Environ. Sci. Technol. **2000**, *34,* 4577−4581

Hydrogen Isotope Fractionation during Methanogenic Degradation of Toluene: Potential for Direct Verification of Bioremediation

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Equilibrium headspace analysis of toluene for δ^2H isotopic composition by continuous flow compound specific isotope mass spectrometry was determined to have an accuracy and reproducibility of ±5‰. Using this analytical approach, the hydrogen isotope fractionation produced by anaerobic biodegradation of toluene was evaluated in laboratory experiments using a mixed methanogenic consortium. A large, reproducible ^{2}H-enrichment in the residual toluene of greater than 60‰ was observed at greater than 95% degradation, reflecting the preferential biodegradation of molecules containing the light (^{1}H) isotope. Recent studies evaluating the magnitude of carbon isotope fractionation produced during biodegradation of aromatic hydrocarbons have documented heavy isotope (^{13}C) enrichment in the residual contaminant approximately an order of magnitude smaller than those reported here for ^{2}H. The very large isotopic enrichment in ^{2}H suggests that under anaerobic conditions compound specific hydrogen isotope analysis may provide a more reliable means of validating intrinsic bioremediation of aromatic hydrocarbons than stable carbon isotope analysis. Combined application of stable carbon and hydrogen isotope analysis in an anaerobic groundwater has the potential to provide two important diagnostic tools. Relatively insensitive to biodegradation by mixed consortia, stable carbon isotope values may provide information about different sources of contaminant, while hydrogen isotope values provide an assessment of the degree of attenuation due to biodegradation.

Introduction

Gasoline products, in particular BTEX (benzene, toluene, ethyl benzene, and *m*-, *p*-, and *o*-xylene), are major groundwater pollutants. Many cleanup efforts have focused on bioremediation and, in particular, on in situ or intrinsic biodegradation—the ability of indigenous microbial populations to degrade organic contaminants (*1*). Current protocol designed to verify the occurrence of intrinsic biodegradation of BTEX at contaminated field sites involves documenting the loss of contaminants at the field scale, demonstrating the presence of biogeochemical indicators, and direct microbiological evidence of contaminant degradation (*2*). Difficulties in obtaining accurate mass balances of contaminant, electron acceptors, and breakdown products, and difficulties in distinguishing between contaminant mass loss due to physical processes such as sorption however, can make it difficult to provide conclusive proof of intrinsic bioremediation. Hence there has been considerable interest in a new technique—compound specific stable isotope analysis (CSIA), with the potential to provide a direct indication of biodegradation.

The elements making up the BTEX compounds, C and H, each contain two stable isotopes, one light and one heavy. Carbon consists of ^{12}C and ^{13}C, comprising 98.89% and 1.11% of the total carbon abundance, respectively. Hydrogen consists of 99.98% ^{1}H and 0.00157% ^{2}H (*3*). Because of the size of this abundance gap between the light and heavy isotopes, the ratios of heavy to light isotopes are expressed as δ values, or ‰ differences relative to a standard, where

$$\delta^{13}\mathrm{C} = (((^{13}\mathrm{C}/^{12}\mathrm{C}\ \text{sample})/(^{13}\mathrm{C}/^{12}\mathrm{C}\ \text{standard}) - 1) \times 1000 \quad (1)$$

and where

$$\delta^{2}\mathrm{H} = ((^{2}\mathrm{H}/^{1}\mathrm{H}\ \text{sample})/(^{2}\mathrm{H}/^{1}\mathrm{H}\ \text{standard}) - 1) \times 1000 \quad (2)$$

In this study δ^{13}C values are reported relative to the V-PDB standard, and δ^2H values are reported relative to V-SMOW (*3*).

Fractionation is a measure of the change in the ratio of heavy to light isotopes and hence in the δ value. Fractionation results because the differences in mass between the isotopes result in slight differences in the activation energies during reactions (*4*). Each isotope will therefore participate in reactions at slightly different rates. Bacterial degradation in particular can result in preferential degradation of the lighter isotope. This preference can result in a substrate that systematically becomes more enriched in heavy isotopes as degradation proceeds. This phenomenon is an example of the kinetic isotope effect and has been shown to occur for stable carbon isotopes during laboratory batch experiments involving degradation of both chlorinated hydrocarbons (*5*−*7*) and aromatics (*8*−*11*). For chlorinated hydrocarbons, biodegradation induced carbon isotope fractionation has also been identified in field studies (*12, 13*).

