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Carbon Isotope Fractionation during Aerobic Biodegradation of Trichloroethene by *Burkholderia cepacia* G4: a Tool To Map Degradation Mechanisms

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The strain *Burkholderia cepacia* G4 aerobically mineralized trichloroethene (TCE) to CO_2 over a time period of ~20 h. Three biodegradation experiments were conducted with different bacterial optical densities at 540 nm (OD_{540}s) in order to test whether isotope fractionation was consistent. The resulting TCE degradation was 93, 83.8, and 57.2% (i.e., 7.0, 16.2, and 42.8% TCE remaining) at OD_{540}s of 2.0, 1.1, and 0.6, respectively. ODs also correlated linearly with zero-order degradation rates (1.99, 1.11, and 0.64 μmol h^{-1}). While initial nonequilibrium mass losses of TCE produced only minor carbon isotope shifts (expressed in per mille $\delta^{13}C_{VPDB}$), they were 57.2, 39.6, and 17.0‰ between the initial and final TCE levels for the three experiments, in decreasing order of their OD_{540}s. Despite these strong isotope shifts, we found a largely uniform isotope fractionation. The latter is expressed with a Rayleigh enrichment factor, ε, and was −18.2 when all experiments were grouped to a common point of 42.8% TCE remaining. Although, decreases of ε to −20.7 were observed near complete degradation, our enrichment factors were significantly more negative than those reported for anaerobic dehalogenation of TCE. This indicates typical isotope fractionation for specific enzymatic mechanisms that can help to differentiate between degradation pathways.

As a suspected carcinogen, trichloroethene (TCE) is a serious pollutant in soils and groundwaters (30). Among other methods of remediation (17, 20, 22), natural and/or engineered bioattenuation has significant potential to degrade TCE from soil and groundwater systems. The biodegradation of TCE can occur either anaerobically through reductive dechlorination (7, 29) or aerobically (8, 13, 15, 18, 24, 27). A bacterial strain that is capable of the latter process is *Burkholderia cepacia* G4. Dehalogenation with this strain is cometabolic and requires a primary substrate such as toluene or phenol in order to generate the toluene *ortho*-monooxygenase enzyme that is necessary for aerobic TCE degradation (27). The literature describes various degrees of this type of TCE degradation (8, 13, 15, 18, 24), and here we investigated its relationship between cell density and removal rate. As a novel aspect of this work, we investigated associated carbon isotope fractionations. We expected them to be largely independent of the cell density, because isotope selection should be constant for a given enzymatic process. Nevertheless, variations of the degree of fractionation may depend on the completeness of the reaction, with more substrate being used when more organisms are present. If isotope fractionation is unique and largely independent of optical density, it may serve as a tool to distinguish this from other degradation pathways.

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Several studies of organic contaminant degradation show significant changes in stable carbon isotope ratios of the remaining substrate, intermediates, and final products, thus offering the potential to use them as monitoring tools (1, 2, 3, 5, 6, 9, 12, 14, 28). For halogenated hydrocarbons, stable carbon isotope changes have been found for abiotic (2, 3, 6) and anaerobic (4, 10, 23) microbiological degradation. This study presents the first carbon isotope data set for aerobic degradation of TCE with *B. cepacia* G4. With data for anaerobic TCE removal already published, our first objective was to determine carbon isotope differences between these two biodegradation processes. If carbon isotope fractionation during aerobic degradation of TCE by *B. cepacia* G4 is a reality and if it also differs from that observed during anaerobic degradation, it can provide a comparative tool to help differentiate between degradation types (aerobic versus anaerobic).

