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Pathway Dependent Isotopic Fractionation during Aerobic Biodegradation of 1,2-Dichloroethane

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1,2-Dichloroethane (1,2-DCA) is a widespread groundwater contaminant known to be biodegradable under aerobic conditions via enzymatic oxidation or hydrolytic dehalogenation reactions. Current literature reports that stable carbon isotope fractionation of 1,2-DCA during aerobic biodegradation is large and reproducible (−27 to −33‰). In this study, a significant variation in the magnitude of stable carbon isotope fractionation during aerobic biodegradation was observed. Biodegradation in experiments involving microcosms, enrichment cultures, and pure microbial cultures produced a consistent bimodal distribution of enrichment factors (ϵ) with one mean ϵ centered on −3.9 ± 0.6‰ and the other on −29.2 ± 1.9‰. Reevaluation of ϵ in terms of kinetic isotope effects $^{12}k/^{13}k$ gave values of $^{12}k/^{13}k$ = 1.01 and 1.06, which are typical of oxidation and hydrolytic dehalogenation (S_N2) reactions, respectively. The bimodal distribution is therefore consistent with the microbial degradation of 1,2-DCA by two separate enzymatic pathways. This interpretation is further supported in this study by experiments with pure strains of *Xanthobacter autotrophicus* GJ10, *Ancylobacter aquaticus* AD20, and *Pseudomonas* sp. Strain DCA1 for which the enzymatic degradation pathways are well-known. A small fractionation of −3.0‰ was measured for 1,2-DCA degradation by *Pseudomonas* sp. Strain DCA1 (monooxygenase enzyme), while degradation by the hydrolytic dehalogenase enzyme by the other two pure strains was characterized by fractionation of −32.3‰.

Introduction

Chlorinated ethanes are commonly employed as solvents and can become widespread groundwater contaminants due to accidental spills and leaks and improper disposal methods. In particular, 1,2-dichloroethane, an intermediate in plastics production, has contaminated groundwater worldwide, with an estimated 310 000 kg released off-site to air, water, and land in 1999 alone (*2*). The density and solubility of 1,2-dichloroethane (1,2-DCA) are 1.25 g/cm^3 at 20 °C and 8.7 g/L, respectively (*3, 4*). As a dense nonaqueous phase liquid (DNAPL), 1,2-DCA can provide a long-term source of contamination. Moreover, 1,2-DCA is a suspected carcinogen with drinking water standards set by the U.S. EPA at 5 μg/L. The development of reliable and efficient remediation strategies for this EPA priority pollutant is therefore a matter of importance (*5*−*10*).

At some sites, groundwater contaminant concentrations decrease with time or distance as a result of a combination of physical, chemical, and biological attenuation processes. Biodegradation can result in complete mineralization of 1,2-DCA to CO_2. Monitored natural attenuation (MNA) has become appealing over the past decade as an option to manage contaminated sites, provided that the occurrence of intrinsic biodegradation can be unequivocally demonstrated. Traditional monitoring strategies for documenting bioremediation rely on documenting loss of contaminant in the field, the presence of biogeochemical indicators, and direct microbial evidence (*11*). However, it can often be difficult to distinguish between mass loss due to actual degradation or mass loss due to physical processes such as sorption, dissolution, and volatilization. Further, assessing the biodegradation of 1,2-DCA can be particularly challenging as common products of 1,2-DCA degradation, such as CO_2 or ethene, can be generated from many sources (*5*−*10*, *12*−*14*). Much interest is therefore directed at compound specific isotope analysis (CSIA) as a new analytical technique to monitor contaminated sites (*15*−*17*).

Compound specific isotope analysis measures the ratio of heavy to light elements (i.e., $^{13}C/^{12}C$) in a sample versus that of a standard (V-PDB in the case of carbon). Values are reported as δ values in units of permil (‰) using the following equation:

$$\delta^{13}C\ ‰ = 1000[R_{sample}/R_{std} - 1] \quad (1)$$

where $R = {}^{13}C/{}^{12}C$. For chlorinated hydrocarbons, nondegradative processes such as volatilization, dissolution, and sorption have been found to be nonfractionating under equilibrium conditions (i.e., the isotope ratio has been found to remain unchanged within the typical error associated with compound specific isotope analysis (±0.5‰ for $\delta^{13}C$) (*17*−*19*)). In contrast, both biotic and abiotic degradation of chlorinated hydrocarbons has been observed to be mass-discriminating, with an enrichment of the heavy isotopes in the remaining substrate (*19*−*23*). Such isotope fractionation makes CSIA a useful technique to distinguish between concentration decreases due to degradative versus nondegradative processes and therefore demonstrates the occurrence of intrinsic biodegradation in the field.

