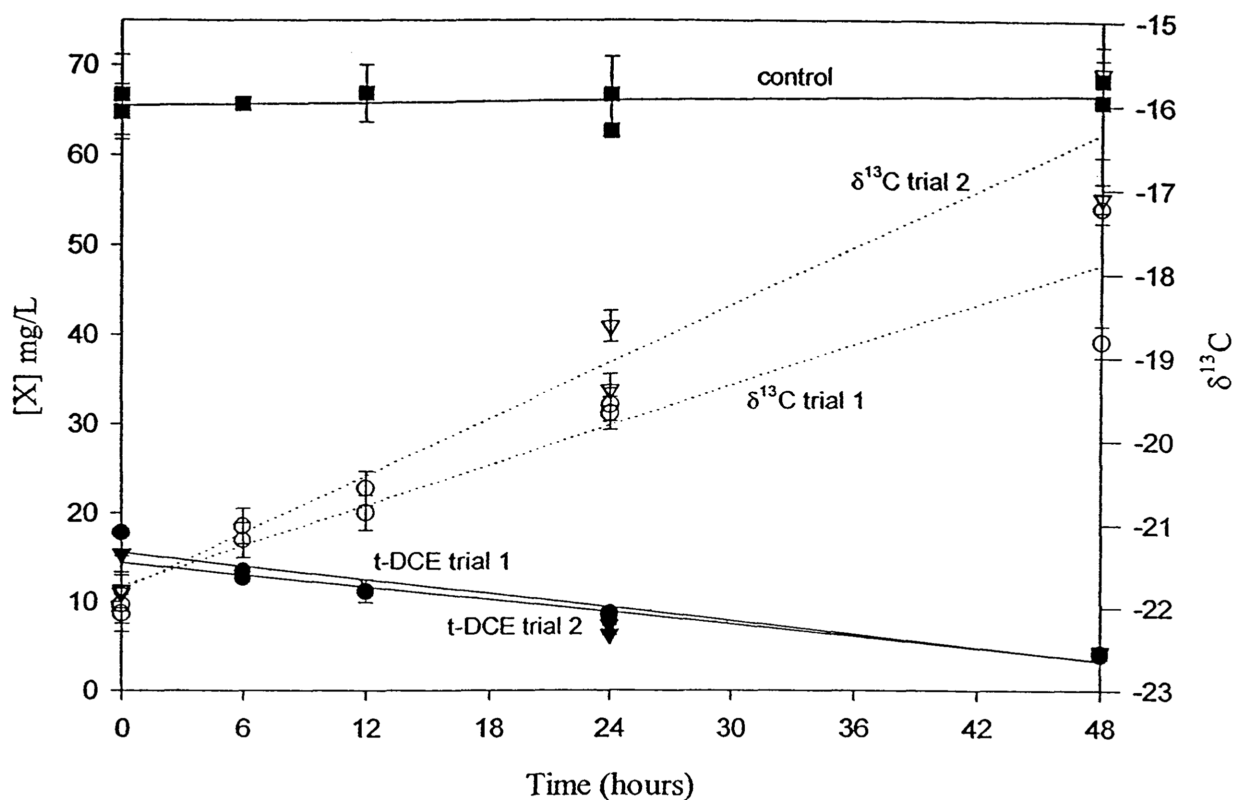


Figure 2-3: The degradation of t-DCE by *Methylomonas methanica*, a type I methanotroph, over a 48 hour incubation period, combined with $\delta^{13}C$ values for the residual t-DCE. Error bars represent standard deviations. Data are represented by the following symbols: ■ control; ● t-DCE trial 1; ▼ t-DCE trial 2; O $\delta^{13}C$ trial 1; ∇ $\delta^{13}C$ trial 2.