Studies to date indicate that for chlorinated hydrocarbons, large and reproducible carbon isotope fractionation associated with biodegradation can be used as an effective indicator to confirm intrinsic biodegradation. For BTEX compounds, however, the evidence for applying stable carbon isotope in the same way is less compelling. All results to date indicate that carbon isotope fractionation due to anaerobic biodegradation of aromatic hydrocarbons is small. Anaerobic biodegradation of toluene by mixed consortia under methanogenic and sulfate-reducing conditions was found to result in only a small enrichment in the carbon isotope value of the residual toluene (2.0 and 2.4‰, respectively) (*11*). Similar batch experiments carried out with pure cultures by Meckenstock et al. (*8*) documented only up to 6‰ enrichment in ^{13}C in residual toluene under denitrifying, iron-reducing, and

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10.1021/es001128j CCC: $19.00
Published on Web 09/28/2000

sulfate-reducing conditions. During aerobic biodegradation of toluene, Sherwood Lollar et al. (*6*) found no significant fractionation of carbon isotopes using a mixed consortium. Stehmeier et al. (*10*) documented only a 2‰ enrichment in ^{13}C in benzene in similar experiments. In contrast, during aerobic biodegradation of toluene carried out by the pure culture *Pseudomonas putida* mt-2, a 10‰ enrichment in ^{13}C was observed (*8*). Clearly, the extent of overall carbon isotope fractionation is culture specific. Nonetheless, the weight of evidence indicates that carbon isotopic enrichment in residual BTEX contamination during biodegradation by mixed consortia is on the order of a few ‰. Since the extent of overall carbon isotope fractionation during biodegradation of BTEX compounds by mixed consortia is relatively small, applying stable carbon isotope analysis to assess biodegradation at field sites will be a challenge. Isotope variability at the site due to the presence of different sources of contaminant with different initial $\delta^{13}C$ values (*14*, *15*) could confound the small carbon isotopic signal produced by biodegradation.

The objective of this study was to evaluate the hypothesis that stable hydrogen isotope values would provide a more definitive indicator of biodegradation for aromatic hydrocarbons. The mass difference between the two stable isotopes of carbon (^{12}C and ^{13}C) is 1/12, or approximately 8%. In contrast, the mass difference between the two stable isotopes of hydrogen (^{1}H and ^{2}H) is 1/2, or 50%. Due to this larger mass difference between the heavy and light isotopes, for any given fractionation process, hydrogen isotope fractionation is often significantly larger than carbon isotope fractionation. Here we report the results of experiments designed to characterize hydrogen isotope fractionation during anaerobic toluene degradation and compare the data with carbon isotope fractionation results that were previously determined by Ahad et al. (*11*), using the same methanogenic culture. The feasibility of equilibrium headspace analysis (sampling of the vapor phase or headspace above an aqueous solution) for measuring δ^2H values is also demonstrated.

Methodology

Experiment 1 − Assessing Reproducibility and Accuracy. The δ^2H value of the Stable Isotope Laboratory toluene working standard or "free product" (−94 ± 5‰) was determined by running 20 1.0 μL injections of toluene diluted to a concentration of 10 mg/L in pentane on the continuous flow compound specific hydrogen isotope mass spectrometer with a split setting of approximately 5:1. The Stable Isotope Laboratory mass spectrometry system consists of an HP 6890 gas chromatograph (GC) interfaced with a micropyrolysis furnace (1450 °C), in line with a Finnigan MAT Delta$^+$-XL gas source isotope ratio mass spectrometer (GC-IRMS). The mass spectrometer provides real time measurement of the $^2H/^1H$ ratio in the H_2 peak resulting from pyrolysis of the organic compound and uses an external H_2 reference gas to obtain highly precise isotopic compositions or δ^2H values. The GC column on the GC-IRMS used for analysis of the hydrogen isotope values for toluene was a Poraplot Q column (27.5 m × 0.32 mm ID), with a He flow of 2.2 mL/min. For each headspace injection the method was set at starting temperature 200 °C for 2 min and raised to 235 °C @ 10 °C/min with a hold time of 8 min.