Stable isotope fractionation (i.e., change in the $^{13}C/^{12}C$ ratio) during biodegradation occurs due to differences in the reaction rates and activation energies of heavy versus light atoms present at a reacting bond, with light isotopic bonds in general reacting more quickly (*24, 25*). During biodegradation of 1,2-DCA, the preferential breakage of the bond containing the lighter isotope (producing an enrichment of the heavy isotope in the remaining substrate), can be described by the Rayleigh equation:

$$R/R_0 = f^{(\alpha-1)} \quad (2)$$

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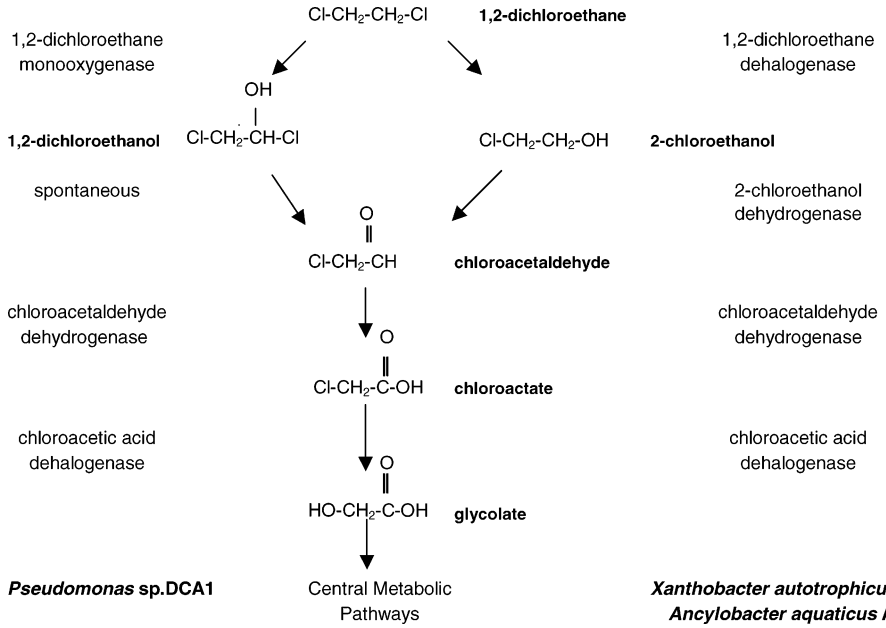

FIGURE 1. Outline of enzymatic pathways for aerobic 1,2-DCA degradation by *X. autotrophicus* GJ10, *A. aquaticus* AD20, and *Pseudomonas* sp. Strain DCA1 (modified from Hage et al. (*5*)).

where R is the isotopic composition ($^{13}C/^{12}C$) of a substrate at any given time with respect to the initial isotopic composition of the substrate (R_0) and the fraction of substrate remaining (f).The fractionation factor α in this equation is a measure of the difference in reaction rates of heavy versus light isotopic molecules and is a constant value throughout the conversion (*26*). Experimentally, the fractionation factor (α) is determined by plotting ln f versus $\ln(R/R_0)$ and determining the slope (m) of the linear regression, where $m = (\alpha - 1)$. The fractionation can also be described by the isotopic enrichment factor, ϵ, where

$$\epsilon = 1000(\alpha - 1)\,[‰] \qquad (3)$$

If in laboratory experiments the enrichment factor, ϵ, is found to be robust and reproducible for a specific transformation reaction, this ϵ value can be used to determine the extent of biotransformation at field sites. Using measured $\delta^{13}C$ values, laboratory derived ϵ, and the Rayleigh equation, the calculated fraction of substrate remaining (f) can then be interpreted as the extent of biotransformation in field samples. This concept has been successfully applied in several field studies investigating both chlorinated aliphatic and petroleum hydrocarbon contamination (*16*, *17*, *30*, *31*).

Since differences in degradation mechanisms can result in different ϵ values, reproducible fractionation is not self-evident for all systems. It has been found that isotope fractionation can vary greatly depending on the type of bond being broken (e.g., C−H vs C−Cl (*32*)), the reaction mechanism (e.g., S_N1 vs S_N2 hydrolysis (*33*)), and the extent of bond cleavage in the transition state. Two pathways have been described for the initial steps in the aerobic biodegradation of 1,2-DCA (*5*−*10*). Theoretically, given the differences in reaction mechanisms between these two pathways (Figure 1), significantly different carbon enrichment factors should be observed in each case. One known degradation pathway, utilized by *Xanthobacter autotrophicus* GJ10 and *Ancylobacter aquaticus* AD20, involves a hydrolytic dehalogenase enzyme. The initial hydrolytic dehalogenation step involves the cleavage of a C−Cl bond (S_N2 reaction), producing 2-chloroethanol (*7*, *10*). The second proposed pathway, utilized by *Pseudomonas* sp. Strain DCA1, is thought to involve a monooxygenase enzyme, resulting in an initial C−H bond cleavage (*5*).

