

Stable Carbon Isotope Fractionation during Aerobic Biodegradation of Chlorinated Ethenes

KUNG-HUI CHU,[†] SHAILY MAHENDRA,[‡]
DONALD L. SONG,[§]
MARK E. CONRAD,^{||} AND
LISA ALVAREZ-COHEN^{*·‡}

Department of Civil and Environmental Engineering,
110B Perkins Hall, University of Tennessee,
Knoxville, Tennessee 37996-2010, Department of
Civil and Environmental Engineering, 726 Davis Hall,
University of California, Berkeley, California 94720-1710,
Brown and Caldwell, 201 North Civic Drive,
Walnut Creek, California 94596, and Center for Isotope
Geochemistry, MS 70A-4418, Lawrence Berkeley National
Laboratory, Berkeley, California 94720

Stable isotope analysis is recognized as a powerful tool for monitoring, assessing, and validating in-situ bioremediation processes. In this study, kinetic carbon isotope fractionation factors (ϵ) associated with the aerobic biodegradation of vinyl chloride (VC), *cis*-1,2-dichloroethylene (cDCE), and trichloroethylene (TCE) were examined. Of the three solvents, the largest fractionation effects were observed for biodegradation of VC. Both metabolic and cometabolic VC degradation were studied using *Mycobacterium aurum* L1 (grown on VC), *Methylosinus trichosporium* OB3b (grown on methane), *Mycobacterium vaccae* JOB5 (grown on propane), and two VC enrichment cultures seeded from contaminated soils of Alameda Point and Travis Air Force Base, CA. *M. aurum* L1 caused the greatest fractionation ($\epsilon = -5.7$) while for the cometabolic cultures, ϵ values ranged from -3.2 to -4.8 . VC fractionation patterns for the enrichment cultures were within the range of those observed for the metabolic and cometabolic cultures ($\epsilon = -4.5$ to -5.5). The fractionation for cometabolic degradation of TCE by *Me. trichosporium* OB3b was low ($\epsilon = -1.1$), while no quantifiable carbon isotopic fractionation was observed during the cometabolic degradation of cDCE. For all three of the tested chlorinated ethenes, isotopic fractionation measured during aerobic degradation was significantly smaller than that reported for anaerobic reductive dechlorination. This study suggests that analysis of compound-specific isotopic fractionation could assist in determining whether aerobic or anaerobic degradation of VC and cDCE predominates in field applications of in-situ bioremediation. In contrast, isotopic fractionation effects associated with metabolic and cometabolic reactions are not sufficiently dissimilar to distinguish these processes in the field.

* Corresponding author e-mail: alvarez@ce.berkeley.edu; phone: (510)643-5969; fax: (510)642-7483.

[†] University of Tennessee.

[‡] University of California, Berkeley.

[§] Brown and Caldwell.

^{||} Lawrence Berkeley National Laboratory.

Introduction

Chlorinated ethenes, including trichloroethene (TCE), *cis*-1,2-dichloroethene (cDCE), and vinyl chloride (VC), are among the most commonly detected subsurface contaminants in the United States (1). TCE and cDCE are suspected carcinogens (2), and VC is a known human carcinogen (3). These compounds can be rapidly oxidized to nontoxic end products (chloride ions and carbon dioxide) by several different oxygenase-expressing cultures. Some of the oxygenase-catalyzed degradations are metabolic, yielding energy and carbon for cell growth, while others are cometabolic, providing no energy to the cells. *Methylosinus trichosporium* OB3b, a well-studied methane-oxidizing strain, exhibits high degradation rates for TCE, cDCE, and VC via cometabolism (4, 5). Similarly, *Mycobacterium vaccae* JOB5 is a propane-oxidizing pure culture capable of degrading TCE, cDCE, and VC cometabolically (6). These organisms are a subset of known cometabolic degraders of chlorinated ethenes. Although no aerobic microorganism capable of using TCE as a growth substrate has yet been reported, a recent study reported an aerobic strain capable of using cDCE as a growth-supporting substrate (7), and VC has been shown to serve as a growth substrate by a variety of aerobic pure cultures (8–10) including *Mycobacterium aurum* L1.

