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Stable carbon isotope fractionation of *trans*-1,2-dichloroethylene during co-metabolic degradation by methanotrophic bacteria

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Abstract

Changes in the carbon isotope ratio (δ^{13}C) of *trans*-1,2-dichloroethylene (*t*-DCE) were measured during its co-metabolic degradation by *Methylomonas methanica*, a type I methanotroph, and *Methylosinus trichosporium* OB3b, 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 co-metabolism, expressed as ε, was measured to be − 3.5‰ for the type I culture and − 6.7‰ for the type II culture. This fractionation effect and subsequent enrichment in the δ^{13}C of the residual *t*-DCE can thus be applied to determine the extent of biodegradation of DCE by these organisms. Based on these results, isotopic fractionation clearly warrants further study, as measured changes in the δ^{13}C values of chlorinated solvents could ultimately be used to monitor the extent of biodegradation in laboratory or field settings where co-metabolism by methanotrophs occurs.
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1. Introduction

Of the 25 most commonly encountered groundwater contaminants at hazardous waste sites, 10 are chlorinated volatile organic compounds (VOCs) (Gillham, 1996), including the chlorinated ethenes trichloroethylene (TCE), dichloroethylene (DCE), and vinyl chloride (VC). Chlorinated ethenes are known or suspected carcinogens (Maltoni and Lefemine, 1974; Miller and Guengerich, 1983). They are readily transported by groundwater and are difficult to reduce to a safe level for human consumption (Gillham, 1996). As a result, they represent a significant health hazard to a large portion of the human population, and therefore require some form of remediation.

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 (Aggarwal et al., 1997). However, in order to determine its effectiveness, a means of accurately tracking and quantifying the biotransformation process must be developed (Aggarwal et al., 1997).

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Wilson and Wilson (1985) were the first to demonstrate that a chlorinated ethene could be degraded aerobically to carbon dioxide in the presence of air and methane. Presumably, addition of the methane and air stimulated a background population of methanotrophic (methane-oxidizing) bacteria that, as subsequent studies have confirmed, degrade various chlorinated organics (Fogel et al., 1986; Little et al., 1988; Janssen et al., 1988; Oldenhuis et al., 1989; Tsien et al., 1989). Methanotrophs degrade chlorinated ethenes without producing harmful degradation products such as vinyl chloride, an end product of biotic reductive dehalogenation. They are classified as type I or type II, depending on their internal cell membrane structures and carbon assimilation pathways (Hanson and Hanson, 1996). Methanotrophs are widespread in both terrestrial and aquatic environments (Hanson and Hanson, 1996). Their ubiquity combined with the ability to degrade various contaminants suggests that methanotrophs could be an important component of natural attenuation or bioremediation.

Methanotrophic bacteria degrade chlorinated organics through co-metabolic processes that use the methane monooxygenase enzyme (MMO) (Colby et al., 1977; Oldenhuis et al., 1989; Fox et al., 1990; Lontoth and Semrau, 1998). The MMO enzyme catalyzes the oxidation of methane to methanol during the first step of methane metabolism. All methanotrophs express a particulate MMO (pMMO; membrane-bound) enzyme under copper-sufficient conditions. In addition, numerous type II methanotrophs (and a type I *Methylococcus* species) contain both a pMMO and a soluble MMO (sMMO; cytoplasmic) enzyme, the latter generally expressed when available copper is limiting (Murrell et al., 1998). The sMMO has a broad substrate specificity and is therefore able to oxidize or dehalogenate a variety of organic contaminants; the pMMO has a narrower substrate range but will co-oxidize a number of chlorinated aliphatic hydrocarbons (CAHs) (Stirling et al., 1979; Burrows et al., 1984; DiSpirito et al., 1992; Anderson and McCarty, 1997). It has been proposed that chlorinated solvents are initially degraded by MMO enzymes to an unstable epoxide intermediate, which then decomposes abiotically (Little et al., 1988).