Slater et al. (*16*) demonstrated that for toluene and TCE, during formation of an equilibrium headspace from an aqueous solution of pure organic phase liquid, the $\delta^{13}C$ isotopic composition remains unchanged within 0.5‰ (the typical accuracy and reproducibility for stable carbon isotope analysis for continuous flow compound specific analysis). Equilibrium headspace extraction (sampling the vapor phase or headspace above a free product or aqueous solution) is now a routine extraction technique for carbon isotopic analysis of dissolved VOCs. To test the validity of using the same method for hydrogen isotope analysis, experiments were carried out to measure the δ^2H values for toluene headspace over aqueous toluene standards of varying concentration. Using the Stable Isotope Laboratory toluene working standard previously characterized for δ^2H, a set of toluene standards with concentrations ranging from 2 to 100 mg/L were prepared. Each standard was made by adding a known amount of neat toluene (Fisher Scientific, 99.9779% purity) to 50 mL of distilled water in 250 mL bottles and sealed with screw-cap Mininert valves (Precision Sampling Corp.). All standards were left to equilibrate for at least 24 h prior to running. Split settings between 0.1:1 and 8:1 were used, and injection volumes varied between 50 and 1000 μL to test the extent to which these additional variables might affect δ^2H values. All headspace samples were injected into the Delta$^+$-XL mass spectrometry system using either 0.5 mL or 1.0 mL Pressure-Lok gastight syringes (Precision Sampling Corp.). All samples were within the tested H_3^+ linearity range for the Delta$^+$-XL mass spectrometry system (approximately 2−6 V).

Experiment 2 − Hydrogen Isotope Fractionation during Biodegradation of Toluene. Anaerobic biodegradation of toluene was carried out under methanogenic conditions using the same consortium for which stable carbon isotope fractionation during toluene biodegradation was previously characterized by Ahad et al. (*11*). This well-defined consortium consists of a mixed methanogenic culture enriched from creosote-contaminated aquifer sediments from Pensacola, FL that mineralizes toluene to carbon dioxide and methane (*17, 18*). Anaerobic medium was prepared as described by Edwards and Grbic-Galic (*18*) and dispensed into four sterilized 250 mL glass bottles inside an anaerobic chamber (Coy Laboratory Products). Two control incubations contained 60 mL of medium, while two active cultures received 30 mL of medium inoculated with 30 mL of the methanogenic toluene-degrading culture in suspension. All four bottles were sealed with screw-cap Mininert valves. The pH of the medium was maintained at approximately 7, and the temperature remained at 25 ± 2 °C. Each control and culture was amended with 0.075 mmol (8.0 μL) of neat toluene (Fisher Scientific, 99.9779% purity) resulting in an aqueous toluene concentration of approximately 62 mg/L.

Toluene levels were determined by comparing peak intensities on the Delta$^+$-XL mass spectrometer for toluene samples (C) to peak intensities of controls (C_o). Results are expressed as C/C_o or fraction of toluene remaining (f) and have an error of ±7%. Controls were run on day 7, 15, 18, and 19. Hence, toluene concentration levels were only determined on the days that controls were run. Hydrogen isotope analysis of the headspace toluene for cultures was carried out on day 6, 7, 12, 15, 16, 18, and 19. Split settings between 0.1:1 and 1.1 were used, and injection volumes varied between 400 and 1000 μL giving total concentration on column that ranges between 4 and 25 nmol. Reproducibility on δ^2H values is ±5‰ based on the standard analysis carried out in Experiment 1.

Results and Discussion

Equilibrium Headspace Analysis. Figure 1shows the results from Experiment 1. δ^2H values determined for toluene headspace samples in equilibrium with aqueous solutions of the Stable Isotope Laboratory toluene working standard in concentrations from 2 to 100 mg/L are shown. The amount of toluene injected onto the GC column incorporates different injection volumes, concentrations, and split settings and ranges from 6 to 67 nmoles. The mean δ^2H value for all headspace samples is −100 ± 5‰ (1 standard deviation). The estimate of total uncertainty (±5‰) incorporates all the samples regardless of injection volume, concentration, split

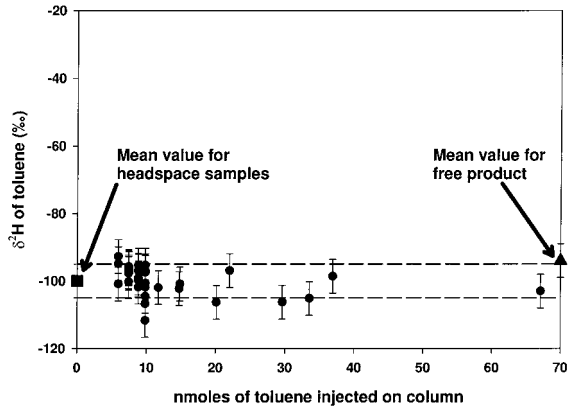

FIGURE 1. Reproducibility and accuracy for δ^2H values obtained for injections of equilibrium headspace produced over aqueous solutions of the toluene working standard in Experiment 1. Toluene headspace injections are shown in solid circles. The mean value for all injections is shown by the solid square (-100 ± 5‰). The dashed lines mark the standard deviation ($\pm$5‰) around the mean. Based on this experiment, vertical error bars representing a reproducibility ($\pm$5‰) are assigned for each injection. The mean δ^2H value for the toluene working standard in the free product phase is shown by the solid triangle (-94 ± 5‰) and is within error of the mean value obtained by headspace extraction (see text).