Furthermore, a fundamental issue in understanding biodegradation of contaminants is the extent to which it has occurred. Isotope systematics may help with this, because previous research has shown that pseudoremoval processes such as sorption of TCE to inorganic material do not significantly fractionate carbon isotopes (3, 26). Therefore, carbon isotope analysis holds potential to complement or even replace the interpretation of concentration data alone, because it responds only to a true breakup of molecules. A specific type of isotope effect that has not been addressed previously may result from sorption to biomass. Therefore, our secondary objective was to investigate this type of sorption with dead-biomass control experiments. If we are to use carbon isotope measurements as a tool to com-

pare specific enzymatic degradation mechanisms, such effects should be considered, particularly when applying isotope systematics to microbial systems. Our data confirm the hypothesis that secondary carbon isotope variations caused by sorption are minor compared to overall enzymatic isotope shifts.

MATERIALS AND METHODS

The strain *B. cepacia* G4 was grown in a sterile minimal salt medium (0.96 g of KH_2PO_4 per liter, 1.23 g of K_2HPO_4 per liter, 3 g of NH_4Cl per liter, 0.4 g of $MgSO_4 \cdot 7H_2O$ per liter, and 1.9 μl of Vishniac trace element solution per liter [pH 6.9]). In a first stage, 2 mmol of phenol per liter was added as the primary carbon source to ensure the accumulation of the TCE-degrading toluene monooxygenase during growth. The bacteria were then harvested on the rising limb of their growth curve at an optical density at 540 nm (OD_{540}) of ~0.25. They were then separated from the supernatant by centrifugation and resuspended in the same minimal salt medium without phenol to the desired OD_{540} (2.0, 1.1, or 0.6). Resuspension in a pure TCE solution without cosubstrate allowed investigations at precisely determined ODs. This mode of operation also permitted analyses of TCE as the primary substrate. Furthermore, the removal of toluene ensured that generation of new toluene *ortho*-monooxygenase was likely very small due to presumably small storage reserves of the bacteria. In this manner we limited potential masking effects on associated isotope fractionations due to shifting ODs. Aliquots of 100 ml were placed in 166-ml glass bottle reactors that were capped with Mininert valves. The experiments were started by adding 50 μmol of TCE in a methanol solution. A spike of hexane was used as an internal standard.

Each experiment, except for the one at an OD_{540} of 0.6, had triplicate bottles as well as controls and blanks. Dead-biomass control experiments were carried out to investigate possible sorption effects on the biomass in the absence of degradation. This was done by boiling a cell suspension of the same OD as used in the relevant experiment before addition of TCE. During all experiments, the temperature was constant at 30°C and the reactors and standards were moved with an orbital shaker at 170 rpm. Headspace aliquots were sampled at regular time intervals with a gas-tight syringe to determine TCE concentration and stable carbon isotope values.

TCE was separated from other gases by using a Thermoquest 8000 Top gas chromatograph and identified with an MD800 mass-selective detector. The injector split flow had a ratio of 5:1, and the chromatographic column was a CP Poraplot column with an inner diameter of 0.32 mm. The temperature for the chromatographic profile was held at 70°C for 0.5 min and then ramped at 79.5°C/min to 200°C, where it was held for 2.5 min. The carbon isotope composition of the TCE was determined with a Micromass Isoprime II isotope ratio mass spectrometer in continuous-flow mode after conversion to CO_2. The combustion interface was filled with copper oxide and silver wool and had a temperature of 800°C. Decreases in the concentration of TCE were calculated from the ratio of the hexane and TCE peak areas and compared to standards of various concentrations. The $^{13}C/^{12}C$ isotope ratios are expressed in the conventional δ notation as a per mille deviation from the Vienna Pee Dee Belemnite (VPDB) standard. This notation allows convenient expression of only minute changes of ^{13}C in the remaining TCE with the formula (11):

$$\delta^{13}C_{VPDB} = \frac{^{13}C/^{12}C_{(sample)} - {}^{13}C/^{12}C_{(standard)}}{^{13}C/^{12}C_{(standard)}} \times 1{,}000 \quad (1)$$

Deviations towards more positive values therefore express enrichment in ^{13}C in the remaining TCE. Measurements of pure house-made TCE standards at various concentrations (i.e., at a variety of peak sizes) revealed an overall precision of better than 0.4‰.