During bacterial oxidation of methane, the stable carbon isotope abundances of the substrates can be significantly changed, or ‘‘fractionated’’ through microbial metabolism. For example, during oxidation of methane to carbon dioxide, the carbon dioxide formed instantaneously at any point in time can be depleted in the heavy carbon isotope (^{13}C) relative to the methane source. This effect results from a faster rate of oxidation reaction for methane composed of ^{12}C relative to methane composed of ^{13}C. As the reaction proceeds, the fractionation effect is compounded over time, and the difference in the measured $\delta^{13}C$ value of the reactant methane and product carbon dioxide becomes greater due to the Rayleigh distillation effect as described elsewhere (Mariotti et al., 1981). Studies have reported that the oxidation of methane by methanotrophs has imparted carbon isotopic fractionations by as much as ~ 30‰, with the residual methane enriched in ^{13}C (Coleman et al., 1981; Zyakun et al., 1985, 1987; Zyakun and Zakharchenko, 1998). In addition to fractionation during methane oxidation, the ability of microorganisms to isotopically fractionate a variety of substrates has been observed at contaminated sites experiencing petroleum hydrocarbon degradation (Aggarwal and Hinchee, 1991; Landmeyer et al., 1996; Conrad et al., 1997), and carbon isotope fractionation of halogenated hydrocarbons during biotic or abiotic degradation has been observed in laboratory experiments (Heraty et al., 1999; Sherwood Lollar et al., 1999; Dayan et al., 1999; Bloom et al., 2000) and in the field (Stehmeier et al., 1999). These studies suggest that stable carbon isotopic measurements may be used as a means for monitoring chlorinated solvent biodegradation based on the measured increase in the $\delta^{13}C$ value of the residual solvent over time as it becomes increasingly degraded.

In this study, we investigated whether carbon isotope fractionation also occurs during co-metabolism of *trans*-1,2-dichloroethylene (*t*-DCE), a common by-product of reductive TCE degradation, by type I and type II methanotrophs. These experiments were designed as a first step toward determining whether this fractionation effect could potentially be used to predict the extent of biodegradation of chlorinated solvents by methanotrophs in field settings. Our results demonstrate that the $\delta^{13}C$ of the residual *t*-DCE can be applied to determine the extent of biodegradation of DCE by these organisms.

Based on these results, isotopic fractionation clearly warrants further study as measured changes in the δ^{13}C values of chlorinated solvents could ultimately be used as a bioremediation monitoring tool in laboratory or field settings.

2. Experimental methods

2.1. Culture of organisms

Pure cultures of *Methylomonas methanica*, Oak Ridge (type I) and *Methylosinus trichosporium* OB3b (type II) were obtained from Dr. Richard Hanson of the University of Minnesota. Bacterial inocula were mixed with 80 ml of methanotrophic nutrient medium (Fogel et al., 1986) and sealed with a gray butyl rubber septum in 160-ml serum bottles under air atmosphere and sterile conditions. 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.

The cultures used in the isotope experiments were prepared by mixing 40 ml of common stock culture with 360 ml of the methanotrophic medium. The mixtures were then sealed in ten 1-l bottles with 60-ml methane overpressure and placed on the orbital shaker at 20 °C for 10 days. Every 3 days, oxygen was added as needed to restore that lost 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 experiments

Degradation experiments with *M. methanica* were performed twice with separate stocks of bacterial cultures, while experiments with *M. trichosporium* were performed once. For each experiment, cultures grown in the 1-l bottles were mixed together in a sterile 5-l beaker after 10 days of growth, in order to eliminate growth discrepancies between bottles. After mixing, a 400-ml aliquot of the culture was poured into each of the ten 1-l bottles (including duplicates) to be used for sampling duplicate cultures at the following times: 0, 6, 12, 24, and 48 h. Control bottles with methanotrophic media and no culture were also prepared. Previous control experiments indicated there was no change in *t*-DCE concentrations in control samples with sodium hydroxide-killed culture (see following) or with no culture (data not shown). All bottles were sealed with a Teflon-lined gray butyl rubber stopper, and injected with 60 ml of methane using a sterile syringe. Bacterial culture bottles and control bottles were injected with $\sim$20 μl of *t*-DCE. Time 0 samples were taken immediately in duplicate for concentration measurements while the other bottles were incubated while shaken at 95 rpm in the 20 °C chamber.

t-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 10 M 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 sample bottle; and (3) to remove a degradation product that increased at a constant rate as *t*-DCE concentrations decreased during the experiment (presumed to be an epoxide based on preliminary evidence from GC-mass spectrometry). The bottles were then inverted and stored in the 20 °C chamber to prevent leakage of *t*-DCE. Control experiments indicated that storage for 1 week resulted in no change in the δ^{13}C value of the NaOH-amended *t*-DCE samples (data not shown).

2.3. t-DCE stable carbon isotope measurements

Samples for isotopic analysis were collected in 20-cm × 6-mm Pyrex tubes that had previously been heated to 550 °C for 1 h and stored in a 105 °C oven. Each tube contained approximately 2-g cop-

per oxide wire and 0.25-g pure silver wool. 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 (Sofer, 1980).

