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Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: Implications for intrinsic bioremediation

B. Sherwood Lollar[a,*], G.F. Slater[a], J. Ahad[a], B. Sleep[b], J. Spivack[c], M. Brennan[c], P. MacKenzie[c]

[a]*Stable Isotope Laboratory, Department of Geology, University of Toronto, 22 Russell Street, Toronto, Canada M5S 3B1*
[b]*Department of Civil Engineering, University of Toronto, 35 St George Street, Toronto, Canada M5S 1AH*
[c]*General Electric Research and Development Centre, Schenectady, NY, USA*

Abstract

In experiments involving anaerobic biodegradation of trichloroethylene (TCE), $\delta^{13}C$ values for residual TCE changed from −30.4‰ to values more enriched than −16‰. All data exhibit a consistent correlation between $\delta^{13}C$ value of the residual TCE and the extent of biodegradation of TCE, described by a fractionation factor (α) of 0.9929. In contrast, during aerobic biodegradation of toluene by two separate mixed consortia, no change in $\delta^{13}C$ value of the residual toluene was observed within analytical uncertainty (0.5‰). Stable carbon isotopes have the potential to be a useful indicator for identification and monitoring of intrinsic bioremediation of chlorinated hydrocarbons such as TCE. Conversely, for aromatic hydrocarbons such as toluene, more conservative isotopic values may instead be more applicable as a means of source differentiation at sites with a history of multiple spills. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Carbon isotopes; Chlorinated solvents; Intrinsic bioremediation; Natural attenuation; Contamination

1. Introduction

Intrinsic bioremediation refers to the action of microorganisms to degrade contaminants in situ under natural conditions. Recently it has gained widespread attention as offering a potentially more efficient and cost effective contaminant remediation strategy than more highly engineered groundwater and soil cleanup technologies (National Research Council, 1993; Hinchee et al., 1995). Current protocol for proving that biodegradation is actually occurring in the field, however, relies on accumulation of a broad basis of evidence from a variety of different scientific approaches, including microbiological and chemical field measurements, field experiments, and modeling experiments. This paper investigates the potential to use carbon isotopic analysis of hydrocarbon contaminants to provide more direct evidence of intrinsic bioremediation.

Stable carbon isotope analysis involves measurement of the two stable isotopes of carbon, ^{12}C and ^{13}C to establish $\delta^{13}C$ signatures where:

* Corresponding author. Fax: +1-416-978-3938.
E-mail address: bsl@quartz.geology.utoronto.ca (B. Sherwood Lollar)

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$$\delta^{13}C = (\text{Rsample}/\text{Rstandard} - 1) \times 1000 \quad \text{(in units of‰)} \tag{1}$$

Rsample is the $^{13}C/^{12}C$ ratio in a given compound, which is normalized to Rstandard, the $^{13}C/^{12}C$ ratio in a standard reference material. In this study all $\delta^{13}C$ values are reported relative to the V-PDB standard (Craig, 1957). The application of stable carbon isotope analysis to provide evidence for biodegradation relies on the fact that processes such as biodegradation may produce a net change in the $\delta^{13}C$ value, or fractionation, in the compound being degraded. Fractionation during microbial oxidation of CH_4 is well-established (Barker and Fritz, 1981; Coleman et al., 1981). Due to preferential degradation of the ^{12}C-containing CH_4 molecules, the residual CH_4 pool becomes enriched in ^{13}C-containing CH_4, resulting in a progressively more positive $\delta^{13}C$ value as a result of this kinetic isotope effect. In contrast, for other organic compounds (particularly those of high molecular weight), no fractionation has been documented. For instance, despite extensive degradation, compounds such as polycyclic aromatic hydrocarbons (O'Malley et al., 1994; Trust et al., 1995) and C13–C35 *n*-alkanes (Mansuy et al., 1997) show no change in $\delta^{13}C$ values. Very little information has been available on fractionation resulting from biodegradation of priority pollutants such as the chlorinated solvents and aromatic hydrocarbons. The first objective of this study is to determine whether stable carbon isotope fractionation occurs during biodegradation of two compounds, trichloroethylene (TCE) and toluene, representative members of these two major groups of contaminants. The second objective is to assess the applicability of using such carbon isotope fractionation to provide evidence of intrinsic bioremediation of these compounds in the field.