The biodegradability of chlorinated ethenes suggests that natural attenuation and enhanced in-situ bioremediation could be attractive groundwater treatment options. However, successful application of these technologies requires reliable monitoring tools to assess their performance in the field. Compound-specific stable isotope analysis has been recognized as a powerful tool for monitoring, assessing, and validating in-situ bioremediation of chlorinated organics (11–14). The enzymatic degradation of organic compounds can cause significant shifts in the isotope ratio of both reactants and products. This phenomenon occurs because of the stronger molecular bonds (with higher activation energies) formed by the heavier isotope and is referred to as kinetic isotope fractionation (15). Consequently, lighter isotopes are transformed more quickly, resulting in the enrichment of heavy isotopes in the residual reactant.

A number of studies have reported fractionation of stable carbon isotopes during anaerobic biodegradation and abiotic breakdown of chlorinated ethenes; for example, the fractionation factor (ϵ) for TCE ranges from -2.5 to -13.8 for biological dehalogenation (11–14, 16), -6 for reduction by H_2 on palladium catalyst (17), -10 to -19 for reduction by iron (18–20), and -21.6 for oxidation by permanganate (21). Furthermore, a recent study reported a 17 – 20.7% shift in carbon isotope ratio during the aerobic cometabolic degradation of TCE by *Burkholderia cepacia* G4 (22). In contrast to chemical and biological processes, the fractionation caused by physical processes is extremely small (23–27). In this study, we quantify isotopic fractionation during aerobic degradation of three chlorinated ethenes and evaluate whether metabolic and cometabolic degradation reactions result in different fractionation patterns. The specific objectives of this study are (i) to evaluate and compare fractionation patterns resulting from both metabolic and cometabolic biodegradation of VC by a variety of oxygenase-expressing pure and enriched cultures and (ii) to compare isotopic fractionation during aerobic degradation of VC with that of TCE and cDCE.

Materials and Methods

Chemicals. TCE (99%; Fisher Scientific), cDCE (Supelco, Inc., Bellefonte, PA), VC (Sigma-Aldrich, Milwaukee, WI), methane

(Matheson Gas Products), and propane (Matheson Gas Products) were used in this study. Stocks of TCE- and cDCE-saturated water were maintained in vials capped with mininert valves as described by Chu and Alvarez-Cohen (28), while other chemicals were used in pure form.

Bacterial Strains and Culture Conditions. *M. aurum* L1 (ATCC 27199, designated L1) and *Me. trichosporium* OB3b (ATCC 35070, designated OB3b) were obtained from American Type Culture Collection. *M. vaccae* JOB5 (designated JOB5) was kindly provided by Professor Daniel J. Arp, Oregon State University. All cultures were grown at ambient temperature in 500-mL side-armed flasks containing 50 mL of nitrate mineral salts medium as described by Chu and Alvarez-Cohen (28). Copper sulfate (2 μ M) was added to the medium for all cultures except OB3b. Methane and propane (10%, v/v) were supplied in the flask headspace as primary substrates for OB3b and JOB5, respectively. L1 was initially activated with a complex medium recommended by ATCC prior to subculturing in the nitrate mineral salts medium with VC (0.8% v/v) as the primary carbon source. Two mixed cultures were enriched with VC as the sole carbon and energy source. These mixed cultures were seeded with soil from chlorinated ethene-contaminated sites at Alameda Point and Travis Air Force Base, CA. Cultures were enriched in copper-containing medium with VC (0.8% v/v) supplied to the headspace and subcultured three times at a 1:50 ratio to remove residual soil. For all experiments, cell suspensions were harvested during exponential growth (optical density ca. 0.7 at A_{600}). Prior to the beginning of experiments, cell suspensions were purged for 5 min with 300 mL/min nitrogen gas to remove any dissolved gases.