For comparison of the TCE fractionation between the various experiments, the isotope enrichment factor, ε, between the initial and remaining TCE was determined according to the Rayleigh model. This term can be defined for the reactant only (16):

$$\varepsilon = \frac{1{,}000 \times \ln \dfrac{10^{-3} \times \delta_S + 1}{10^{-3} \times \delta_{S0} + 1}}{\ln f} \quad (2)$$

where δ_{S0} and δ_S are the isotope compositions of the TCE at the beginning and during the experiment, respectively, and f is the remaining fraction of the original substrate. When plotting $\ln f$ versus the numerator of above equation 2, the slope of a linear regression passing though the origin (i.e., $\delta_S = \delta_{S0}$ at $\ln f = 0$) defines ε.

TABLE 1. Summary of results, with averages from reactors 1, 2, and 3 for the three experiments at different OD_{540}s[a]

OD_{540}	Final $\delta^{13}C_{VPDB}$ (‰)	TCE remaining (%)	Enrichment factor ε	95% confidence on ε
0.6	−13.1	42.8	−18.2	0.7
1.1	9.5	16.2	−20.4	0.4
2.0	27.1	7.0	−20.7	0.4

[a] The initial $\delta^{13}C$ value of the TCE was −30‰, and standard deviations between measurements from triplicate bottles during degradation were <1.3‰ except for a few measurements at the end of the experiment at an OD_{540} of 2.0. The enrichment factors ε were calculated by successively expanding data point ranges from all three experiments instead of using data sets from individual experiments.

RESULTS

Most of the TCE biodegradation occurred during the first 20 h of each experiment (Fig. 1A). For calculation of zero-order rates of TCE degradation, only the linear parts of the degradation curves (4.9 to 20.9 h) were used (Fig. 1B). Rate constants over this range were 1.99, 1.11, and 0.64 μmol h^{-1} for the experiments at OD_{540}s of 2.0, 1.1, and 0.6, respectively. These rates positively correlated with the ODs in a linear fashion. With increasing OD and rate, the final amounts of TCE decreased to 42.8, 16.2, and 7% of the original concentration, respectively (Fig. 1; Table 1).

In all experiments, greater initial mass losses were observed during the first few hours. This was most obvious in the dead-biomass experiments (reactors 4). However, even under these extreme conditions that excluded biodegradation, mass balances improved over time. After about 5 h, mass balances in the dead-biomass reactors reached constant values. Note that the calculated rates were not affected by these initial mass defects, because the first few hours were excluded from the rate calculations. After ~5 h, we also noted apparently larger TCE masses than the 50-μmol input in the dead-biomass reactors (Fig. 1). They likely resulted from analytical uncertainties such as deviations in TCE and spiked-hexane masses. The existence of different rates of sorption and release of both TCE and hexane to the much larger surface area of the dead-biomass cells is a plausible explanation. As a result, TCE concentrations could be calculated only with an accuracy of 10% in the control experiments.

Carbon isotope values in the dead-cell experiments showed initial depletions with rare extremes of −4.5‰ from the TCE standard value of −30‰ (Fig. 2). Nevertheless, these $\delta^{13}C$ variations were minor compared to the enzymatically caused isotope shifts in the live-cell experiments (Fig. 2). Overall, $\delta^{13}C$ standard deviations determined over the entire length of the dead-cell experiments were less than 1.3‰, and the final $\delta^{13}C$ values of all dead-biomass reactors were always close to the initial TCE value (Fig. 2).