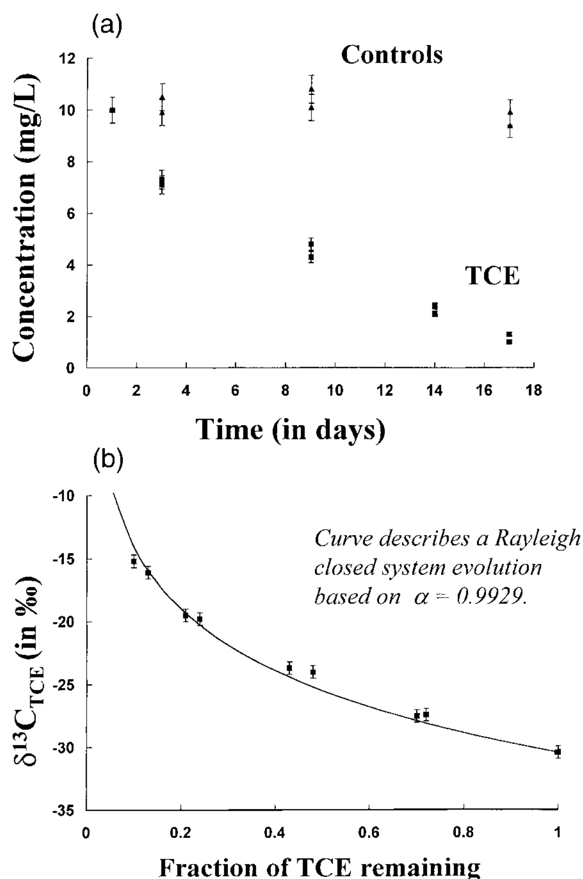

Fig. 1. (a) Concentrations of TCE (in mg/l) in control and sample vials over time during the course of the anaerobic biodegradation experiments. Each point represents a separate independently degrading microcosm vial. Error bars represent ±5% on concentrations. (b) $\delta^{13}C$ values of residual TCE vs the fraction of TCE (f) remaining undegraded. Initial $\delta^{13}C$ of TCE before biodegradation is −30.4‰ at $f = 1$. The Rayleigh closed system evolution curve is based on a value of $\alpha = 0.9929$ calculated by least squares regression of all data points on a plot of lnf vs $\ln((\delta^{13}C_{TCE}/1000+1)/(\delta^{13}C_o/1000+1))$ after Mariotti et al. (1981) (see text). Error bars represent an accuracy and reproducibility of <0.5‰ on $\delta^{13}C$ values. $\delta^{13}C$ values for controls are shown in Table 1 and are always within error (0.5‰) of the initial $\delta^{13}C$ value for TCE of −30.4‰.

2. Experimental methods

2.1. Anaerobic TCE biodegradation experiment

Biodegradation of TCE was carried out anaerobically in two laboratory batch vial experiments carried out at General Electric Research and Development Centre using a mixed consortium of bacteria cultured from contaminated soil at the Pinellas site. The Pinellas consortium is facultatively anaerobic, but does contain obligate anaerobes such as sulphate reducing bacteria. For each experiment, five 250 ml serum bottles were filled with mineral media (RAMM) (Shelton and Tiedje, 1984), and with TCE at a concentration of 10 mg/l. Lactate was added to serve as an electron donor. The bottles were then inoculated with N_2 purged Pinellas culture, capped with Teflon faced crimp seal septa and allowed to degrade over 17 days. For each experiment, 3 control bottles were also set up, consisting of 10 mg/l TCE in sterilized mineral media (RAMM).

Throughout the experiment, one sample bottle was analyzed for TCE concentration to monitor degradation progress. Concentrations of TCE and degradation products were determined by removing 1 ml of liquid for analysis by GC/MS at General Electric. When the monitoring bottle indicated the reaction had

degraded 30% of the TCE, a sample vial was analyzed to determine both the concentration and the isotopic composition of the residual TCE. The same procedure was followed at 60, 80 and 90% degradation for each of the 3 remaining sample vials. Sampling consisted of removing 1 ml of liquid for analysis of TCE concentration as described above. This was followed by releasing of excess pressure from the bottle, opening it and using a sterile 0.22 micron syringe filter to quickly transfer 40 ml of liquid into 50 ml serum bottles containing 15 g of NaCl. This bottle was capped with a Teflon crimp seal septa and kept cold and dark during transport to University of Toronto for carbon isotope analysis. At 14 days and 17 days, (80 and 90% degradation, respectively) the samples were frozen to ensure all biodegradation stopped while samples were in transit to the University of Toronto for isotope analysis. Controls indicate that this step introduced no change in the isotopic composition of the TCE. Furthermore, each step in the sampling and sample transfer procedure was rigorously tested prior to the experiment to ensure no sample handling artifacts affected either the concentrations or the isotopic data. Fig. 1(a) and (b) shows the results of the 2 experiments carried out using the above protocol.