Experimental Approach. Degradation experiments were conducted at ambient temperature in 26-mL vials containing 5 mL of cell suspension. Sodium formate (20 mM) was added to provide reducing energy during cometabolic degradation of TCE, cDCE, and VC by OB3b and JOB5. A total of 150 μ L of TCE-saturated water (1.2 μ mol of TCE), 100 μ L of cDCE-saturated water (0.3 μ mol of cDCE), or 100 μ L of pure VC gas (2.45 μ mol of VC) was injected into vials capped with mininert valves to begin experiments. The aqueous concentrations of chlorinated ethenes in the vials were approximately 100 μ mol/L. Amended vials were vigorously shaken by hand for 30 s prior to the first headspace measurement. Vials were incubated on a benchtop shaker at 150 rpm during the course of experiments. The decrease in concentration of chlorinated solvents in vials was monitored over time; vials were sacrificed for isotopic analysis. Degradation of chlorinated ethenes in vials was stopped at different time intervals for isotopic analysis by adding 25 μ L of concentrated sulfuric acid to lower pH below 2. Acidified vials were then stored at 4 $^{\circ}$ C for later $\delta^{13}\text{C}$ analysis. Experiments using JOB5 were conducted slightly differently; vials were basified by adding 25 μ L of 10 M NaOH and then stored at 4 $^{\circ}$ C for later $\delta^{13}\text{C}$ analysis. Duplicate samples and cell-free controls were used in all experiments. Abiotic loss of chlorinated compounds in controls was consistently less than 5%.

Gas Chromatography. Concentrations of TCE, cDCE, and VC in vials were quantified by 25- μ L headspace injections onto a Hewlett-Packard series II 5890 gas chromatograph (GC) equipped with a splitless injector, flame-ionization detector (FID), and VOCOL capillary column (Supelco Inc., Bellefonte, PA). The temperatures of injector, column, and detector were 220, 150, and 250 $^{\circ}$ C, respectively. Calibration curves were developed as described in Chu and Alvarez-Cohen (29), and Henry's constants (30) were used to determine liquid-phase concentrations of the chlorinated ethenes. All experiments were run in duplicates. Typical ranges of duplicate concentration values were within 5%.

Stable Carbon Isotope Analysis. Compound-specific isotope ratios were determined by direct headspace injection

using a gas chromatograph-combustion-isotope ratio mass spectrometry system (GC-C-IRMS) as described by Song et al. (14). Briefly, the GC-C-IRMS system consists of a Hewlett-Packard 6890 gas chromatograph equipped with a Supelco Supel-Q-Plot capillary column (0.32 mm \times 30 m) for the separation of chlorinated ethenes, a Micromass combustion interface operated at 850 $^{\circ}$ C, and a Micromass Isoprime isotope ratio mass spectrometer (Micromass, Manchester, U.K.). Before injection into the GC-C-IRMS, the gas sample (0.1–0.3 mL) was first cryogenically trapped in a stainless steel loop that was submerged in liquid nitrogen and attached to a six-port valve. The chlorinated ethene was carried by a helium gas stream and trapped on the steel loop. Once the valve was activated and liquid nitrogen was removed, the sample loop was then heated with a heat gun in order to inject the sample into the GC-C-IRMS.

Carbon isotopic ratios, $R = {}^{13}\text{C}/{}^{12}\text{C}$, are expressed in the conventional δ notation and reported in per mil (‰):

$$\delta^{13}\text{C} (\text{‰}) = ((R_{\text{sample}}/R_{\text{standard}}) - 1) \times 1000 \quad (1)$$

where R_{sample} and R_{standard} are carbon isotopic ratios for the sample and the Vienna Pee Dee Belemnite (V-PDB) standard, respectively. The reference CO_2 gas standard for the GC-C-IRMS was calibrated using a dual-inlet mass spectrometer (VG Prism series II). Reference standards for TCE, cDCE, and VC were analyzed as described by Song et al. (14) using both direct headspace injection and off-line combustion and dual-inlet mass spectrometry using a Prism IRMS for comparison to the online system. The uncertainty associated with these measurements is $\pm 0.5\text{‰}$ (2*c*), based on repeated analyses of laboratory standards.