To date, only one study has looked at stable carbon isotope fractionation during aerobic biodegradation of 1,2-DCA. Transformation of 1,2-DCA by *X. autotrophicus* GJ10 was investigated in laboratory experiments, and a reproducible enrichment factor of $\epsilon = -27$ to -33‰ was observed (*1*). The goal of this study was to determine the reproducibility of stable carbon isotope fractionation during aerobic biodegradation of 1,2-DCA using microcosms constructed with aquifer material from two different field sites and enrichment cultures and two pure bacterial strains originating from one of the two field sites. To test whether differences in the initial degradation step can lead to differences in observable fractionation, experiments were conducted with pure cultures of *X. autotrophicus* GJ10 and *A. aquaticus* AD20 (initial hydrolytic dehalogenation reaction) and *Pseudomonas* sp. Strain DCA1 (initial oxidation reaction).

Experimental Procedures

All experiments were conducted using 250 mL glass bottles containing 95 mL of liquid and 155 mL of air and sealed with screw-cap Teflon-lined Mininert caps (Precision Sampling Corp.). Bottles were maintained in a dark fumehood at room temperature on a Thermolyne orbital shaker at 100 rpm. Cultures and controls were amended with 8 μL of 1,2-DCA from the same aliquot of neat 1,2-DCA (Fisher Scientific, 99.8% purity) to produce an aqueous concentration of 100 mg/L prior to all degradation and isotope experiments. Sterile control microcosms were prepared similarly but were autoclaved at 120 °C for 20 min. Water controls contained only 95 mL of autoclaved water.

Microcosm Preparation. Cores and groundwater samples were collected from two aquifers in East and West Louisiana, both highly contaminated with 1,2-DCA. The East Louisiana site was a shallow aquifer at a former landfill and ammonium nitrate impoundment at a chemical manufacturing facility, where 1,2-DCA was the only chlorinated solvent present as a groundwater contaminant. The West Louisiana site was a multilayered aquifer at a former chlorinated solvent disposal facility where a variety of other chlorinated solvents were present in the site groundwater in addition to 1,2-DCA, including trichloroethene (TCE), 1,1,2-trichloroethane (1,1,2-TCA), and chloroform. Microcosms contained approximately 20 g of wet aquifer material. Stable carbon isotope fraction-

ation was measured in batch microcosms from the East Louisiana (EL) (duplicate experiments) and West Louisiana (WL) (duplicate experiments) field sites.

Enrichment and Pure Cultures. Enrichment cultures T1, T2, and T3 were prepared by transferring 5 mL from the East Louisiana microcosms into 95 mL of medium[34] (in mM: 13.00 KH_2PO_4, 12.99 K_2HPO_4, 100.07 NH_4Cl, 3.26 $CaCl_2 \cdot 2H_2O$, 1.01 $FeCl_2 \cdot 4H_2O$, 0.01 H_3BO_3, 0.002 ZnCl, 0.001 $Na_2MoO_4 \cdot 2H_2O$, 0.006 $NiCl_2 \cdot 6H_2O$, 0.01 $MnCl_2 \cdot 4H_2O$, 0.001 $CuCl_2 \cdot 2H_2O$, 0.013 $CoCl_2 \cdot 6H_2O$, 0.0002 Na_2SeO_3, 0.0003 $Al_2(SO_4)_3 \cdot 18\ H_2O$, 0.51 $MgSO_4 \cdot 7H_2O$, and 10 mg/L yeast extract) adjusted to a pH of 7. Isotope fractionation was measured in enrichment cultures T1 and T3 in August 2000. Subsequently, enrichment cultures T1, T2, and T3 were amended with 1,2-DCA and air bimonthly over a period of one year. Isotope fractionation during degradation of 1,2-DCA by these three enrichment cultures was reinvestigated in August 2001. Pure cultures capable of degrading 1,2-DCA were obtained from the enrichment cultures using streak plates prepared with the same medium as stated previously supplemented with agar (1.5%) (*34*). On the basis of 16S rRNA sequences, two distinct organisms have been isolated and designated EL-2 (GenBank Accension Number AY526573) and EL-5 (GenBank Accension Number AY526573) (*34*). El-2 is grouped closest with *Ralstonia* sp. JMP 134 with 96% similarity, and El-5 is grouped closest with *Arthrobacter oxidans* with 93% similarity (*34*). Cultures of *X. autotrophicus* GJ10 (DSMZ 3874) and *A. aquaticus* AD20 (DSMZ 9000) were purchased, and *Pseudomonas* sp. Strain DCA1 was a gift from J. Dijk and A. Stams (Wageningen University). Isotope fractionation during aerobic 1,2-DCA degradation by pure cultures of EL-2, EL-5, *X. autotrophicus* GJ10, *A. aquaticus* AD20, and *Pseudomonas* sp. Strain DCA1 was investigated.