2.2. Aerobic toluene biodegradation

Biodegradation of toluene was carried out in a laboratory batch vial experiment at the University of Toronto using sand from a Saskatchewan aquifer that had been previously shown to contain microbes capable of aerobic biodegradation of toluene (Richards, 1997). Three 160 ml sample vials were filled with 60 g of this sand (at 15% moisture content). To provide sufficient nitrogen and phosphorus to the microorganisms present in the sand, inorganic nutrients (1.02×10^{-5} moles of KNO_3, 1.03×10^{-6} moles of K_2HPO_4 and 1.03×10^{-6} moles of KH_2PO_4) were added to each sample bottle in the form of a nutrient solution prepared in distilled water. 6 mg of pure phase toluene was added to each sample vial to produce a starting headspace concentration of approximately 32 mg/l. Vials were then sealed with a Mininert valve. The microcosms were manually shaken for 1 min to ensure partitioning between the air, water and solid phases. In order to simulate natural groundwater conditions and to slow down the rate of biodegradation, the microcosms were kept in a refrigerator at 9 ± 1°C throughout the course of the experiment. The three vials were allowed to degrade for approximately 150 h and each vial was sampled at 8 sampling points to determine the carbon isotope value and concentration of the residual toluene vapor.

Two types of controls were set up for this experiment. Control 1 consisted of duplicate vials of 60 g of autoclaved, sterile, acid washed 250 μm glass beads and 6 mg toluene. Control 1 was used to determine if any isotope effects were associated with the vial or Mininert septa, or developed over time in the system due to leakage, sorption to the glass, etc. Control 2 consisted of duplicate vials of 60 g of autoclaved, sterile sand, combined with 5 μl HgCl (as a biocide) and 6 mg toluene. Control 2 was used to determine if, in the absence of biodegradation, sorption onto the or-

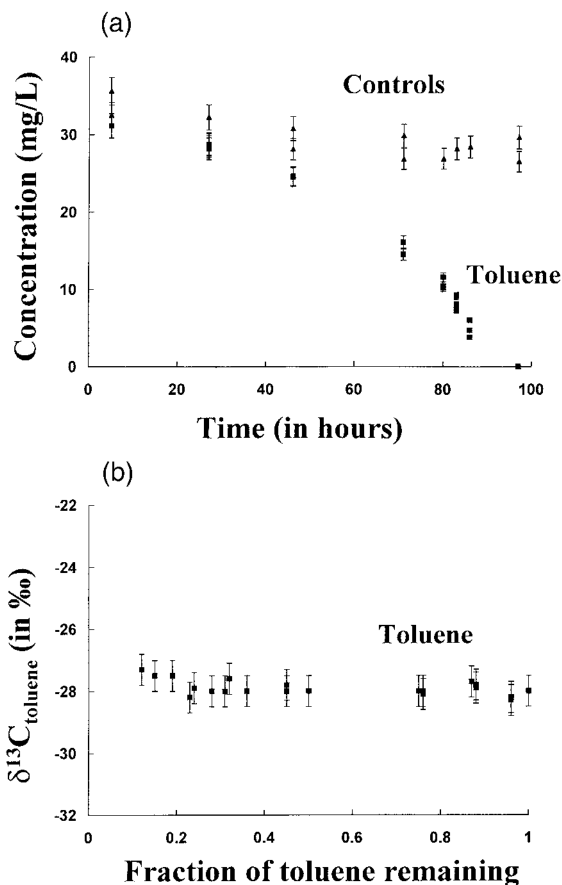

Fig. 2. (a) Concentrations of toluene (in mg/l) in control and sample vials over time during the course of the aerobic biodegradation experiment. Results for each control are the mean of duplicate vials. For sample vials, results are shown for each of the 3 vials separately. All vials are measured repeatedly over the course of the experiment. Error bars represent ±5% on concentrations. (b) $\delta^{13}C$ values of residual toluene vs the fraction of toluene remaining undegraded. Initial $\delta^{13}C$ of toluene before biodegradation is −28.0‰ at $f = 1$. Error bars represent an accuracy and reproducibility of < 0.5‰ on $\delta^{13}C$ values. $\delta^{13}C$ values for controls are shown in Table 2 and are always within error (0.5‰) of the initial $\delta^{13}C$ value for toluene of −28.0‰. The fraction of toluene remaining (f) is calculated by assuming the concentration in each of the sample vials at $t = 0$ is equal to that of Control Vial C2 (32.5 mg/l ± 5%).