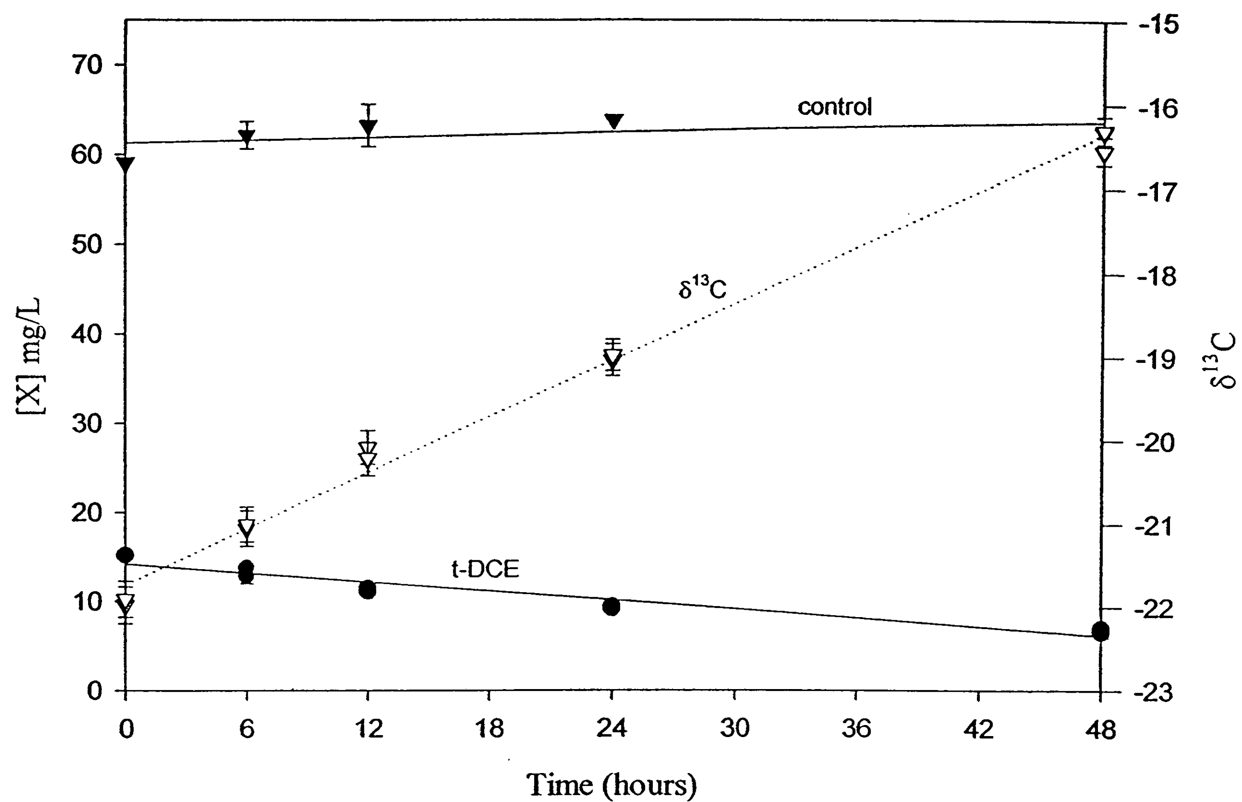


Figure 2-4: The degradation of t-DCE by *Methylosinus trichosporium* OB3b, a type II methanotroph, over a 48 hour incubation period, combined with $\delta^{13}C$ values for the residual t-DCE. Error bars represent standard deviations. Data are represented by the following symbols: ▼ control; ● t-DCE; ∇ $\delta^{13}C$.