1,2-DCA Concentration Analysis. Samples were collected using a VICI Pressure-Lok gastight syringe (Supelco). For compositional analysis, 500 μL of filtered laboratory air was first injected into the bottle, and then a 500 μL headspace sample was removed to keep the bottles slightly overpressurized with respect to atmospheric pressure. Concentrations of VOCs were measured by injecting 500 μL headspace samples into a Varian 3300 Gas Chromatograph equipped with a FID detector and a 30 m $\times$ 0.53 mm DB-624 column (J&W Scientific). The oven temperature was held at 40 °C for 1 min, increased at 15 °C/min to 120 °C, and held for 5 min. 1,2-DCA concentrations were determined using a three-point calibration curve. Reproducibility on analysis of standards was $\pm$6% (standard deviation).

Stable Carbon Isotope Analysis. Isotope measurements were done by headspace analysis on a GC/C/IRMS (gas chromatograph/combustion/isotope ratio mass spectrometer) after Slater et al. (*21*). The system consisted of a Varian 3300 GC coupled to a Finnigan MAT 252 mass spectrometer via a combustion interface. A 30 m $\times$ 0.32 mm DB-624 column was used, with an oven temperature program of 40 °C for 1 min, increasing at 15 °C/min to 120 °C, and holding for 5 min. Split settings were held constant at 4:1. Samples (300$-$1000 μL) were collected using a VICI Pressure-Lok gastight syringe (Supelco) as in 1,2-DCA concentration analysis and injected onto the GC. Total mass removed during sampling was calculated to be <5 μM and did not have any significant impact on molar or isotopic balances. Total error, incorporating both reproducibility and accuracy, was $\pm$0.5‰ (*17*$-$*19*) as determined by $\delta^{13}C$ analysis of aqueous 1,2-DCA standards over a range of concentrations, signal sizes, and split settings. The mean $\delta^{13}C_0$ in 1,2-DCA of replicate bottles was always within error of the laboratory 1,2-DCA working standard (-27.3 ± 0.5‰) and $\delta^{13}C_{mean}$ of controls remained within $\pm$0.5‰ of $\delta^{13}C_0$ throughout the experiments.

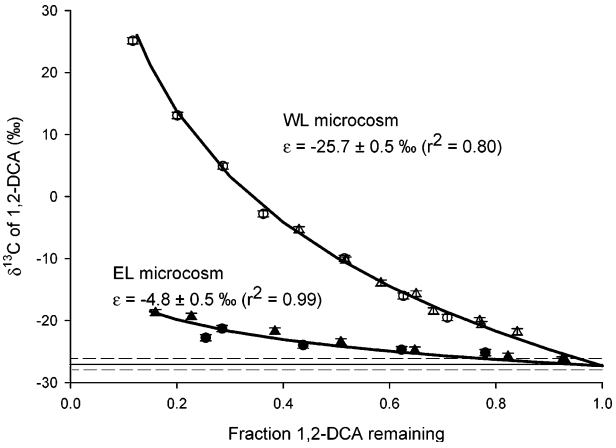

FIGURE 2. $\delta^{13}C$ values for field microcosms. Closed symbols represent replicates for the East Louisiana microcosm. Open symbols represent replicates for the West Louisiana microcosm. Hatched lines represent $\delta^{13}C$ of the initial 1,2-DCA (-27.3 ± 0.5‰). The solid curve represents the Rayleigh fractionation curve based on $\delta^{13}C$ values for the data from each site (see text—eq 2). Rayleigh model agreed well with the data ($r^2 = 0.99$ and 0.80 for EL and WL, respectively). Vertical error bars represent the error (reproducibility and accuracy) of $\pm$0.5‰ associated with $\delta^{13}C$ analysis.