ganic carbon of the soil would cause any isotopic fractionation. All controls were handled in a manner identical to the sample vials, and were analyzed for concentrations and isotopic compositions throughout the course of the experiment. This experiment was repeated 3 times with identical results so Fig. 2(a) and (b) show the results of only one of the 3 experiments.

2.3. Analytical methods

Toluene concentrations were analyzed at the University of Toronto on a Varian 3300 gas chromatograph equipped with a 30 m × 0.53 mm i.d. DB-624 column and FID (flame ionization detector). Toluene samples were run isothermally at 80°C. TCE concentrations were analyzed at General Electric by headspace GC/MS with a Tekmar 7000 Headspace Analyzer in tandem with a HP 5890 GC and a Fisons Trio 1000 Mass Spectrometer equipped with a GS-Q (30 m × 0.32 mm i.d.) fused silica capillary column. Reproducibility on concentrations measurements for TCE and toluene are ±5%. Compound specific carbon isotope analysis of toluene and TCE was done at the University of Toronto using a gas chromatograph/combustion/isotope ratio mass spectrometer (GC/C/IRMS). This technology and recently developed sample extraction techniques are the key to routine application of $\delta^{13}C$ isotopic analysis to dissolved organic contaminants typically present at ppm to ppb concentrations in groundwater (Dempster et al., 1997; Dias and Freeman, 1997; Slater et al., 1999). The University of Toronto system consists of a Varian 3400 gas chromatograph equipped with a 30 m × 0.25 mm i.d. DB-624 column and Finnigan MAT 252 gas source isotope ratio mass spectrometer. For toluene the column was held isothermally at 80°C. For TCE the temperature was held at 40°C for 4 min, followed by a ramp to 90°C at 10°C/min, with a final holding time at 90°C of 3 min.

Sample extraction from solution was by headspace equilibration following the protocol described in Slater *et al.* (1999). Samples are transferred from sample vials by gas-tight syringe and injected into a split/splitless injector on the GC/C/IRMS where samples are run against external CO_2 isotopic standards. A range of split settings on the split/splitless injector was required to control the moles of carbon entering the system and to avoid over-saturating the source. While internal reproducibility based on triplicate sample injection is generally < 0.3‰, differences between samples (error bars) are assigned a value of no less than 0.5‰ to incorporate not only variation due to reproducibility but variation due to different split settings. Errors of 0.5‰ for carbon isotope values thus incorporate both reproducibility and accuracy of the measurement.

Table 1
Concentration (in mg/l) and $\delta^{13}C$ values (in ‰) for TCE in control and sample vials vs sampling time over the course of the anaerobic biodegradation experiments[a]

Vial	Time (d)	Concentration (mg/l)	$\delta^{13}C$ (‰)
Experiment 1			
Sample A1	3	7.1	−27.5
Sample A2	9	4.8	−24.0
Sample A3	14	2.4	−19.8
Sample A4	17	1.0	−15.2
Control A1C	3	10.5	−30.4
Control A2C	9	10.8	−30.6
Control A3C	17	9.9	−30.4
Experiment 2			
Sample B1	3	7.3	−27.4
Sample B2	9	4.3	−23.7
Sample B3	14	2.1	−19.5
Sample B4	17	1.3	−16.1
Control B1C	3	9.9	−30.4
Control B2C	9	10.1	−30.4
Control B3C	17	9.4	−30.5

[a] Errors in concentration are ±5%. Accuracy and reproducibility for $\delta^{13}C$ values is < 0.5‰. No controls were run at sampling interval t = 14 d.