Within 3 days of measuring *t*-DCE concentrations, the stored culture bottles were sampled for isotopic compositions. Using a 100-ml gastight 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. Condensable gases (*t*-DCE and trace carbon dioxide) 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. Finally, the *t*-DCE was collected in a sample tube containing the copper and silver wool, using a liquid nitrogen bath. The tube was then sealed and removed from the vacuum line. Duplicate samples were collected from each culture bottle. The sealed tubes were heated to 550 °C for 4 h 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 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. Results are expressed using the standard δ notation relative to the Peedee Belemnite (PDB) carbonate standard as follows:

$$\delta(‰) = (R_x/R_{std} - 1) \times 1000 \quad (1)$$

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 PDB standard. Replicate analysis ($n=4$) of the *t*-DCE stock solution provided a $\delta^{13}C$ value of -22.0 ± 0.1‰.

3. Results

3.1. Degradation of t-DCE by M. methanica and M. trichosporium OB3b

Comparable rates of *t*-DCE degradation were observed for both the type I and type II methanotrophs (Fig. 1a and b, respectively). During 48-h incubation, *M. methanica* reduced *t*-DCE concentrations from ~ 17 to ~ 5 mg/l, while *M. trichosporium* OB3b reduced concentrations from ~ 15 to ~ 7 mg/l. The corresponding first-order reaction rate constants were 0.65 day^{-1} ($r^2=0.96$) for the type I culture and 0.39 day^{-1} ($r^2=0.98$) for the type II culture. Degradation by both organisms ended at ~ 48 h of incubation due to cell death, as evidenced by the termination of gas consumption in the sample bottles and by lack of cell movement as determined by microscopy.

3.2. Stable carbon isotopes

The $\delta^{13}C$ values and concentrations of the residual *t*-DCE during degradation by the type I and type II cultures are shown in Fig. 1a and b. Degradation by both types resulted in an increase in $\delta^{13}C$ over time, indicating that bacterial fractionation of the carbon isotopes occurred.

For reactions that behave as first order where the isotope fractionation is controlled by a single step in the reaction sequence, and the reaction is unidirectional, the kinetic isotopic fractionation factor can be calculated using the Rayleigh model as derived by Mariotti et al. (1981):

$$10^3 \ln \frac{10^{-3}\delta^{13}C_{T_1} + 1}{10^{-3}\delta^{13}C_{T_0} + 1} = \varepsilon \ln \frac{X_{T_1}}{X_{T_0}} \quad (2)$$

or in simplified form,

$$\delta^{13}C_{T_1} - \delta^{13}C_{T_0} = \Delta^{13}C = \varepsilon \ln \frac{X_{T_1}}{X_{T_0}} \quad (3)$$

where $\delta^{13}C_{T_0}$ and $\delta^{13}C_{T_1}$ are the isotopic composition of the reactant at time $(T)=0$ and T_{final} of the reaction, respectively, and X_{T_0} and X_{T_1} are the concentrations of the reactant at T_0 and T_{final}. The ratio X_{T_1}/X_{T_0} represents F, the fraction of reactant remaining, and is also a measure of the extent of the reaction. ε is the isotopic enrichment factor of the product relative to the sub-