A log-based Rayleigh model for closed systems was used to describe the isotopic shifts during biodegradation in terms of ϵ , an enrichment factor (31):

$$\epsilon \ln(f_R) = 1000 \times \ln\left(\frac{\delta^{13}\text{C}_R + 1000}{\delta^{13}\text{C}_{R_0} + 1000}\right) \quad (2)$$

where f_R is the fraction of reactant remaining at some time during the reaction (given in concentration units as C_R/C_{R_0}), and $\delta^{13}\text{C}_{R_0}$ and $\delta^{13}\text{C}_R$ are the isotopic ratios of the initial and remaining reactant, respectively. At the start of an experiment, $f_R = 1$, and both sides of eq 2 equal zero. Assuming that the enrichment factor is constant throughout the experiment, it can be estimated by plotting the expression on the right side of equation 2 against $\ln(f_R)$ and using a linear regression to solve for the slope, ϵ . The Rayleigh equation is expected to yield a constant enrichment factor in a batch system when the reactant pool is homogeneous and the products are stable (31).

Results

Isotopic Fractionation Caused by the Metabolic and Cometabolic Degradation of VC. *M. aurum* L1 metabolically degrades VC and grows rapidly on VC as a carbon and energy source whereas both *Me. trichosporium* OB3b and *M. vaccae* JOB5 cometabolically degrade VC after growing on methane and propane, respectively. The results of carbon isotope fractionation of VC during metabolic and cometabolic degradation by L1, OB3b, and JOB5 are shown in Figure 1 and Table 1. This figure also shows isotopic fractionation measured during the degradation of VC by two mixed cultures seeded with contaminated soils from Alameda Point and Travis Air Force Base, CA, and enriched on VC. The measured $\delta^{13}\text{C}$ values of VC are plotted against the fraction of VC remaining in batch bottles, C/C_0 . Over 80% of VC was biodegraded by L1 via a metabolic process. In this case, VC fractionated approximately 8‰ (from -27 to -19‰) with

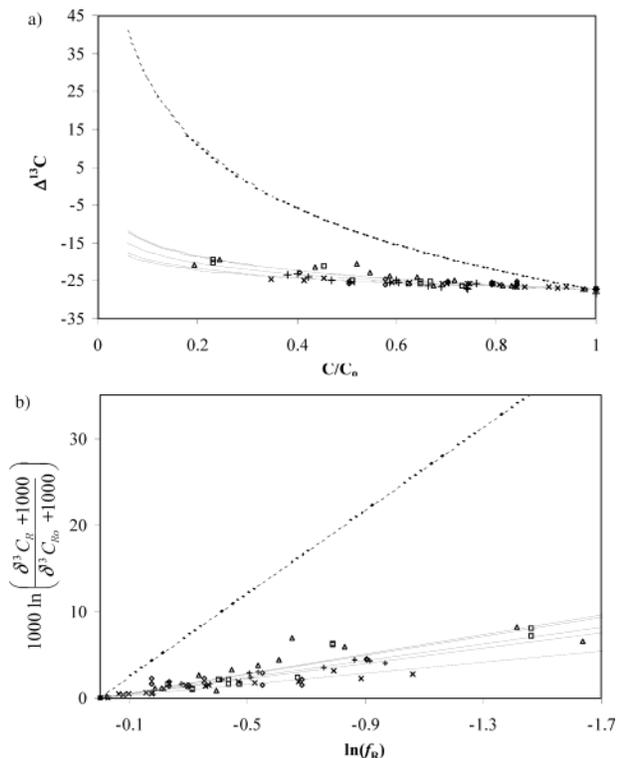


FIGURE 1. (a) Stable carbon isotope fractionations during VC biodegradation by pure cultures (L1 (Δ), OB3b (\times), JOB5 ($+$)) and mixed cultures (Travis (\diamond) and Alameda (\square)). The data points denote $\delta^{13}\text{C}$ values averaged from duplicate samples. In most cases, the error bars delineating the range of data were smaller than the symbol. (b) Rayleigh model of stable carbon isotope fractionation of VC during aerobic biodegradation. Data for the anaerobic line were taken from ref 11.