3. Results

Fig. 1 shows the results of the 2 experiments on anaerobic biodegradation of TCE. At day 1, the starting concentration and $\delta^{13}C$ value for TCE were 10.1 mg/l and −30.4‰, respectively. In Fig. 1(a), each data point represents one of the 8 independently degrading microcosm vials or 6 control vials involved in the 2 experiments. No change in either concentration (Fig. 1(a)) or isotopic composition (Table 1) occurred in the controls. In contrast, in both experiments, the concentration in the sample vials decreased by an order of magnitude over the course of the biodegradation experiment from 10.1 mg/l to 1.0 and 1.3 mg/l, respectively (Fig. 1(a)). Isotopic values for the residual TCE changed from −30.4 to −15.2‰ (at 90% degradation in experiment 1) and −16.1‰ (at 87% degradation in experiment 2) respectively. This trend of increasing isotopic enrichment in ^{13}C in the residual TCE reflects the preferential biodegradation of ^{12}C-^{12}C vs ^{12}C-^{13}C containing TCE molecules.

Fig. 1(b) indicates the highly reproducible nature of the kinetic isotope effect associated with anaerobic biodegradation of TCE by the Pinellas consortium. The relationship between the concentration of the residual TCE (or fraction of TCE remaining, f) and the isotopic composition of the residual TCE ($\delta^{13}C_{TCE}$) can be described by a simple Rayleigh closed system set of equations expressed in δ‰ notation after Mariotti et al. (1981):

Table 2
Concentration (in mg/l) and $\delta^{13}C$ values (in ‰) for toluene in control and sample vials vs sampling time over course of aerobic degradation experiment. Results for controls 1 and 2 each represent the mean of duplicate vials. For samples, individual results are shown for each of the 3 vials measured repeatedly over the course of the experiment[a]

	Controls				Samples					
	C1		C2		Vial 1		Vial 2		Vial 3	
Time (h)	Conc. (mg/l)	$\delta^{13}C$ (‰)	Conc. (mg/l)	$\delta^{13}C$ (‰)	Conc. (mg/l)	$\delta^{13}C$ (‰)	Conc. (mg/l)	$\delta^{13}C$ (‰)	Conc. (mg/l)	$\delta^{13}C$ (‰)
5	35.6	−28.1	32.5	−28.0	31.1	−28.3	31.1	−28.3	31.1	−28.2
27	32.2	−27.9	28.8	−28.0	28.5	−27.8	28.7	−27.9	28.2	−27.7
46	30.8	−28.0	28.2	−28.0	24.5	−28.0	24.6	−28.0	24.6	−28.1
71	29.9	−28.3	26.9	−27.9	14.6	−28.0	16.1	−28.0	14.5	−27.8
80	NA	NA	26.9	−27.8	10.5	−27.6	11.6	−28.0	10.2	−28.0
83	NA	NA	28.2	−27.9	8.0	−27.9	9.1	−28.0	7.3	−28.2
86	NA	NA	28.4	−28.3	4.7	−27.5	6.0	−27.5	3.8	−27.3
97	29.6	−27.9	26.5	NA	0.05	NA	< 0.006	NA	< 0.006	NA

[a] NA = not analyzed. Errors in concentration are ±5%. Accuracy and reproducibility for $\delta^{13}C$ values are < 0.5‰.

$$(\alpha - 1)\ln f = \ln((\delta^{13}C_{TCE}/1000 + 1)/(\delta^{13}C_o/1000 + 1)) \quad (2)$$

where α is the isotopic fractionation factor and $\delta^{13}C_o$ is the initial isotopic composition of the TCE (−30.4‰). Fig. 1(b) shows f (x axis) vs $\delta^{13}C_{TCE}$ (y axis) and a Rayleigh closed system evolution curve based on $\alpha = 0.9929$. Expressed as an enrichment factor, this corresponds to $\epsilon = -7.1$ where $\epsilon = 1000\ (\alpha - 1)$. This value for α is calculated by plotting the data for the 2 experiments on a plot of lnf vs $\ln((\delta^{13}C_{TCE}/1000+1)/(\delta^{13}C_o/1000+1))$ after Mariotti et al. (1981) and determining the slope ($\alpha - 1$) by least squares regression of the data. An r^2 value of 0.98 for this fit attests to the strength of the correlation. All eight independently degrading microcosms vials for the 2 experiments exhibit the same correlation between $\delta^{13}C$ value of the residual TCE and the extent of biodegradation of that TCE, described by the fractionation factor $\alpha = 0.9929$.