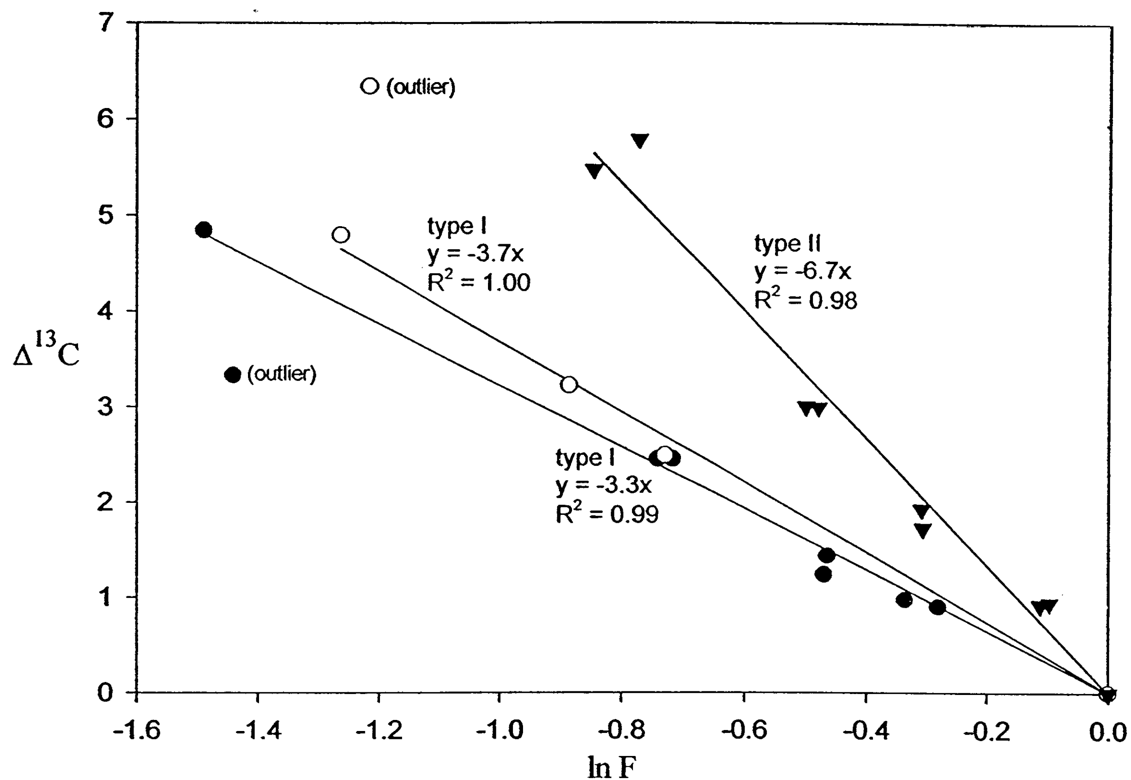


Figure 2-5: Rayleigh model expressing ε for type I and type II methanotrophs. Two outliers have been excluded in determining values for ε. Y-axis values were calculated using Equation 2; however, are represented by the simplified parameter from Equation 3. Data are represented by the following symbols: ● type I trial 1; O type I trial 2; ▼ type II.

Table 2-4. Data for the derivation of ε from laboratory methanotrophic incubation experiments

Cell Type	Time (hours)	ln F	$\Delta^{13}C$
Type I, experiment	0	0	0
	0	0	0
	6	-0.281	0.899
	6	-0.337	0.971
	12	-0.469	1.237
	12	-0.464	1.430
	24	-0.742	2.451
	24	-0.717	2.451
	48	-1.441*	3.328*
	48	-1.490	4.845
Type I, experiment	0	0	0
	0	0	0
	24	-0.887	3.225
	24	-0.729	2.491
	48	-1.263	4.793
	48	-1.217*	6.339*
Type II	0	0	0
	0	0	0
	6	-0.098	0.940
	6	-0.113	0.920
	12	-0.309	1.931
	12	-0.307	1.726
	24	-0.499	3.002
	24	-0.479	2.991
	48	-0.773	5.791
	48	-0.847	5.475

Note: * denotes an outlier

determination of ε allows for easy comparisons between the type I and type II cultures. Figure 2-5 shows that the duplicate experiments with the type I methanotroph resulted in ε values of –3.3 and –3.7‰, which contrast with the value of –6.7‰ estimated for the experiment with the type II methanotroph. Thus, it appears that under identical growth conditions, the two types of methanotrophs have different kinetic isotopic effects.

2.6 Discussion

Based on the results of this study and previous studies involving methanotrophs and stable carbon isotope fractionation of methane (53,55-57), the possibility of monitoring methanotrophic biodegradation of chlorinated solvents (TCE, DCE, and VC) via kinetic isotopic fractionation seems plausible. Previous studies have demonstrated that stable carbon isotope fractionation varies from ~10 to 34‰ during the oxidation of methane by type I and type II methanotrophic bacteria (53,55-57). The fractionation factors resulting from the current study (3.5±0.2‰, and 6.7‰ for type I and type II methanotrophs, respectively) are lower than the range of fractionation values measured for methanotrophic methane oxidation. This could be a result of the response of the MMO enzyme to significant chemical and physical property differences between the t-DCE and methane molecules.

2.6.1 Liquid-vapor Partitioning

The fractionation of ^{13}C appears to be related predominantly to enzyme-mediated effects and not to liquid-vapor partitioning. The measured $\delta^{13}C$ value of –22.3±0.1 for

liquid t-DCE stock solution was consistent with measured $\delta^{13}C$ values of -22.4±0.4 for t-DCE from the headspace of control incubation bottles at concentrations ranging from ~1,200 mg/L to ~12 mg/L. These results are generally consistent with isotopic results for TCE and dichloromethane (DCM) that showed little or no isotopic fractionation associated with evaporation (64,72-74). Based on these results, significant isotopic fractionation does not occur as a result of mass transfer between liquid and vapor.

2.6.2 pMMO and sMMO Enzymes

During investigations of the activity of the sMMO and pMMO, Jahnke et al. (59) observed that the pMMO enzyme (i.e. membrane bound) isotopically fractionates methane to a further extent than the sMMO enzyme. In comparison, Zyakun and Zakharchenko (1998) reported identical maximum carbon isotope enrichment factors of -30.1‰ for methane oxidation by both *M. methanica* and *M. trichosporium* (57) even though the type II methanotroph was expressing the sMMO enzyme. They concluded that the pMMO in cells is associated with a higher intensity of carbon isotope discrimination of methane (57). The results of the current study show a greater carbon isotopic fractionation of t-DCE during its co-oxidation by *M. trichosporium* than by *M. methanica* (Figure 2-5).

The fractionation difference between the two types is not likely to have resulted from the expression of different MMO enzymes since the expression of sMMO under the incubation conditions of the experiments was unlikely, as confirmed by the lack of

response using the naphthalene assay of Brusseau et al. (75) for the detection of the sMMO activity. In addition, sMMO expression has been observed in *M. trichosporium* at 0.3-µmol copper per gram of cells (40), and the level of copper in the current experiment was 0.37-µmol copper per gram of cells.