TABLE 1. Table of ϵ Calculated during Aerobic 1,2-DCA Biodegradation Experiments

culture	ϵ (‰)	CI (‰)[a]	r^2
Analysis Yielding Low ϵ			
EL (1)	-4.2	0.5	0.96
EL (2)	-5.3	1.2	0.63
T1 (August 2000)	-3.3	0.5	0.94
T3 (August 2000)	-3.5	0.4	0.98
Pseudomonas sp. Strain DCA1 (*1*)	-3.0	0.2	0.99
mean:	**-3.9**	**0.6**	**0.90**
Analysis Yielding High ϵ			
WL (1)	-25.0	0.6	0.96
WL (2)	-26.2	1.1	0.99
T1 (August 2001)	-23.5	3.0	0.98
T2 (August 2001)	-24.1	2.7	0.98
T3 (August 2001)	-21.5	4.3	0.89
EL-2 (A)	-31.6	1.5	1.00
EL-2 (B)	-30.5	1.0	1.00
EL-2 (C)	-30.4	1.2	1.00
EL-5 (A)	-31.2	1.5	1.00
EL-5 (B)	-32.6	1.9	0.99
EL-5 (C)	-32.3	1.9	1.00
X. autotrophicus GJ10 (*1*)	-31.7	1.4	1.00
X. autotrophicus GJ10 (*2*)	-33.0	2.2	0.99
A. aquaticus AD20 (*1*)	-32.4	1.1	1.00
A. aquaticus AD20 (*2*)	-31.9	2.4	0.99
mean:	**-29.2**	**1.9**	**0.98**

[a] 95% confidence interval.

Results

Field Microcosms. As degradation proceeded in the East Louisiana (EL) microcosm, the $\delta^{13}C$ values of the remaining 1,2-DCA became progressively more enriched, producing isotope values as enriched as -18.7‰ at 0.16 fraction of 1,2-DCA remaining. For the EL microcosm replicates, the mean ϵ value was -4.8 ± 0.5‰ (95% confidence interval) (Figure 2). Values of ϵ for each replicate, EL (1) and EL (2), are in Table 1. In contrast, in the West Louisiana (WL) microcosm, a much stronger enrichment in ^{13}C in the remaining 1,2-DCA was observed (Figure 2). $\delta^{13}C$ values reached positive values up to $+25.1$‰ at 0.12 fraction of 1,2-DCA remaining. The mean ϵ for the WL microcosm replicates was -25.7 ± 0.5‰. Values of ϵ for each replicate, WL (1) and WL (2), are in Table 1.

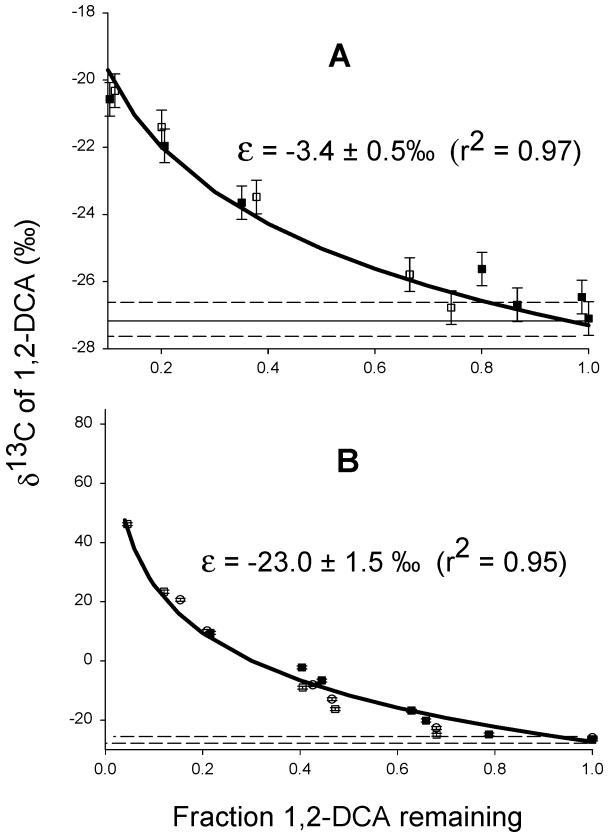

FIGURE 3. δ^{13}C values for 1,2-DCA during biodegradation by enrichment cultures. (A) δ^{13}C values for cultures T1 and T3 (August 2000) (open squares represent T1, and closed squares represent T3). (B) δ^{13}C values for T1, T2, and T3 enrichment cultures (August 2001) (T1 and T3 as in panel A; T2 is represented by open circles). Note differences in *y*-axis scale for plot A vs B. Error bars on all data points are as in Figure 2. Hatched lines represent the δ^{13}C of the initial 1,2-DCA (−27.3 ± 0.5‰). The solid curve represents the Rayleigh fractionation curve based on the mean of all δ^{13}C data for each plot (see text—eq 2). r^2 values of 0.97 (A) and 0.95 (B) indicate the data are consistent with a Rayleigh model. Note: for each panel, the calculated Rayleigh curves are the same within 95% confidence intervals regardless of whether each replicate is plotted separately, and separate curves are calculated or all the replicates are plotted together to calculate one curve.