TABLE 1. Measured Enrichment Factors (ϵ) for Each Pure and Mixed Culture Observed in This Study

compound	culture	ϵ (mean \pm 95% CI) ^a
VC	L1	-5.7 ± 1.1
VC	OB3b	-3.2 ± 0.3
VC	JOB5	-4.8 ± 0.3
VC	Travis	-4.5 ± 1.0
VC	Alameda	-5.5 ± 0.8
TCE	OB3b	-1.1 ± 0.3
cDCE	OB3b	-0.4 ± 0.5

^a Enrichment factor ϵ is calculated based on the Rayleigh model, where ϵ is the slope determined by plotting $\ln f_R$ vs $1000 \times \ln((\delta^{13}\text{C}_R + 1000)/(\delta^{13}\text{C}_0 + 1000))$ for the listed cultures.

an average ϵ value of -5.7 . The 95% confidence interval for ϵ was calculated as -6.8 to -4.5 . Fractionations during cometabolic degradation were smaller. During 65% degradation of VC by OB3b, the fractionation observed was just 3‰ (from -27 to -24 ‰) with a calculated ϵ of -3.2 (95% confidence interval -3.6 to -2.9). JOB5 degraded 62% of total VC in the vials, causing the isotope ratio to shift by 5‰ (from -28 to -23 ‰) with a calculated ϵ of -4.8 (95% confidence interval -5.1 to -4.5).

Fractionation patterns associated with VC degradation by the mixed cultures were within the range of those observed for the metabolizing and cometabolizing pure cultures. The Alameda culture fractionated VC by 8‰ during 77% degradation, with an ϵ of -5.5 (95% confidence interval -6.3 to -4.7), whereas the Travis culture fractionated only 4‰ while degrading 60% of the total VC. The average ϵ for VC degradation by the Travis culture was -4.5 (95% confidence

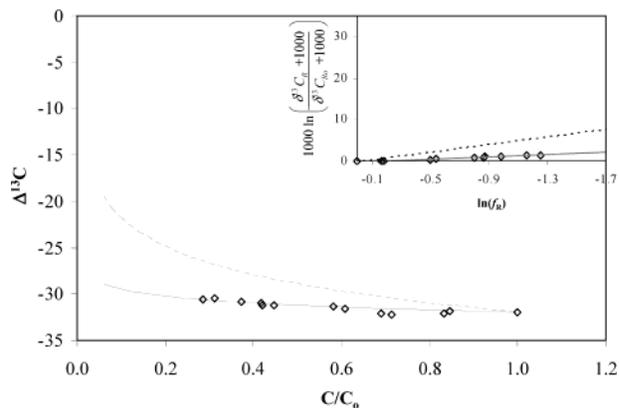


FIGURE 2. Stable carbon isotope fractionation of TCE degraded by OB3b via cometabolic reaction. The data points denote $\delta^{13}\text{C}$ values averaged from duplicate samples. In most cases, the error bars delineating the range of data were smaller than the symbol. The modeled curve represents Rayleigh model determined from all data points. Data for the anaerobic line were taken from ref 11.

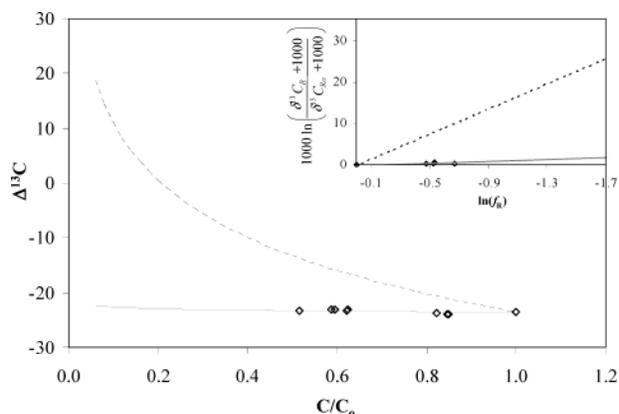


FIGURE 3. Stable carbon isotope fractionation of cDCE degraded by OB3b via cometabolic reaction. The data points denote $\delta^{13}\text{C}$ values averaged from duplicate samples. In most cases, the error bars delineating the range of data were smaller than the symbol. The modeled curve represents Rayleigh model determined from all data points. Data for the anaerobic line were taken from ref 11.

interval -5.5 to -3.5). Also shown in Figure 1 for comparison is the fractionation previously reported for anaerobic reductive dechlorination of VC ($\epsilon = -24$) (11).