2.6.3 Kinetic Parameter Effects

Earlier studies have shown that the carbon isotope fractionation accompanying methane oxidation can be affected by a number of kinetic parameters including temperature (53,55-57) and growth phase of the bacteria (60). Coleman et al. (1981) showed that carbon isotope fractionation by a mixed methane-oxidizing consortium was ~13‰ at 11.5°C and ~25‰ at 26°C, contrary to the general rule that kinetic fractionations tend to decrease with increasing temperature (53). Zyakun et al. (1987), using pure cultures of methanotrophs, confirmed the results of Coleman et al. (1981), reporting a greater isotopic fractionation in residual methane and cell biomass at 37°C than at 22°C (56). Zyakun et al. (1998) concluded that the carbon isotope composition of residual methane and cell biomass is controlled by the rate of methane dissolution in the liquid and its active consumption by the bacteria (57), both of which can be sensitive to temperature. Throughout the current experiments with *M. methanica* and *M. trichosporium*, cultures were maintained at 20°C to eliminate such differences in fractionation.

Summons et al. (60) reported a maximum fractionation of 30‰ for methane oxidation during the exponential growth phase of *Methylococcus capsulatus* and 16‰ as the cells approached the stationary phase. All cells in the current study were assumed to be in the stationary phase, because cell densities of approximately 4.9 x 10^{10} cells/ml were close to the maximum values measured previously at stationary growth in batch cultures. The isotopic fractionation produced by mixed cultures of methanotrophs in the field would most likely be represented by a composite fractionation factor resulting from variable combinations of cells in different growth stages, varying rates of degradation, and the expression of both sMMO and pMMO enzymes.

2.6.4 Methylotroph Fractionation Comparison

During an aerobic biodegradation experiment, Heraty et al. (1999) used a methylotrophic organism closely related to *Methylobacterium* or *Ochrobactrum* to degrade dichloromethane (DCM) and found the fractionation factor, α, to be 0.9576 (ε = -42.4‰) (64). This value of ε is large relative to the values of –3.5‰ to –6.7‰ that were determined here for methanotrophs. The large fractionation factor could be characteristic of DCM, of the type of bacteria used, or a combination of both. Methylotrophs can metabolize a greater variety of C-1 compounds, including methylamines (47). In contrast, methanotrophs rely primarily on methane for metabolism. Because the specific species of bacteria used by Heraty et al. is unknown, it is difficult to make a direct comparison between their results and the current results using methanotrophs.

2.6.5 Reductive Dehalogenation Fractionation Comparison

A study conducted by Sherwood-Lollar et al. (1999) reported a carbon isotope fractionation factor (α) of 0.9929 (ε = -7.1‰) resulting from reductive dehalogenation of TCE by a mixed anaerobic consortium (65). Another study by Bloom et al. (2000) obtained isotope enrichment factors (ε) of –6.6 and –2.5‰ for a mixed methanogenic culture degrading TCE (66). These results are in relatively close agreement with the ε values of -3.5‰ to -6.7‰ from the current study, suggesting that a similar fractionation of different chlorinated ethenes may exist between anaerobic and aerobic bacterial species. On the other hand, Dayan et al. (1999) reported α values of 0.9747, 0.9914, and 0.9856 (ε = -25.3‰, -8.6‰, -14.4‰) for PCE, TCE and c-DCE, respectively, for abiotic reductive dehalogenation by metallic iron (67). Clearly, $\delta^{13}C$ fractionation of chlorinated ethenes by metallic iron is compound-specific. However, a comparison of ε for TCE degradation by metallic iron and by the anaerobic microorganisms studied by Sherwood-Lollar et al. and Bloom et al. suggests that enrichment factors for chlorinated ethenes may be similar during abiotic and biotic degradation conditions. The results of these four studies demonstrate that more data are needed to make an accurate comparison between $\delta^{13}C$ fractionation during biotic and abiotic processes.

2.7 Field Application Example

The relatively large and reproducible isotopic fractionation of carbon by methanotrophic degradation of t-DCE, and by biotic and abiotic degradation of other

CAHs through reductive dehalogenation, may provide a means to monitor the extent of (bio)degradation occurring in a wide variety of engineered or natural field settings. Figure 2-6 presents a simplified, hypothetical example of the application of the current results to a field site contaminated with t-DCE that is subsequently degraded by methanotrophs. Using Equation 3 and the measured values of ε, estimates of F (the extent of degradation) can be obtained by measuring the changes in $\delta^{13}C$ of the residual contaminant as it is degraded. The initial $\delta^{13}C$ measurement of a contaminant is taken near the source of a contaminated plume, while final $\delta^{13}C$ measurements are taken from down-plume locations. Measuring the actual initial $\delta^{13}C$ of the original manufactured compound would be difficult to confirm. Instead, the change in contaminant $\delta^{13}C$ between source areas and down-plume locations are measured, and from this, F, the extent of degradation, can be estimated from measured values of ε. Therefore, F reflects changes in contaminant concentration within a plume due to (bio)degradation and is not a measure of total loss, which could include dilution, sorption, and vaporization. The range of ε values calculated for type I and II methanotrophs provides a range of estimates of F for methanotrophic systems degrading t-DCE. Figure 2-6 displays the variability in the estimated value of F for systems with combinations of type I and type II methanotrophs.