Enrichment Cultures. In August 2000, stable carbon isotope fractionation was measured during 1,2-DCA degradation by enrichment cultures T1 and T3. The mean enrichment factor (ϵ) for both T1 and T3 was −3.4 ± 0.5‰ (Figure 3A). Isotope fractionation during 1,2-DCA biodegradation by enrichment cultures was reinvestigated in August 2001 (Figure 3B). The mean enrichment value for T1, T2, and T3 of −23.0 ± 1.5‰ (95% confidence interval) is almost an order of magnitude larger than that observed for the same cultures one year earlier. For each sampling date, the ϵ values for each of the replicates are the same within 95% confidence intervals (Table 1). The fact that there was this excellent reproducibility in the ϵ values for replicates in Figure 3A (−3.4 ± 0.5‰) and Figure 3B (−23.0 ± 1.5‰) indicates that the observed change in the magnitude of isotopic fractionation is systematic and not simply due to random variation.

Pure Cultures. Degradation of 1,2-DCA by pure cultures EL-2 and EL-5 (three replicates of each strain) resulted in significant enrichment of ^{13}C in the remaining substrate (Figure 4). Calculated ϵ were within 95% confidence intervals for all three bottles of each strain and between strains (Table 1). Therefore, a mean enrichment factor of −31.4 ± 0.8‰ was calculated for all six bottles (Figure 4).

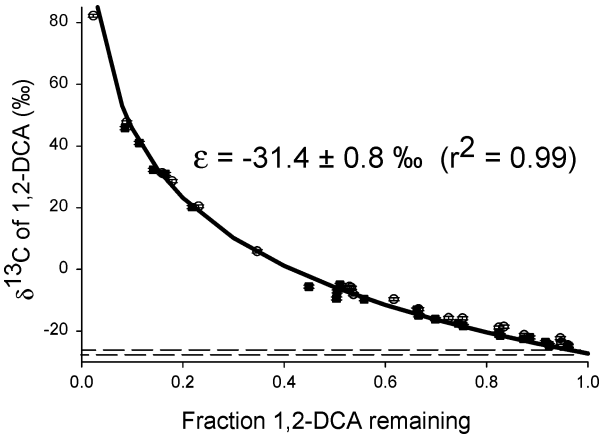

FIGURE 4. δ^{13}C values for 1,2-DCA during biodegradation by pure cultures El-2 and EL-5. Open circles represent EL-2 (triplicate bottles). Closed squares represent EL-5 (triplicate bottles). Error bars on all data points are as in Figure 2. Hatched lines represent the δ^{13}C of the initial 1,2-DCA (−27.3 ± 0.5‰). The solid curve represents the Rayleigh fractionation curve based on the mean of all δ^{13}C data for EL-2 and EL-5 (see text—eq 2). Mean r^2 value of 0.99 indicates the data are consistent with a Rayleigh model.

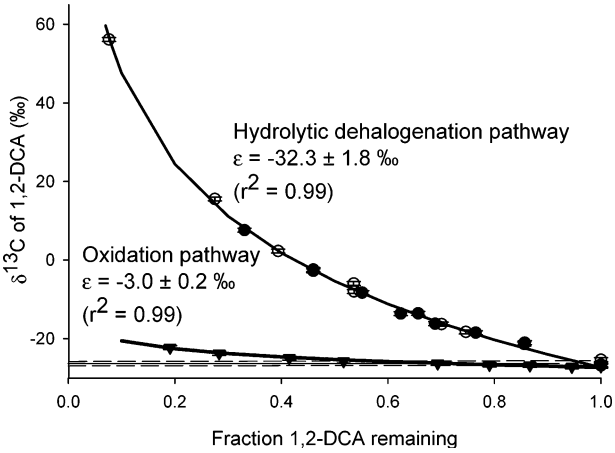

FIGURE 5. δ^{13}C values for 1,2-DCA during biodegradation by *X. autotrophicus* GJ10, *A. aquaticus* AD20, and *Pseudomonas* sp. Strain DCA1. Closed circles represent replicates of *X. autotrophicus* GJ10. Open circles represent replicates of *A. aquaticus* AD20. Closed triangles represent *Pseudomonas* sp. Strain DCA1. Hatched lines represent the δ^{13}C of the initial 1,2-DCA (−27.3 ± 0.5‰). The upper solid curve represents the Rayleigh fractionation curve calculated based on the mean of all δ^{13}C data for pure cultures using the hydrolytic dehalogenation pathway. The lower curve represents the Rayleigh fractionation curve for the pure culture using the oxidation pathway. r^2 values of 0.99 for both curves indicate the data are consistent with a Rayleigh model.