setting, or syringe size since none of these variables had a systematic effect on δ^2H values within the limits tested. This estimate of uncertainty is in good agreement with the mass spectrometer manufacturer's specification for continuous flow compound specific hydrogen isotope analysis. Clearly, there is a small absolute shift in δ^2H values between the direct injection of the toluene working standard or "free product" (-94 ± 5‰), and the mean value determined by headspace extraction (-100 ± 5‰). This difference may reflect a small isotopic shift occurring during volatilization, similar to that noted for δ^{13}C values by Harrington et al. (*15*), Slater et al. (*16*), and Poulson and Drever (*19*). As was the case for these earlier carbon isotope studies however, the total isotopic shift in $\delta^2H_{controls}$ seen in Figure 1 is less than the typical error ($\pm$5‰ reproducibility) associated with continuous flow compound specific isotope analysis. Whatever the source of this minor isotopic shift, the mean δ^2H value determined for toluene by headspace extraction (-100 ± 5‰) is within error of the value determined for the toluene working standard or "free product" (-94 ± 5‰).

Hydrogen Isotope Fractionation during Methanogenic Biodegradation of Toluene. Having established that equilibrium headspace analysis is accurate and reproducible within $\pm$5‰, this technique was applied throughout Experiment 2. In Figure 2, the fraction of residual toluene remaining is plotted for both culture bottles. The experiment was continued until toluene reached the lowest concentration limit that could be analyzed for δ^2H values by the equilibrium headspace technique (approximately 2 mg/L). Complete degradation of toluene by the methanogenic consortium took approximately 19 days. Methane levels in the culture bottles increased over the course of the experiment, confirming that toluene degradation was proceeding via methanogenesis.

Figure 3 shows δ^2H values for both culture bottles and controls versus time throughout the 19 days of the experiment. All δ^2H values were assigned a value of $\pm$ 5‰ based on the accuracy and reproducibility determined in Experiment 1. The isotopic value at time zero (-100 ± 5‰) was based on the mean value for the Stable Isotope Laboratory working standard determined by equilibrium headspace extraction in Experiment 1. While the controls are always within error of the starting δ^2H value, and the reproducibility between the two controls is excellent at each time point,

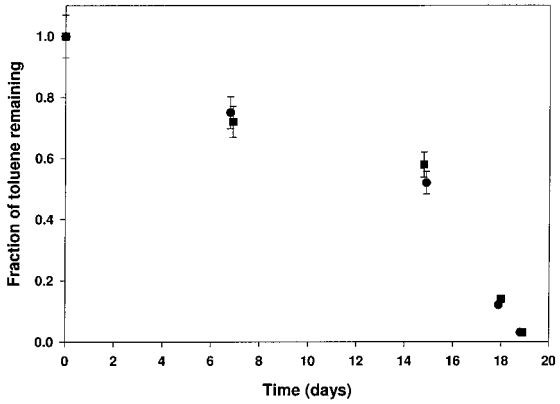

FIGURE 2. Fraction of toluene remaining versus time (days) during anaerobic biodegradation of toluene by the methanogenic consortium. Toluene culture bottles undergoing degradation are shown by solid circles and squares. Toluene degradation took place at approximately the same rate in both culture bottles. Error bars represent ($\pm$7%).

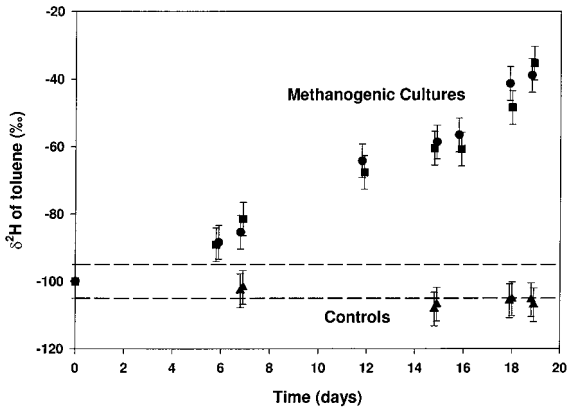

FIGURE 3. Plot shows δ^2H values for residual toluene versus time (days) during anaerobic biodegradation of toluene by the methanogenic consortium. Toluene culture bottles undergoing degradation are shown by solid circles and squares. The δ^2H value for toluene at time zero is based on the mean value determined for headspace analysis of the Stable Isotope Laboratory toluene working standard in Experiment 1 (-100 ± 5‰). Dashed lines represent the standard deviation ($\pm$5‰) around this starting value (δ^2H_o). Controls are shown in solid triangles. While the δ^2H values for the controls do shift over the course of the experiment, they remain within error of δ^2H_o (see text). Error bars on controls and samples are $\pm$5‰ (see text).