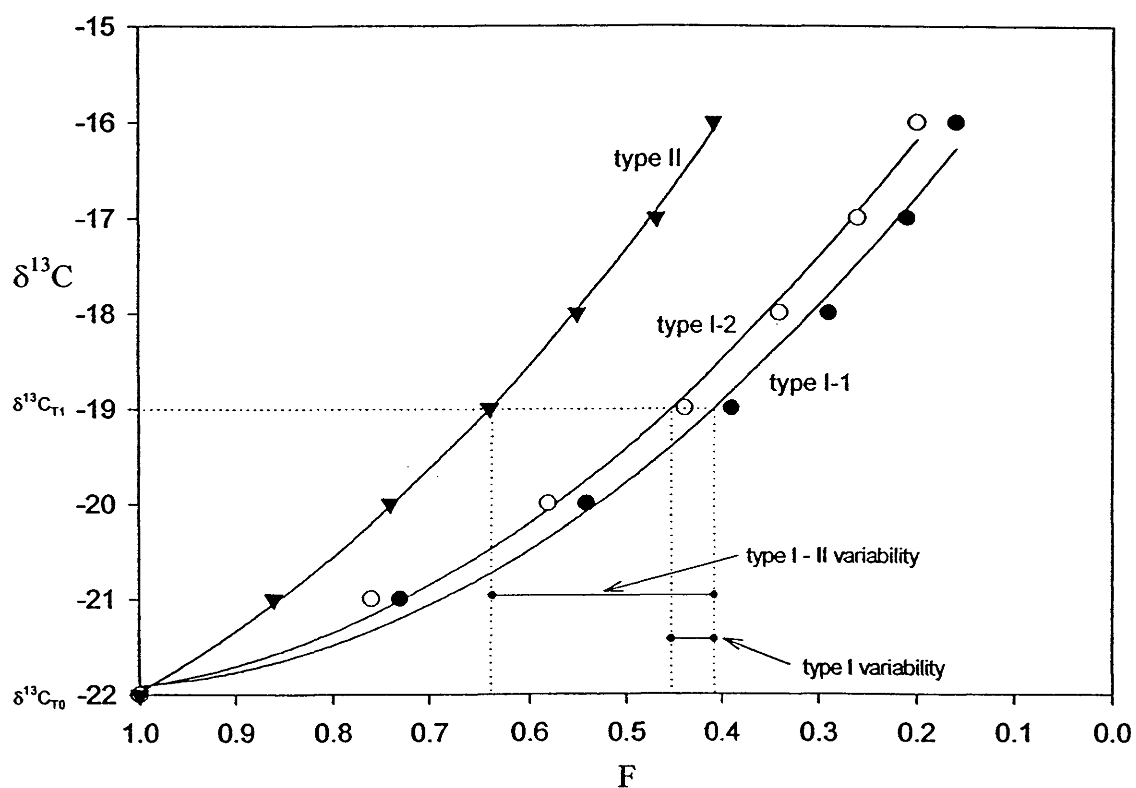


Figure 2-6: An example showing the use of methanotrophic t-DCE degradation and $\delta^{13}C$ fractionation effects (ε) to monitor the extent of biodegradation occurring in field settings. Data are represented by the following symbols: ● type I trial 1; O type I trial 2; ▼ type II.

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At a final $\delta^{13}C$ of –19‰, the variability between systems with a combination of type I and type II methanotrophs is larger (± 1.7 variance in ε corresponds to a ± 0.115 variance in F) than the variability within a system with only type I methanotrophs (± 0.2 variance in ε corresponds to a ± 0.025 variance in F) based on the results of the current study. More specifically, a final $\delta^{13}C$ of –19‰ would correspond to 41 to 46% of the initial reactant remaining in a system dominated by type I, whereas 63% of the initial reactant would remain in a system dominated by type II methanotrophs. Thus, estimates of F will vary depending on the relative amounts of the different types of methanotrophs that exhibit different ε values in a system.