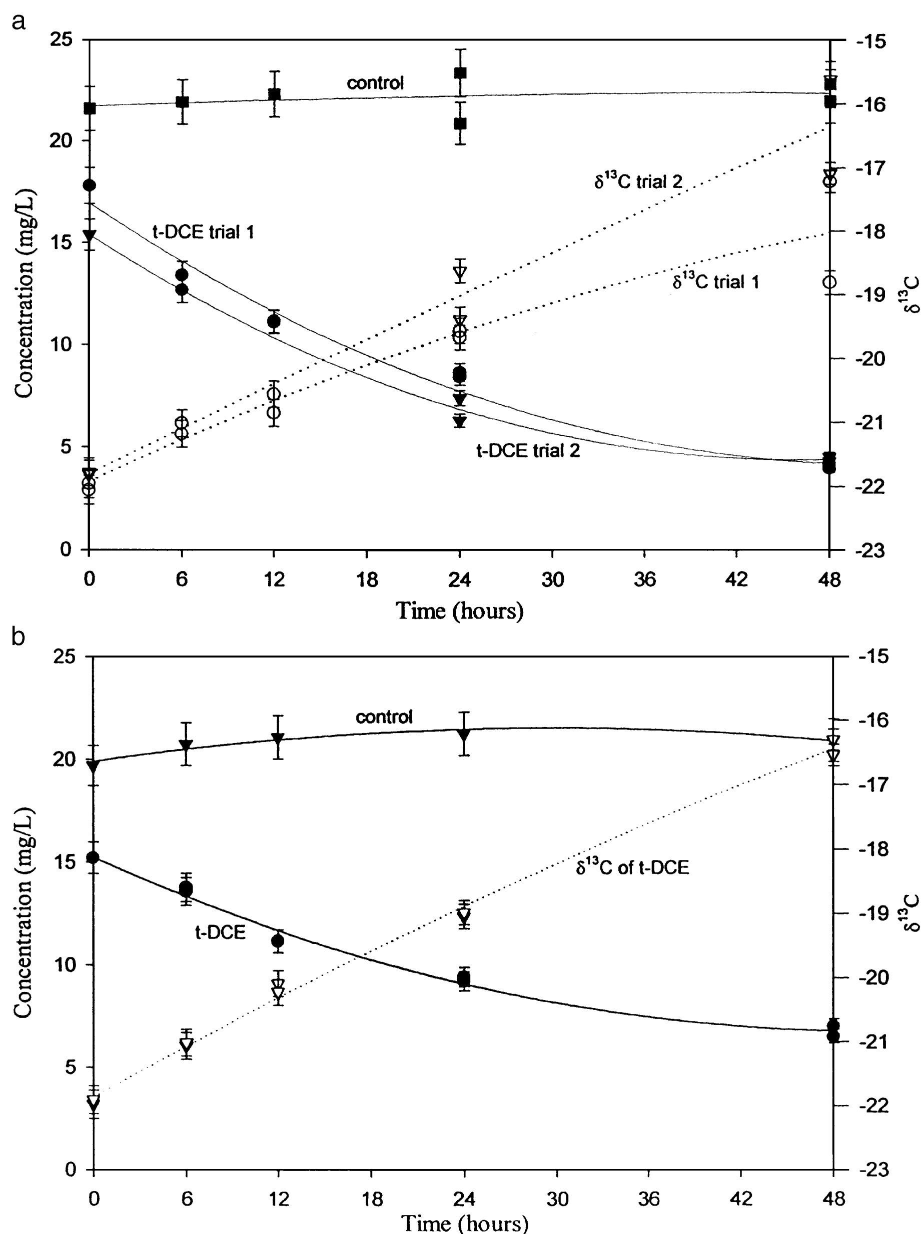


Fig. 1. (a) The degradation of *t*-DCE during two different experimental trials with the same species of *M. methanica*, Oak Ridge (a type I methanotroph) over a 48-h incubation period, combined with δ^{13}C values for the residual *t*-DCE. Each *t*-DCE concentration data point represents the average ± standard deviation of triplicate samples from single incubation bottles. Each bottle was then sacrificed by NaOH addition and subsequently analyzed for the δ^{13}C of *t*-DCE (see text). Data are represented by the following symbols: control [*t*-DCE] (■); trial 1 [*t*-DCE] (●); trial 2 [*t*-DCE] (▾); δ^{13}C trial 1 (○); δ^{13}C trial 2 (▿). (b) As in (a) for the degradation of *t*-DCE by *M. trichosporium* OB3b (a type II methanotroph), over a 48-h incubation period, combined with δ^{13}C values for the residual *t*-DCE. Data are represented by the following symbols: control [*t*-DCE] (▾); culture [*t*-DCE] (●); δ^{13}C (▿).