Average carbon isotope differences from the triplicate reactors between initial and final TCE values were 57.2, 39.6, and 17.0‰ for each experiment in decreasing order of OD (Fig. 2; Table 1). This indicates more pronounced enrichment of ^{13}C in the remaining TCE at higher cell densities. Except for a few outliers towards the end of the experiment at an OD_{540} of 2.0

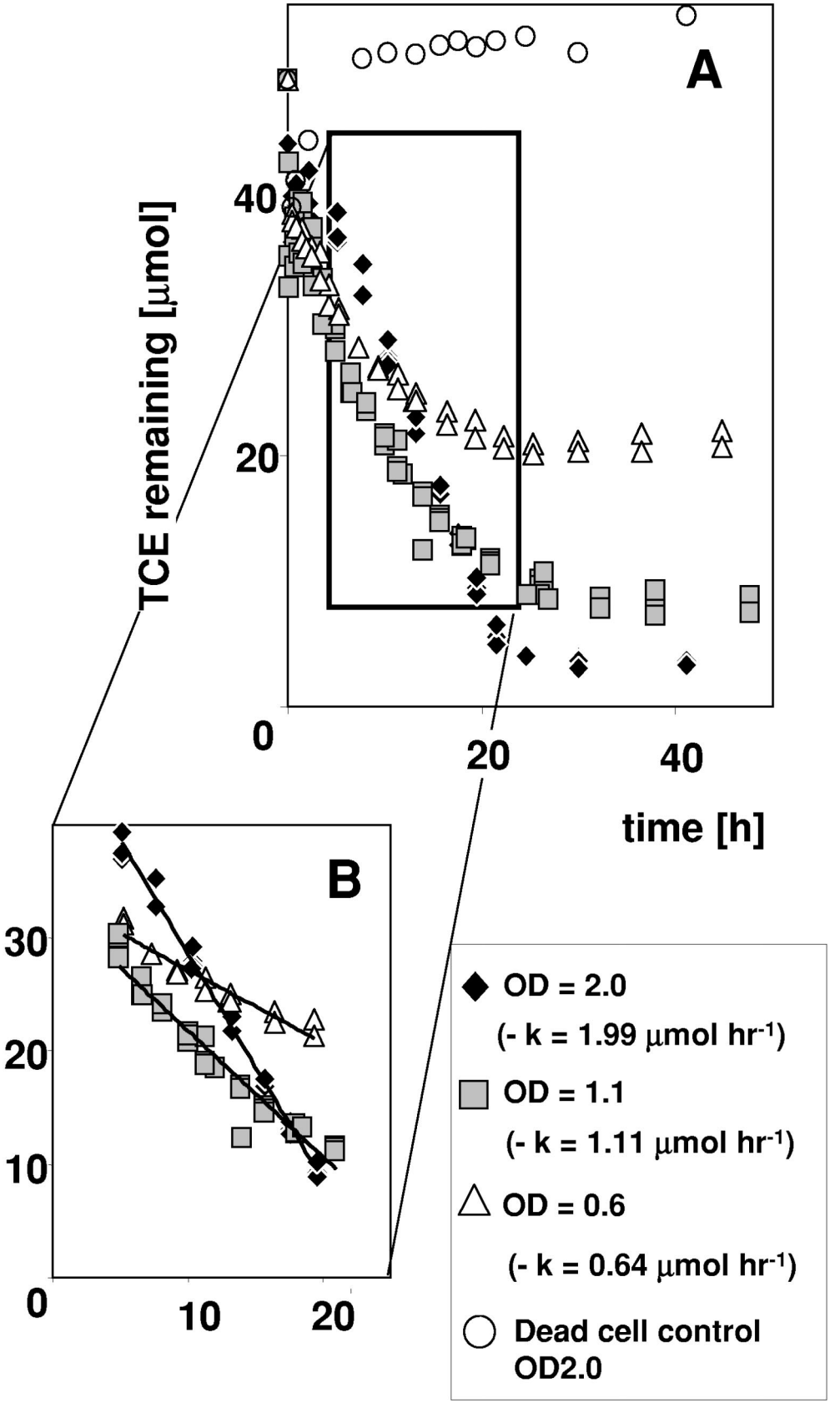

FIG. 1. Fraction of TCE remaining versus time (A) and calculation of zero-order rates (−k) over the linear parts of the reaction curves (B).