Section 3.0

IDENTIFICATION OF THE *trans* 1,2-DCE DEGRADATION PRODUCT

Experiments were conducted to identify the chemical produced during *trans*-1,2-DCE degradation by mixed and pure cultures of methanotrophs, and to remove this degradation product from the gas headspace prior to $\delta^{13}C$ analyses. The following section provides a description of the materials and methods that were used, the results of experimentation, and a discussion of the results.

3.1 Materials and Methods

Initially, a mixed culture of methanotrophic bacteria was enriched from a landfill soil sample collected at the Rooney Road Landfill near Golden, Colorado. Using aseptic techniques, approximately 3-g of landfill soil was mixed with 80-ml methanotrophic nutrient media (refer to Table 2-1) and sealed with gray butyl rubber septa in 160-ml serum bottles under an air atmosphere. Using a sterile syringe, 10-ml of methane was injected into the headspace of each bottle. These stock cultures were maintained over time by periodically injecting methane and oxygen, and transferring 10-ml of the broth enrichment culture to 160-ml serum bottles containing 80-ml of fresh media.

Using sterile technique, 35-ml aliquots of the methanotrophic enrichment culture were transferred to 65-ml bottles and sealed with Teflon-lined gray butyl rubber septa

under an air and methane atmosphere. The bacterial culture bottles were injected with 13-µl of t-DCE and shaken at 95 rpm on an orbital shaker in a 20°C environmental chamber for 48 hours. From gas headspace samples, concentration measurements of t-DCE were made with a Perkin Elmer Auto System gas chromatograph (GC) with an electron capture detector and a J&W Scientific DB-VRX, 60-m, 0.25-mm inner diameter column. GC analysis confirmed that degradation of t-DCE had occurred and that there was a volatile t-DCE degradation product in the headspace.

As a first step for identifying the degradation product, a culture bottle that had been incubated with t-DCE for two days was taken to Quanterra Environmental Laboratories in Arvada, Colorado for mass spectral analysis. The bottle was stored for four days before analysis in a 40°F chamber as an attempt to keep a significant proportion of the unknown volatile compound in the liquid phase. Measurements to identify the compound were taken after injecting a 1-ml sample of the liquid culture into a Hewlett-Packard 5890II gas chromatograph with a Hewlett-Packard 5972 mass spectrometer detector and a J&W Scientific DB-624, 75-m, 0.53-mm inner diameter column via a Tekmar 2000 concentrator purge and trap system. The purge and trap system was also equipped with a Tekmar 2016 auto sampler and a Supelco VOCARB trap.

As an attempt to further characterize the degradation product, different reagents were added to three additional culture bottles that had been incubated with 13-µl t-DCE for approximately 1 hour. After confirming the presence of the degradation product on the Perkin Elmer Auto System gas chromatograph, an injection of approximately 0.1 moles

of sodium azide was added to the first serum bottle, 1-ml of 10M sodium hydroxide to the second bottle, and 1-ml concentrated sulfuric acid to the third bottle. Two additional 200-μl gas headspace samples were measured after injection of the three reagents. After gas headspace measurements were taken, the pH of each of the three solutions was measured.

3.2 Results

A chromatogram produced during GC analysis showed that the retention time of the degradation product peak is 12.5 minutes, while the t-DCE peak retention time is 9.6 minutes (Figure 3-1). This suggests that the unknown compound could have a greater mass than t-DCE. The product produced a peak that was large relative to the t-DCE peak, and steadily increased with time as the t-DCE was degraded, suggesting that the unknown compound is a degradation product of the t-DCE (Figures 2-1 and 2-2).

The mass spectrum produced by the t-DCE degradation product (Figure 3-2) could not be matched with spectra stored in an electronic National Institute of Standards and Technology (NIST) spectra library; however the 112-118 mass peaks suggest that the molecule contains two chlorine atoms (76-77).