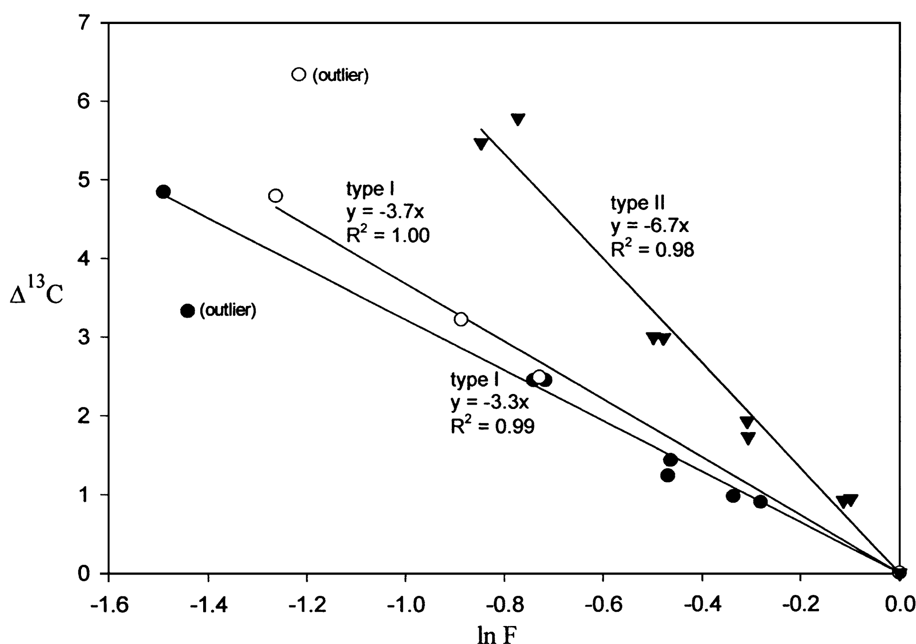


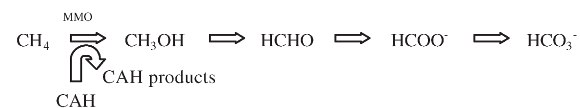
Fig. 2. Rayleigh model expressing ε for type I and type II methanotrophs. Two outliers have been excluded in determining values for ε. *Y*-axis values were calculated using Eq. (2), but are represented by the simplified parameter from Eq. (3). Data are represented by the following symbols: type I trial 1 (●); type I trial 2 (○); type II (▼).

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

It follows from Eq. (3) that linear regression of the experimental data on the plot shown in Fig. 2 gives the value of the enrichment factor (ε) corresponding to each series of experiments. The determination of ε allows for easy comparisons between the type I and type II cultures. Fig. 2 shows that the two sets of experiments with separate stocks of the type I methanotroph resulted in ε values of − 3.3 ‰ and − 3.7 ‰, and an ε value of − 6.7 ‰ for the experiment with type II methanotrophs. Thus, it appears that under identical growth conditions, the two types of methanotrophs have different kinetic isotopic effects.

4. Discussion

Based on the results of this study, and previous studies involving methanotrophs and stable carbon isotope fractionation of methane (Coleman et al., 1981; Zyakun et al., 1985, 1987; Zyakun and Zakharchenko, 1998), the possibility of monitoring methanotrophic biodegradation of chlorinated solvents (TCE, DCE, and VC) via kinetic isotopic fractionation seems plausible. In the metabolic pathway for methane oxidation, CAHs are fortuitously degraded during the first step by the MMO enzyme, as indicated below (Little et al., 1988; Fox et al., 1990):



Previous studies have demonstrated that stable carbon isotope fractionation varies from ~ 10 ‰ to 34 ‰ during the oxidation of methane by type I and II methanotrophic bacteria (Coleman et al., 1981; Zyakun et al., 1985, 1987; Zyakun and Zakharchenko, 1998). The enrichment factors resulting from our current *t*-DCE study (− 3.5 ‰ average of both experimental trials with the type I methanotroph and − 6.7 ‰ for the type II methanotroph) could be lower than the range of fractionation values measured for methanotrophic methane oxidation because of the MMO enzyme

response to significant chemical and physical differences between the *t*-DCE and methane molecules.

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 ~1200 to ~12 mg/l (data not shown). These results are generally consistent with isotopic results for TCE and dichloromethane (DCM) that showed little or no isotopic fractionation associated with evaporation (Huang et al., 1999; Harrington et al., 1999; Poulson and Drever, 1999). Based on these results, isotopic fractionation does not occur as a result of mass transfer between liquid and vapor, and the fractionation observed in this study confirms the lack of mass transfer limitation in the experimental system.

Jahnke et al. (1999) observed that the particulate MMO enzyme (pMMO) (i.e. membrane bound) isotopically fractionates methane to a further extent than the soluble MMO enzyme (sMMO). In comparison, Zyakun and Zakharchenko (1998) reported identical maximum fractionation values of -30.1‰ for methane oxidation by both *M. methanica* and *M. trichosporium* 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. Our results show a difference in the fractionation of *t*-DCE during its co-oxidation by *M. methanica* and *M. trichosporium* (Fig. 2), but a greater fractionation effect for the type II methanotrophs was observed. The fractionation difference between the two types may have resulted from the expression of different MMO enzymes. However, the expression of sMMO by *M. trichosporium* OB3b occurs at a copper to cell ratio of ~0.3 μmol Cu/g cells or less (Tsien et al., 1989). In our experiments, this ratio was as low as 0.37 μmol Cu/g cells. Furthermore, we observed no response in the naphthalene assay of Brusseau et al. (1990) for the detection of the sMMO activity (data not shown).