Results of isotope fractionation during 1,2-DCA degradation by pure cultures *X. autotrophicus* GJ10, *A. aquaticus* AD20, and *Pseudomonas* sp. Strain DCA1 are shown in Figure 5. Cultures of *X. autotrophicus* GJ10 and *A. aquaticus* AD20 (both of which degrade 1,2-DCA by a hydrolytic dehalogenase enzyme) yielded enrichment factors of −32.3 ± 1.8‰ and −32.1 ± 1.7‰, respectively. The mean ϵ for these two pure cultures together is −32.3 ± 1.8‰ (Figure 5). *Pseudomonas* sp. Strain DCA1 (monooxygenase enzyme) yielded fractionation an order of magnitude smaller (ϵ = −3.0 ± 0.2‰).

Discussion

Assessing the variability in stable carbon isotope fractionation during biodegradation is essential for both qualitative and quantitative applications of isotope analysis to investigate

TABLE 2. Table of Theoretical and Calculated Kinetic Isotope Effects Based on Mean ϵ Values in Table 1

	theoretical kinetic isotope effect	calculated kinetic isotope effect
hydrolytic dehalogenase reaction	1.04−1.09[a]	1.06
oxidation reaction	1.00−1.03[b]	1.01

[a] Refs *33* and *36*. [b] Refs *32* and *37*.

biodegradation in the field. Stable carbon isotope fractionation observed during aerobic biodegradation of 1,2-DCA varies over an order of magnitude (Table 1). Rather than demonstrating random variation, however, a bimodal distribution was seen with enrichment factors centered around −3.9 and −29.2‰, with average standard deviations of 0.6 and 1.9‰, respectively (Table 1). The distribution of ϵ values observed substantially differs from those of previous studies addressing biodegradation of chlorinated hydrocarbons, where comparatively small differences in fractionation have been reported (*15*, *27*−*29*).

The most intriguing result of this study is the distinct enrichment factors of −32.1 and −3.0‰ that were observed with pure cultures of *X. autotrophicus* GJ10/*A. aquaticus* AD20 (hydrolytic dehalogenase enzyme) and *Pseudomonas* sp. Strain DCA1 (monooxygenase enzyme), respectively. These distinct values provide strong evidence that different degradation pathways control the bimodal distribution of ϵ observed in the various experiments. On the basis of the ϵ values identified for these pure cultures with known degradation pathways, the degradation mechanism operative in the EL and WL microcosms and in the enrichment cultures (T1, T2, T3) can be deduced. This approach can also be used to support an interpretation that a change in the dominant degradation mechanism over time occurred in the enrichment cultures.

Systematic differences in ϵ can be attributed to the fact that light isotopic bonds containing ^{12}C (rate constant ^{12}k) are more readily broken than heavy bonds containing ^{13}C (rate constant ^{13}k). A kinetic isotope effect ($^{12}k/^{13}k$) results, which is often strongly dependent on the reaction mechanism as well as the strength of the bonds being broken or formed. For example, it is well-documented that S_N2 reactions (hydrolytic dehalogenation) are associated with large carbon kinetic isotope effects between $^{12}k/^{13}k = 1.04$ and 1.09 (*33*). Conversely, available evidence indicates that carbon isotope effects are much smaller for aliphatic C−H bond cleavage in oxidation reactions (Table 2) (*32*). Maximum calculated effects would be expected around 1.02 (*32*), and experimentally determined values have also been found to be rather small. Huang et al. (*36*) observed a carbon kinetic isotope effect of 1.00 in the oxidation of propane by soluble and particulate methane monooxygenase from *Methylococcus capsulatus* (Bath), and a reevaluation of data by Morasch et al. (*37*) gives values between 1.02 and 1.03 for oxidative degradation of toluene by *Pseudomonas putida* (*35*).