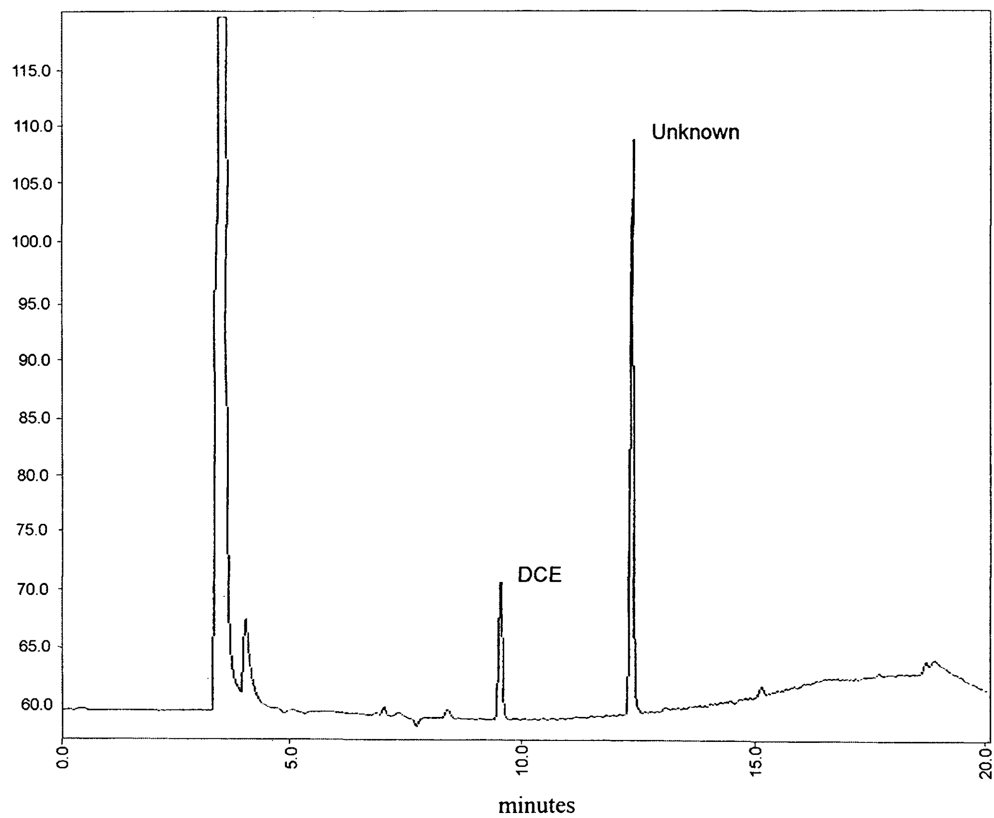

Figure 3-1. Experimental chromatogram for the t-DCE degradation product

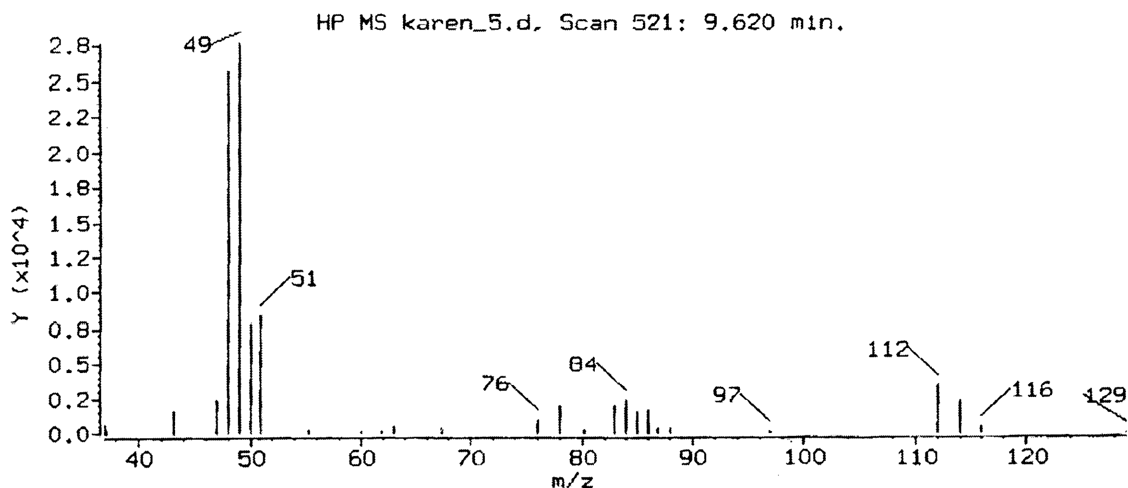

Figure 3-2. Experimental mass spectrum for the t-DCE degradation product

Concentrations of the t-DCE degradation product were not detected in the gas headspace of the culture bottle after sodium azide or sodium hydroxide additions. In contrast, the degradation product was still observed in the headspace after sulfuric acid addition (Figure 3-3). There was no change in t-DCE concentrations after reagent injections for all three experiments. The final pH levels of the three culture bottles sampled were 6.5, 13.5, and 2.0 for the bottles with sodium azide, sodium hydroxide, and sulfuric acid, respectively. Therefore, it appears that the removal of the degradation product from the gas headspace was not a response to extreme pH levels.