Earlier studies have shown that the carbon isotope fractionation accompanying methane oxidation can be affected by a number of kinetic parameters including temperature (Coleman et al., 1981; Zyakun et al., 1985, 1987; Zyakun and Zakharchenko, 1998) and growth phase of the bacteria (Summons et al., 1994). To eliminate such differences in fractionation throughout our experiments with *M. methanica* and *M. trichosporium*, cultures were maintained at 20 °C and all cultures were grown in identical media to stationary phase, where measured cell densities of 4.9×10^{10} cells/ml were close to the maximum values measured previously at stationary growth in batch cultures (data not shown).

Because of the technical expertise and facilities required to perform such measurements, few studies have investigated isotopic fractionation of chlorinated solvents. During an aerobic biodegradation experiment, Heraty et al. (1999) used a methylotrophic organism closely related to *Methylobacterium* or *Ochrobactrum* to degrade dichloromethane (DCM) and reported a fractionation factor, α, to be 0.9576 ($\varepsilon = -42.4$). This value of ε is large relative to the values of -3.5‰ to -6.7‰ that we have 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, but not all are able to oxidize methane. In contrast, methanotrophs rely primarily on methane for metabolism. Because the species of bacteria used by Heraty et al. (1999) is unknown, it is difficult to compare their results for methylotrophs with our results for methanotrophs.

A study conducted by Sherwood Lollar et al. (1999) reported a carbon isotope fractionation factor (α) of 0.9929 ($\varepsilon = -7.1$‰) resulting from reductive dehalogenation of TCE by a mixed anaerobic consortium. Similar experiments with a methanogenic enrichment culture provided ε values for carbon isotopes of -2.5‰ to -6.6‰, -14.1‰ to -16.1‰, and -21.5‰ to -26.6‰ during reductive dehalogenation of TCE, *cis*-DCE (*c*-DCE), and VC, respectively (Bloom et al., 2000). In addition, Dayan et al. (1999) showed similar results for abiotic reductive dehalogenation by metallic iron, with fractionation factors (α) of 0.9747, 0.9914, and 0.9856 ($\varepsilon = -25.3$, -8.6, -14.4) for PCE, TCE and *c*-DCE, respectively. A comparison of ε for TCE degradation by metallic iron and by the anaerobic microorganisms suggests that fractionation of TCE may be similar

under abiotic and biotic degradation conditions. However, additional studies are needed to make such comparisons for the daughter products *c*-DCE and *t*-DCE during biotic (anaerobic and aerobic) and abiotic degradation.

The relatively large and reproducible isotopic fractionation of carbon during 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. If one can determine the proper value of the enrichment factor (ε) to be used for a given contaminant in a particular field setting, then the measured increase in the $\delta^{13}C$ of that contaminant over time or space can be directly related to the extent of degradation (see Eq. (3)). However, numerous issues related to this application clearly require further study. For example, extension of this study to other methanotrophic cultures is necessary to verify the broad application of the type-specific fractionation factors. In addition, the isotopic fractionation produced by mixed cultures of methanotrophs in the field would most likely result from variable combinations of cells in different growth stages, which would result in a composite fractionation factor. Some characterization of the methanotrophic community would thus be required for application of this analysis. Finally, CAH fractionation during degradation by other kinds of microorganisms (e.g. methanogens) and by abiotic means must be quantified in order to apply isotope measurement accurately in the field.

Considering the limited data available, carbon isotopic fractionation during degradation of CAHs such as TCE and DCE may ultimately provide a means of reliably estimating extent of degradation in field studies. The similarity in measured fractionation during degradation by different biotic and abiotic pathways suggests that $\delta^{13}C$ measurements may be useful for monitoring the efficiency of (bio)remediation in a given system but may not reveal the specific degradation pathways. Identification of the pathways would require additional observations of degradation products, environmental conditions (i.e. Eh and pH), and/or microbial communities. Although CAH fractionation via various mechanisms clearly requires additional study before field application, the demonstrated ability to measure methanotrophic fractionation of carbon isotopes in a contaminant is a first step toward the use of microbial isotopic fractionation as a means to estimate on-site contaminant biodegradation or to quantify natural attenuation.

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