Isotopic Fractionation Caused by the Cometabolic Degradation of TCE and cDCE. Since no known aerobic organism is capable of growth on TCE, this study examined the isotopic fractionation caused only by the cometabolic degradation of TCE. Results of TCE degradation by the methanotroph OB3b are presented in Figure 2 and Table 1. Only a small amount of fractionation occurred during this degradation reaction (from -32 ‰ to -30.5 ‰), even when over 70% of total TCE was degraded. The associated enrichment factor was correspondingly low, $\epsilon = -1.1$ (95% confidence interval -1.3 to -0.8). Fractionation caused by the anaerobic reductive dechlorination of TCE (11) is also plotted in Figure 2 for comparison purposes.

The isotopic fractionation of cDCE during cometabolic degradation by OB3b is shown in Figure 3 and Table 1. Because of the lack of fractionation, the fit of the Rayleigh model to the data is poor. The degree of fractionation calculated from these data was small or nonexistent ($\epsilon = -0.4 \pm 0.5$), with all $\delta^{13}\text{C}$ measurements falling within the uncertainty range for the data (± 0.5 ‰). The variability observed in ϵ was large as evidenced by a 95% confidence interval of -1.0 and 0.1 . For this experiment, we were unable

to make any isotope measurements for >50% degradation, significantly limiting our ability to discern a small fractionation factor (e.g., $\epsilon < 2$). Even at that level, however, it is clear that the fractionation due to aerobic degradation of cDCE is considerably smaller than the fractionation associated with anaerobic reductive dechlorination of cDCE ($\epsilon = -15$) (11).

Discussion

This is the first paper to examine the effects of two different degradation mechanisms, cometabolic and metabolic, on compound-specific isotopic fractionation of chlorinated ethenes. In the VC degradation experiments performed with pure cultures, only a small difference in isotopic fractionation patterns was observed between metabolic degradation by L1 (8‰) and cometabolic degradation by OB3b and JOB5 (3–4‰). Consequently, the observation of intermediate isotopic fractionation patterns produced by the VC-degrading mixed cultures was not unexpected (ranging from 4‰ to 8‰), given the likelihood that a variety of organisms and metabolisms would be present in these consortia. Since the enrichments were subcultured with VC as sole carbon and energy source and since cell growth was observed in each subculture, cells capable of metabolizing VC must have been present in these enrichments. VC-utilizing pure cultures were subsequently isolated from these enrichments (8). However, it is possible that cells capable of cometabolizing VC were also present in the enrichments. Due to the similarity of fractionation patterns caused by the metabolic and cometabolic VC degradation reactions, it would be difficult to distinguish between these mechanisms in field applications.

In contrast, the large differences observed in fractionation patterns caused by aerobic and anaerobic degradation reactions for VC and cDCE suggest that isotopic measurements may be useful for distinguishing the dominant redox conditions for their degradation. The estimated enrichment factors for VC and cDCE degradation under aerobic conditions reported here are from 5- to 35-fold smaller than the enrichment factors observed for anaerobic reductive dechlorination (16). This suggests that measurements of isotopic fractionation could be useful for determining whether observed VC and cDCE disappearance at field sites is due to aerobic or anaerobic degradation, facilitating the optimization of bioremediation processes. In addition to carbon isotope data, other independent data such as background geochemical conditions (e.g., dissolved O₂, redox state, sulfate, methane), relative variations of contaminant concentration with other contaminants (e.g., cDCE/TCE) that are not related to differences in solubility, and concentrations of conservative compounds associated with the contaminant (e.g., Cl⁻) are useful in distinguishing between small amounts of anaerobic degradation and a large extent of aerobic degradation observed in the field. Unlike concentration data, isotope ratios are not affected by groundwater transport and other processes. Therefore, the measurement of both carbon and chlorine isotope fractionation will aid in effectively describing the dominant degradation process in the field (32–34). However, from carbon isotope fractionations alone, it is possible to infer when there has been a significant degree of anaerobic degradation. Furthermore, the high sensitivity of compound-specific isotopic measurements suggests that even small degrees of fractionation such as those associated with aerobic TCE degradation may be detectable in the field when the extent of biodegradation is large (e.g., at 85% consumption, the $\delta^{13}\text{C}$ value of TCE will be shifted by 2‰; at 98% consumption, it will be shifted by 4.5‰).