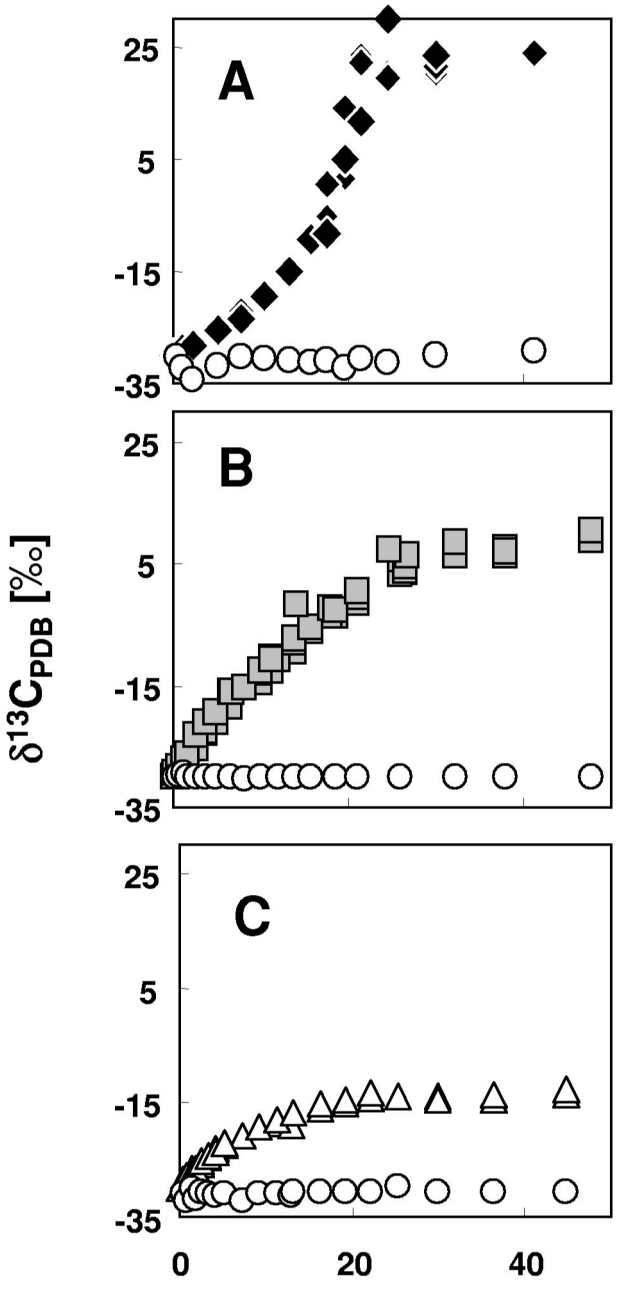

FIG. 2. Carbon isotope composition of TCE in three experiments versus time at OD_{540}s of 2.0 (A), 1.1 (B), and 0.6 (C). The amounts of remaining TCE were 7.0, 16.2, and 42.8%, respectively. Circles represent the TCE isotope values of the dead-biomass control experiments. TCE standards measured at various concentrations revealed a standard deviation of <0.4‰, and triplicate measurements of separate bottles at various points throughout the experiments had a standard deviation of <1.3‰.

(Fig. 2A) the standard deviation between triplicate measurements of separate bottles was smaller than 1.3‰.

The enrichment factor ε was determined by plotting the numerator versus the denominator of equation 2. The slope of the resulting linear regression then represents ε. Over the first half of all experiments, a coherent ε of −18.2 was observed for all three ODs. The associated correlation coefficient, r^2, was 0.89, with a corresponding 95% confidence interval of ±0.7. More negative ε values resulted when data beyond 42.8% TCE remaining were included. With inclusion of all data points to 16.2% TCE remaining, determination of ε yielded a value of −20.4. With further inclusion of all data points down to 7% TCE remaining, ε was −20.7. Both of these enrichment factors were determined with an r^2 of 0.97and had 95% confidence intervals of ±0.4. Even with these slight decreases of ε at more complete TCE degradation, a crucial observation is that these aerobic enrichment factors were significantly more negative than those determined for anaerobic TCE removal. Published data for anaerobic TCE removal range between −2.5 and −13.8 (4, 10, 23).