To interpret the results of this study in the context of kinetic isotope effects, measured enrichment factors ϵ of this study first have to be converted into values of $^{12}k/^{13}k$. In the case of molecules that have only one carbon atom, kinetic isotope effects $^{12}k/^{13}k$ can be directly related to ϵ by the following equation:

$$^{12}k/^{13}k = 1/(1 + \epsilon/1000) \quad (4)$$

where ϵ is the experimentally determined enrichment factor in permil, and $^{12}k/^{13}k$ is the primary kinetic isotope effect of the reaction. Secondary isotope effects (i.e., effects induced by isotopes one or more bonds away from the reactive position) are neglected (*25*, *35*). 1,2-DCA molecules (CH_2-Cl−CH_2Cl) contain two carbon atoms and because of the low natural abundance of ^{13}C, generally only one of them at most will be ^{13}C. Since the reaction occurs only at one of the two carbon atoms, there will be competition between the ^{13}C and the ^{12}C position within a single 1,2-DCA molecule. To correct for this competition, a factor of 2 must be introduced into eq 4 when converting $\epsilon_{(1,2-DCA)}$ to the corresponding kinetic isotope effect

$$^{12}k/^{13}k = 1/(1 + (2\epsilon)/1000) \quad (5)$$

This correction is described in standard textbooks (*25*) and was previously applied to 1,2-DCA isotope data (*1*). If enrichment factors of $\epsilon = -29.2$ and $\epsilon = -3.9$‰ (Table 1) are substituted into eq 5, carbon kinetic isotope effects of 1.06 and 1.01 are obtained, consistent with expected values from the literature for hydrolytic dehalogenation (S_N2) and oxidation, respectively (Table 2). This analysis strongly suggests that different enrichment factors observed in our experiments largely reflect different characteristic primary kinetic isotope effects associated with hydrolytic dehalogenation (S_N2) versus oxidation reactions.

Implications for Field Studies. The results of this study are important for application of compound specific isotope analysis to assess biodegradation at contaminated sites. The consistent bimodal pattern observed during aerobic biodegradation of 1,2-DCA suggests that the fractionation patterns for 1,2-DCA reflect two different types of chemical reactions representing two different degradation pathways. The large enrichment factor around −29.2‰ corresponds to a kinetic isotope effect of 1.06 indicative of an S_N2 reaction such as in the hydrolytic dehalogenase enzyme pathway. Conversely, the ϵ value around −3.9‰ is equivalent to a kinetic isotope effect of 1.01, typical of an oxidation reaction such as in the monooxygenase pathway. This relationship is verified by experiments with pure cultures known to utilize the hydrolytic dehalogenase and monooxygenase enzyme pathways (*X. autotrophicus* GJ10 and *A. aquaticus* AD20, and *Pseudomonas* sp. Strain DCA1, respectively), where observed enrichment factors are equal to predicted values (Table 2). Fractionation does not vary randomly but is representative of different chemical reactions, providing insight into the mechanism of biodegradation that would otherwise be difficult to obtain.

This study also indicates that the appropriate enrichment factor must be known to confidently apply stable isotope analysis to quantify the biodegradation of 1,2-DCA in the field. In the absence of this information, if field samples are analyzed for carbon isotopic ratios and a small ^{13}C-enrichment in the remaining substrate is found, it might be difficult to determine whether 1,2-DCA has been degraded to a small extent in a strongly fractionating reaction (e.g., hydrolytic dehalogenation) or to a large extent in a weakly fractionating reaction (e.g., oxidation). There are two potential solutions to this problem. $\delta^{13}C$ measurements may be made to calculate an enrichment factor for the field samples to compare to laboratory derived ϵ values (*38*, *39*). Alternatively, hydrogen isotope fractionation can be used as well as carbon isotope fractionation to constrain the mechanisms of degradation. This approach was recently successfully applied in a field study addressing MTBE-degradation by Zwank et al. (*40*). As for carbon isotopes, hydrogen isotope fractionation is often characteristic of different degradation pathways. For example, in biodegradation of 1,2-DCA via the hydrolytic dehalogenase pathway (S_N2 reaction), only very small secondary hydrogen isotope effects of around 0.95−1.04 would be expected (*35*, *41*). Conversely, in the monooxygenase pathway (oxidation), a C−H bond would be broken, and a very pronounced

primary effect of 3−8 can be anticipated (*25*, *35*). Using the evaluation scheme proposed by Elsner et al. (*35*), this would result in measurable hydrogen enrichment factors of between −40‰ and slightly positive values in the hydrolytic dehalogenation (S_N2) reaction (calculated from Westerway (*41*)) and between −165 and −220‰ in the oxidative transformation. Therefore, hydrolytic dehalogenation reactions will generally be associated with strong carbon and relatively weak hydrogen isotope fractionation, whereas oxidation reactions should result in strong hydrogen and relatively weak carbon isotope effects. Future studies can therefore profit greatly from analysis of both carbon as well as hydrogen isotope ratios. The complementary information of the two elements may be instrumental in identifying the type of degradation pathway and the appropriate enrichment factor for quantification. Not only does this greatly enhance the precision with which CSIA can be used to determine the occurrence and extent of biodegradation in field situations, but CSIA then also has the potential to provide further insight into in situ subsurface microbial metabolism.

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