The isotope fractionation reported here for aerobic TCE degradation by *Me. trichosporium* OB3b ($\epsilon = -1.1$) was significantly smaller than that recently reported by Barth et al. for *B. cepacia* G4 (referred as G4 below, $\epsilon = -17$ to -20.7) (22). Interestingly, the values reported for G4 are even more

negative than those reported for microbial reductive dechlorination ($\epsilon = -2.5$ to -13.8) (11, 12, 16, 32) and metallic iron oxidation ($\epsilon = -10$ to -19) (18–20). One major difference between OB3b and G4 is the enzyme responsible for the TCE degradation. While OB3b was expressing a soluble methane monooxygenase (sMMO) in this study, G4 expresses a toluene *o*-monooxygenase (TOM). It is possible that the TOM enzyme has an active site that is more specific to the shape of the TCE molecule, resulting in a stronger preference for the lighter isotope. Measured affinity constants of TCE in whole cell studies support this (e.g., 3–4 μM for G4 expressing TOM (35), whereas 126–145 μM for OB3b expressing sMMO (36, 37)). Barth et al. (22) hypothesized that the larger fractionation observed during aerobic degradation was due to the differences of breaking different bonds; a C–C bond as the first step of TCE oxidation by TOM versus a C–Cl bond during anaerobic dechlorination. However, the small fractionation observed for aerobic TCE degradation in this study suggests that there must be an alternate explanation. Although the sMMO and TOM enzyme systems have similar general mechanisms, these enzymes have different specific catalytic properties. For example, when toluene is oxidized by sMMO, different degradation products are observed (benzyl alcohol and *p*-cresol or *m*-cresol and *p*-cresol), while only *o*-cresol is formed from initial oxidation of toluene by TOM (38). In addition, TOM and sMMO systems are different in their ability to reduce di-iron centers and react with peroxide (38). It is possible that different catalytic characteristics of TOM and sMMO contribute to the differences in the isotope fractionation patterns observed during cometabolic degradation of TCE. It is also possible that similar large variations in fractionation factors may occur for VC and cDCE biodegradation catalyzed by TOM and sMMO. If this occurs, the application of isotopic fractionation for differentiating aerobic from anaerobic degradation mechanisms will be more difficult. Several new aerobic cultures capable of utilizing VC and cDCE have been recently reported (7, 8). Studies using a variety of aerobic organisms will reveal whether large differences in fractionation factors are observed during metabolic and cometabolic degradation of VC and cDCE catalyzed by different enzymes.

This study demonstrates that the aerobic degradation of TCE and VC results in fractionation that can be described by the Rayleigh model, while the fractionation associated with cDCE degradation was too small to adequately model, and that metabolic and cometabolic reactions exhibit similar fractionation patterns. The carbon isotopic fractionations measured during aerobic oxidation of VC and cDCE were significantly smaller than those reported for anaerobic reductive dechlorination reactions, suggesting that isotope fractionation may be useful for distinguishing these degradation processes in field applications.

Acknowledgments

The authors thank Professor Daniel Arp for kindly providing JOB5. This work was funded by grants from the University of California Toxics Research and Training Program, the National Science Foundation Grant BES-0104740, and the National Institute of Environmental Health Sciences Grant P42-ES04705.

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Received for review November 7, 2003. Revised manuscript received March 11, 2004. Accepted March 25, 2004.

ES035238C