By following the Rayleigh model, the theoretical $\delta^{13}C$ of the remaining TCE value can be predicted for any value of the remaining fraction f when the initial isotopic composition of TCE is known and a constant ε is available. When plotting the fraction of the remaining TCE versus the measured $\delta^{13}C_{VPDB}$, a theoretical best-fit line was established in this manner, with the initial $\delta^{13}C$ value being −30‰ and ε values at −18.2 and −20.7 (Fig. 3). During the initial stages of degradation, with f from 1.0 to ~0.7, some data points clearly deviated from the ε regression and as a result did not adhere to the modeled ε lines. These outliers are circled in Fig. 3. Note, however, that filtering out these initial data points changed ε of all experiments (to a common point of 42.8% TCE remaining) only to −18.9. This new ε still remained within the 95% confidence limit of ±0.7, and r^2 increased only slightly to 0.95.

Oxygen levels in the headspace were measured only qualitatively, and they decreased slightly during all three experiments but remained sufficient to allow the aerobic operation of the toluene monooxygenase. In contrast to oxygen concentrations, CO_2 concentrations increased by an order of magnitude from their initial values (air background); however, they were not quantified precisely in this study and are not shown here.

DISCUSSION

Before discussing concentration and isotope data, it is important to consider mass losses of TCE that may result from pseudoremoval processes such as volatilization, dispersion, and/or sorption. These effects were already shown to be insignificant for equilibrium partitioning of chlorinated ethenes due to solid sorption and volatilization (3, 21, 25, 26), and our work presents the first data set on possible isotope effects due to sorption of TCE to biomass. Note that the dead-biomass control experiments are an even more rigorous test, because boiling broke the cells into fragments, thus creating more sorption sites than for live cells. The initial steep decreases of TCE concentrations in the live-cell reactors were likely a combination of nonequilibrium sorption, liquid-vapor interaction, and degradation. It is difficult to clearly separate these processes, and further research is necessary to study nonequilibrium effects in detail.

Our data also show some non-equilibrium partitioning effects on the isotope composition of the TCE, as evidenced by variations in the $\delta^{13}C$ of the TCE during the initial stages of the dead-cell experiments. Therefore, caution must be taken when interpreting initial data points during biodegradation