Fig. 2 shows the results for one representative experiment of the 3 experiments carried out on aerobic biodegradation of toluene using a mixed consortia of toluene degraders. At 8 sampling points, all controls and all three sample vials were analyzed for concentrations and carbon isotopic composition. At the final sampling point (t = 97 h), only toluene concentrations were determined in the sample vials as toluene levels were by this point below detection limit for isotopic analysis. Table 2 shows the concentration and $\delta^{13}C$ value for Controls 1 and 2 based on the mean of duplicate vials. The concentration and $\delta^{13}C$ value of residual toluene in each of the 3 sample vials are shown individually for vials 1, 2 and 3. A small decrease in concentration levels in both controls in the first few hours of the experiment (Fig. 2(a)) is due to continuing partitioning of toluene from the vapor to the aqueous phase. After approximately 20–30 h, equilibrium partitioning is reached and concentrations in the controls remain constant within reproducibility (±5%). The isotopic compositions of the control vials show no significant change over the course of the experiment (Table 2). In contrast, the sample vials degraded steadily, with concentrations decreasing to ≤ 0.05 mg/l in approximately 97 h. Despite this extensive biodegradation, Fig. 2(b) shows that the isotopic composition of the residual toluene remained identical within analytical uncertainty to its starting composition of −28.0‰. At 97 h, the $\delta^{13}C$ value of vials 1, 2 and 3 are −27.5, −27.5 and −27.3‰, respectively, all still within error of the starting isotopic composition of −28.0‰. Thus unlike TCE, residual toluene showed no significant isotopic fractionation associated with biodegradation. Identical results were found during aerobic biodegradation of toluene carried out in our laboratory using a second mixed consortia containing toluene degraders cultured from a clean unsaturated zone sand (Mulcahy, 1996). Based on 6 sets of experiments on 2 different mixed consortia of toluene degraders, aerobic biodegradation of toluene involves no significant fractionation of $\delta^{13}C$ values.

4. Discussion

Application of stable carbon isotope analysis to provide direct evidence for intrinsic bioremediation by indigenous microbial populations in the field is dependent on a number of criteria.

1. Systematic changes in $\delta^{13}C$ values, or fractionation, must occur during biodegradation.

2. Fractionation must be greater than analytical uncertainty.
3. Under a given set of conditions, the fractionation must be reproducible.
4. The effects of isotopic fractionation during biodegradation must be readily discernable from isotopic effects associated with other subsurface processes of mass attenuation such as volatilization, dissolution, and sorption.

Transport processes such as advection/dispersion/diffusion have not been shown conclusively to produce significant isotopic fractionation effects in the field even for low molecular weight hydrocarbons such as CH_4 (Stahl, 1977; Ricchuito and Schoell, 1988; Pernaton et al., 1996). Based on these results the assumption was made that transport processes are unlikely to produce an isotope effect for the larger molecular weight compounds that are the subject of this study. In previously published work, any isotopic effects associated with equilibrium volatilization and dissolution of TCE have been shown to be $< 0.5‰$ (Slater et al., 1999). Similarly, results from experiments carried out at the University of Toronto show no significant isotopic fractionation associated with sorption under equilibrium conditions. In contrast, as shown in Fig. 1(b), experiments on anaerobic biodegradation of TCE involve systematic fractionation (Criteria 1) more than an order of magnitude greater than both analytical uncertainty (Criteria 2) and greater than isotopic fractionation associated with processes such as volatilization, dissolution and sorption (Criteria 4). Furthermore, this fractionation is highly reproducible (Criteria 3). Based on a least squares regression ($r^2 = 0.98$) all data can be modelled using a simple Rayleigh closed system set of equations and a fractionation factor of $\alpha = 0.9929$. While the application of these results to either column experiments or to the field will involve modification of the model to incorporate open rather than closed system behaviour, the essential criteria is the highly reproducible correlation of $\delta^{13}C$ values of the residual TCE to the extent of biodegradation of TCE under this set of experimental conditions. Similar trends of isotopic enrichment with increasing biodegradation have been found in experiments carried out on anaerobic biodegradation of PCE in our laboratory. Results indicate a smaller degree of fractionation overall, with a change in isotopic composition of PCE from an initial isotopic composition of $-30‰$ to a final composition of $-20.8‰$ at greater than 75% degradation. Slater et al. (1997) and Dayan et al. (this volume) reported that similar Rayleigh models with larger fractionation factors can be used to describe the isotopic fractionation associated with abiotic dechlorination of TCE with zero valent iron. For chlorinated hydrocarbons such as TCE, the large, reproducible fractionation involved in anaerobic biodegradation demonstrates the clear potential of stable carbon isotope analysis as a means of identifying and monitoring intrinsic bioremediation.