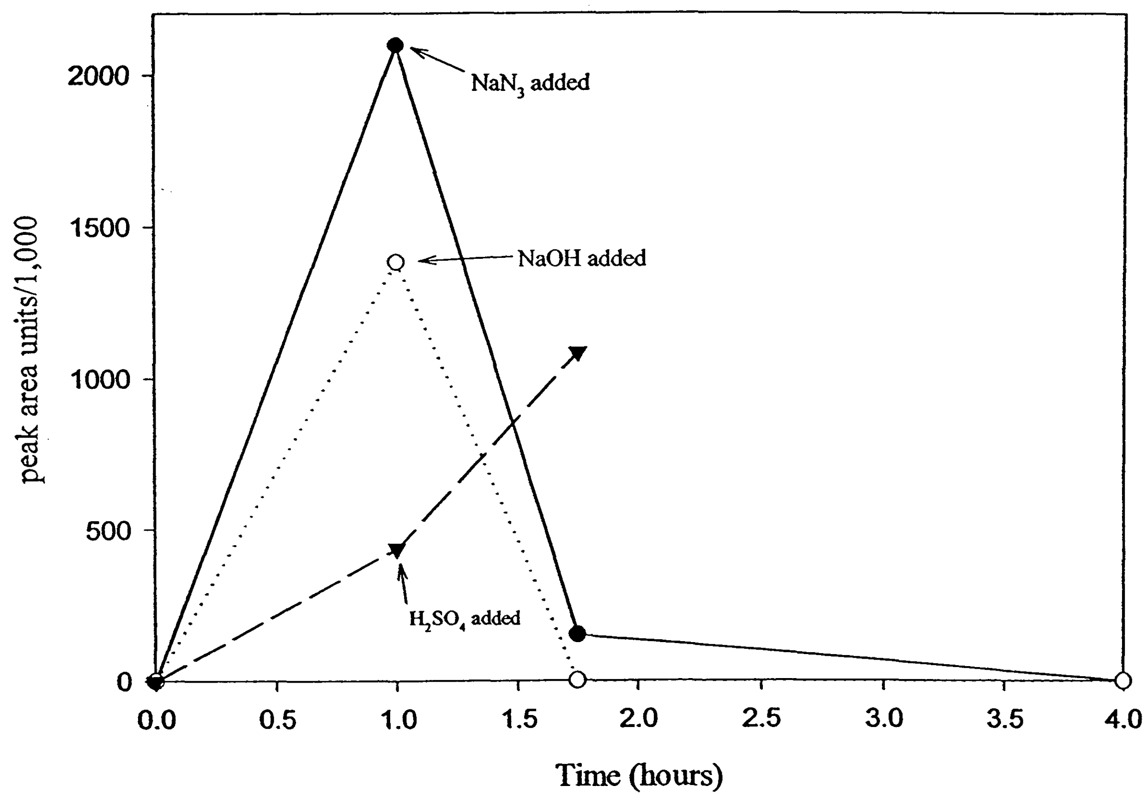

Figure 3-3: Measured values of the t-DCE degradation product after addition of reagents. Data is represented by the following symbols: ● NaN_3; O NaOH addition; ▼ H_2SO_4 addition.

3.4 Discussion

Past research has demonstrated that methanotrophs have the ability to convert ethene to ethene epoxide (70), and that the MMO enzyme catalyzes the epoxidation of alkenes (31,48,71). Results of studies involving the degradation of TCE and t-DCE by methane-oxidizing bacteria show the production of an intermediate volatile degradation product that accumulated during substrate removal (28,43), as was observed in the current study. Based upon a mass spectrometric analysis of the isolated metabolite, Janssen et al. proposed that the intermediate compound formed during degradation of t-DCE by a mixed culture of methanotrophs was *trans*-2,3-dichlorooxirane (t-DCE epoxide) (30). The mass spectrum in Figure 3-2 is consistent with, but does not unambiguously establish the identity of the degradation product in the study as the epoxide (M^+ = 112,114,116) of t-DCE.

CONCLUSION

The overall similarity in measured carbon isotopic fractionation for CAHs such as TCE and t-DCE during degradation by different biotic and abiotic pathways provides a means of estimating the extent of degradation in field studies. This similarity suggests that $\delta^{13}C$ measurements are useful only for monitoring the efficiency of (bio)remediation in a given system, and will not reveal the specific degradation pathways. Identification of the pathways would require additional observations of degradation products, environmental conditions (e.g. Eh and pH), and/or microbial communities. Although it appears that biotic and abiotic isotopic fractionation of CAHs in field settings cannot be discerned at this time, the ability to measure changes in $\delta^{13}C$ values of contaminants as a result of degradation, as demonstrated in this study and others, supports the use of stable isotopic measurements as a means to estimate the extent of on-site contaminant biodegradation and to quantify natural attenuation.

Further research is necessary in order to better compare and understand results derived from biotic and abiotic $\delta^{13}C$ fractionation of CAHs during degradation. Suggested future work involves: 1) the $\delta^{13}C$ fractionation of t-DCE by other biotic (anaerobic) or abiotic processes for comparison to the work performed with methanotrophs; 2) the $\delta^{13}C$ fractionation of other CAHs by methanotrophs; and 3) the

$\delta^{13}C$ fractionation of other CAHs by various biotic (anaerobic and aerobic) and abiotic processes conducted in similar, parallel studies.

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