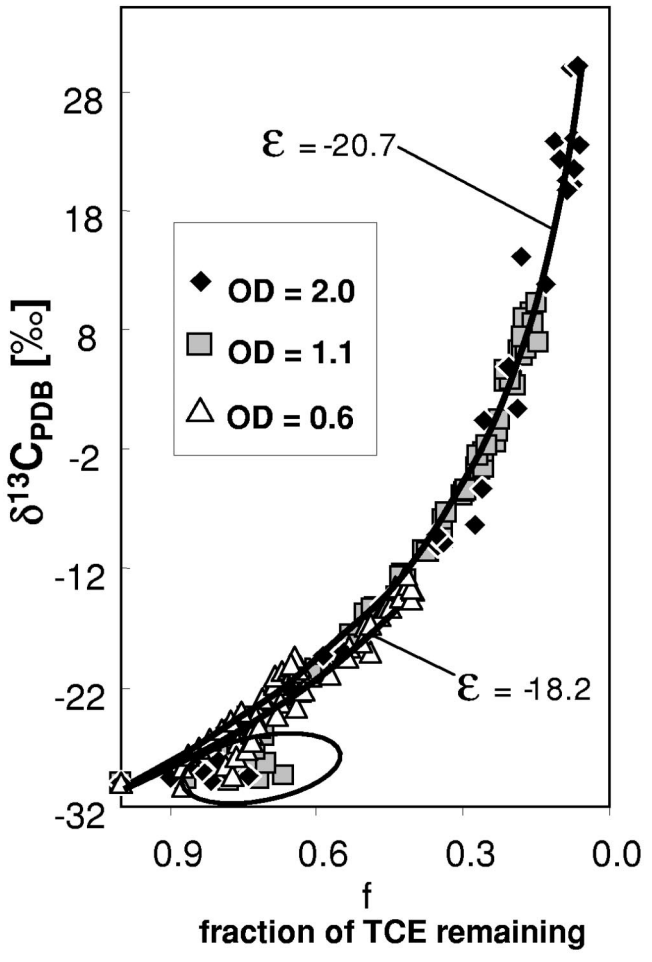

FIG. 3. Carbon isotope composition of TCE during the three experiments versus fraction remaining. The linear regression line that defines the enrichment factor ε was determined with a ε value of −18.2 to a common point of 42.8% TCE remaining. At further degradation down to 7% TCE remaining, ε was determined with a value of −20.7. The r^2 values of the linear regressions were 0.89 and 0.97, respectively. Data points that deviate from the Rayleigh model are circled. These deviations are caused by excessive concentration decreases during initial phases of the experiments.

experiments. Nevertheless, over the experimental time periods, the isotope composition of TCE in the dead-cell control vials went back to the original value (Fig. 2), thus indicating that isotope effects due to equilibrium sorption of TCE to biomass are negligible. This comparison of dead- and live-cell experiments confirms that carbon isotope fractionations result primarily from enzymatic degradation and are largely independent of non-degradative partitioning processes.

The enrichment factor (ε) is an elegant monitoring tool that combines concentration declines with isotope shifts. It describes the relationship between $\delta^{13}C$ and the amount of residual TCE and follows a Rayleigh-type fractionation. The latter also applies to aerobic degradation of TCE, as shown by the agreement of our data with the model (Fig. 3 and equation 2). The ε of −18.2 during biodegradation of the first 42.8% of the TCE remaining suggests that, within these limits, the enzymatic degradation mechanism is the same and is independent of cell density and rate. Furthermore, ε is largely independent of initial sorption to biomass or any other nonequilibrium partitioning processes, as demonstrated by maintenance of an almost identical ε value of −18.9 that remained within the 95% confidence limit after the initial data points of the experiments were filtered out. The isotopic compositions of the remaining TCE were enriched in ^{13}C in a systematic manner, even during the first few hours of the experiments (Fig. 2), which shows that nonequilibrium concentration decreases had little influence on ε. Therefore, the deviation of data points from the modeled ε curve during the initial stages of the experiments (circled in Fig. 3) were caused by more rapid concentration decreases of TCE. A separate evaluation of concentration data with more drastic concentration decreases at the beginning of experiments (Fig. 1) confirms this influence.

Even when considering the noticeable variations in ε (−18.2 to −20.7), the most crucial observation of this work is that the enrichment factors in aerobic degradation are significantly more negative than those in anaerobic degradation. The latter range from −2.5 to −13.8 (4, 10, 23). This difference demonstrates that other degradation pathways with utilization of different enzymes can cause unique isotope fractionation patterns. The aerobic TCE degradation of our study with the toluene monooxygenase enzyme produces the nonvolatile secondary product epoxide at the beginning of the reaction (19; M. Whittaker, D. Monroe, and D. J. Oh, http://www.labmed.umn.edu/umbbd/tce/tce_map.html). This involves the breakup of a C-C bond in the first fractionating reaction step. The disintegration of this bond by toluene *ortho*-monooxygenase produces a more negative ε value. Significantly more positive ε values during anaerobic dehalogenation coincide with the breakup of a C-Cl bond during the formation of dichloroethene in the first reaction step (7). These typical ε values in response to disintegration of different bond types indicate relationships between process-specific enzymatic isotope fractionations and bond strengths, thus providing a tool that can differentiate between degradation mechanisms.