In findings similar to those for chlorinated hydrocarbons, studies focusing on aromatic hydrocarbons have established that at equilibrium, processes such as volatilization, dissolution and sorption (Criteria 4) involve no fractionation of carbon isotope values $> 0.5‰$ (Slater et al., 1999; Harrington et al., this volume). Taking advantage of the greater precision ($< 0.1‰$) associated with conventional carbon isotope mass spectrometry, Harrington et al. (this volume) was able to identify fractionation of $< 0.2‰$ associated with volatilization of benzene, toluene and xylene. Unfortunately, conventional mass spectrometry and its greater precision is not routinely feasible for carbon isotope analysis of contaminants in groundwaters, where concentrations typically are in the low ppm and ppb range. For practical applications in the field, the 4–5 orders of magnitude greater sensitivity of continuous flow compound specific mass spectrometry is essential. Hence, for practical applications, the analytical uncertainty associated with continuous flow mass spectrometry (0.3‰ for reproducibility or 0.5‰ to incorporate both accuracy and reproducibility) will be the measure against which the significance of kinetic isotope effects must be assessed. In this context then, volatilization, dissolution and sorption of aromatic hydrocarbons involve no significant carbon isotope fractionation (Slater et al., 1999; Harrington et al., this volume).

Results reported by different groups investigating fractionation associated with biodegradation of aromatic hydrocarbons are less conclusive. Aerobic biodegradation of toluene by the two mixed consortia used in this study involved no significant fractionation. Two other studies report small fractionation signals ($\leq 2‰$ isotopic enrichment in contaminant residual) during toluene biodegradation under anaerobic conditions (Kelley et al., 1997) and during benzene biodegradation under aerobic conditions (Francis et al., 1997) respectively. These results imply that different microbial communities or environmental conditions may exert controls on whether or not significant fractionation is associated with biodegradation of aromatic hydrocarbons. Based on results of this study and others however, a clear pattern is emerging. While degradation of chlorinated hydrocarbons appear to be characterized by changes in $\delta^{13}C$ values of the residual contaminant on the order of many ‰, carbon isotope fractionation associated with biodegradation of aromatic hydrocarbons is much more subtle, and in many cases may be irresolvable within analytical uncertainty.

5. Conclusions

The application of stable carbon isotopes to provide direct evidence of the effectiveness of intrinsic bioremediation has generated significant interest in the field of contaminant hydrogeology. In this paper, we summarize the experimental evidence for fractionation of carbon isotope values during biodegradation and emphasize the highly compound specific nature of this application of carbon isotope analysis. Results presented in this paper indicate that it is premature to conclude stable carbon isotopes will provide definitive evidence of biodegradation of toluene. While some studies have shown small fractionation signals associated with biodegradation of aromatic hydrocarbons (Francis et al., 1997; Kelley et al., 1997), the lack of any significant isotopic fractionation signal in the aerobic biodegradation experiments carried out in this study indicate that considerably more research is required before the application of this approach to identify intrinsic bioremediation of this group of compounds can be fully assessed. If under certain microbiological/environmental conditions, the isotopic values of aromatic hydrocarbons such as toluene are found to behave conservatively instead of fractionating during biodegradation, stable carbon isotope signatures may in fact have more applicability as a means of source differentiation at sites where different spills may contribute to the overall contamination (Dempster et al., 1997). For chlorinated hydrocarbons such as TCE, however, large and reproducible fractionation signals associated with biodegradation indicate that stable carbon isotopes indeed provide a new tool for confirming that intrinsic bioremediation is taking place and for monitoring its effectiveness. It is not anticipated that this technique will replace existing protocols for validating in situ bioremediation. However, the results presented here indicate that for chlorinated hydrocarbons, successful application of compound specific stable carbon isotopes may significantly improve our ability to reliably evaluate the success of intrinsic bioremediation projects. Protocols under development for validation of in situ bioremediation strategies for chlorinated hydrocarbons should consider incorporation of compound specific carbon isotope analysis along with the more conventional microbiological and chemical criteria.

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