The wider range of ε observed during anaerobic degradation possibly reflects a more complex system, with a consortium of bacteria accomplishing dehalogenation with various combinations of bacterial strains. The different bacterial strains involved may use various metabolic pathways and thus may cause different isotope fractionations. On the other hand, in the case of *B. cepacia* G4, toluene monooxygenase is the only known enzyme for TCE dehalogenation. This may explain the comparatively small variation in the resulting enrichment factors. In experiments at OD_{540}s of 1.1 and 2.0, where the remaining TCE was less than 42.8%, our data yielded a decrease of ε. In these two experiments the decrease in the concentration of TCE continues and is combined with an ongoing isotope change of the remaining substrate. The associated changes of rates, in both concentration and isotope data (Fig. 1 and 2), likely reflect the dying of cells. Possible associated reduction of the reductive power of the toluene monooxygenase enzyme may stem from substrate and nutrient limitations and/or possible toxicity effects. Another possibility for decreasing ε values is a reduction of active sites on the toluene monooxygenase enzymes at different cell densities during later stages of the experiments. It is unclear at this point how often one enzymatic

molecule is recycled before it becomes inactive. Further experiments with pure enzymes as well as studies that precisely quantify the relationship between enzymatic activity, OD, and isotope fractionation may lead to a closer understanding of these questions.

The good agreement of the data points with the modeled Rayleigh curves (Fig. 3) demonstrates the potential to determine the fraction of TCE remaining with only two isotope measurements: one of the TCE before and the other at any stage during the degradation. The variations in ε make it more difficult to precisely evaluate the extent of TCE removal without OD data. However, with less than 2.5‰, these variations are relatively small and the fraction remaining is easily determined during the first half of the experiment. If other cell densities yield similar enrichment factors, this method has potential as an excellent monitoring tool that could complement concentration measurements. The latter can be uncertain at times when masked by sorption, mixing of water masses, or volatilization.

Nevertheless, a number of factors will have to be considered before this method can be applied with confidence to a realistic spill scenario in the field. First, the in vitro experiments do not reflect field conditions because they were carried out at unrealistically high cell densities and temperatures. Second, if *B. cepacia* G4 is present in a soil or groundwater system, it likely occurs in competition with other bacterial strains and substrates. Furthermore, aerobic degradation usually happens mostly at the fringes of plumes where concentrations are low due to previous anaerobic degradation, dispersion, and dilution. Further experiments with conditions that are closer to real field and groundwater conditions are necessary to illuminate these relationships.

In conclusion, we found that under in vitro conditions, carbon isotope changes during aerobic TCE degradation are systematic. The most important result of our investigations with respect to biosorption is that the dead-biomass control experiments revealed only minor isotope effects compared to the overall enzymatic isotope changes. Therefore, enzymatic degradation must be the primary cause of observed isotope effects. Another crucial finding of this work is that the isotope fractionation (expressed with the enrichment factor ε) is significantly more negative during this type of aerobic TCE degradation than during anaerobic degradation. This difference indicates process-specific degradation mechanisms where different types of chemical bonds break up, with typical associated carbon isotope fractionations. Therefore, carbon isotope measurements of TCE are a useful monitoring tool. Together with concentration measurements and microbiological investigations, they hold potential to differentiate between degradation mechanisms. Further investigations may reveal whether stable carbon isotope fractionations allow differentiation of these two types of degradation from abiotic mechanisms such as TCE degradation on zero-valent iron or palladium. The systematic nature of ε also shows that isotope analyses may provide qualitative evaluation of aerobic TCE degradation. The quantification of aerobic TCE removal becomes more precise if the OD of *B. cepacia* G4 is known. At present, the only applications that could directly benefit from our results are engineered applications, such as aerobic bioreactors. In order to use this method as a monitoring tool in subsurface spill scenarios, further experiments that are more representative of real field conditions are necessary. Temperature dependencies of the determined enrichment factors, degradation in a mixture of bacteria and other organic molecules, and work at lower concentrations need further investigation.

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