there is clearly a small isotopic depletion in the control bottles during the 19 day experiment. This shift was within error of the measurement and also in the opposite direction to the isotopic shifts in the culture bottles and therefore does not negate the results from the culture bottles.

As shown in Figure 3, the isotopic fractionation in the culture bottles during the experiment was substantial and involved an enrichment in ^{2}H in the residual toluene of > 60‰, reflecting the preferential biodegradation of ^{1}H-containing molecules. Culture bottles showed a progressive enrichment in ^{2}H of the residual toluene from -100‰ at time zero, to -37.1‰ (based on the average of both culture bottles at the last sampling point). Consistent with the experimental hypothesis, hydrogen isotope fractionation is more than an order of magnitude greater than that previously found for carbon isotope fractionation (2.0 ± 0.5‰) during anaerobic biodegradation of toluene by the same culture in Ahad et al. (*11*) (Figure 4).

For both chlorinated hydrocarbons and aromatic hydrocarbons such as toluene, the relationship between carbon

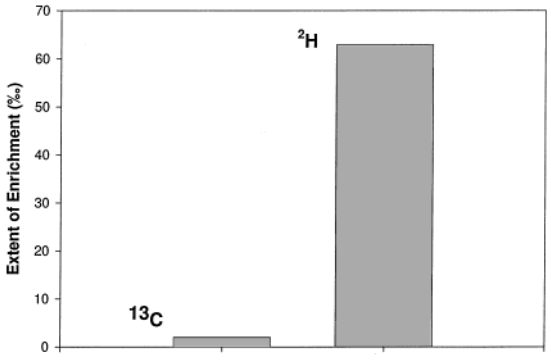

FIGURE 4. Comparison of the extent of heavy isotope enrichment (in ‰) in residual toluene during anaerobic biodegradation by the same methanogenic consortium. ^{13}C enrichment data obtained from Ahad el al. (*11*) and ^{2}H data is from this study.

isotope fractionation and the extent of biodegradation has been modeled by a simple isotopic model, known as the Rayleigh model (*6, 8, 11, 20*). The Rayleigh model assumes a constant isotopic preference during a reaction, which is described by a fractionation factor α, where

$$\alpha = (1000 + \delta a)/(1000 + \delta b) \qquad (3)$$

and where δa and δb are the isotopic values of the initial substrate and residual substrate, respectively (*3*). The Rayleigh model can be applied to a single order one-step process where α is determined by a least squares regression of a plot of ln(f) versus $\ln((\delta^2H_{toluene}/1000 + 1)/(\delta^2H_o/1000 + 1))$ with a slope of $(\alpha - 1)$ (*21*−*23*). For the results of Experiment 2, the r^2 for such a regression is poor, suggesting that hydrogen isotope fractionation during methanogenic biodegradation of toluene is not controlled by a single order one step reaction. Values of α calculated by substituting measured δ values into eq 3 for each sampling point indicate that the extent of hydrogen isotope fractionation during methanogenic biodegradation changes (from $\alpha = 0.988$ to $\alpha = 0.935$) as a function of the fraction of toluene remaining. Values of α are in excellent agreement at each sampling point between the two culture bottles. This consistency indicates that variability in α is not a function of analytical error but reflects an actual decrease in α. Hydrogen isotope fractionation is apparently controlled by a non-Rayleigh, multistep process reflected by a changing fractionation factor (α). This result does not mean that the system is unpredictable however, since α values obtained at each sampling point from duplicate culture bottles are highly reproducible.

Implications for Intrinsic Bioremediation. The large ^{2}H-enrichment in residual toluene of >60‰ produced by methanogenic anaerobic degradation of toluene confirms the hypothesis that for aromatic hydrocarbons hydrogen isotopes will show a much larger isotopic degradation signal than stable carbon isotopes. The details of carbon isotope fractionation during degradation, however, have been seen to be a function of different microbial species, of mixed versus pure cultures, and of different electron acceptors (*6, 8, 10, 11*); therefore, it will be necessary to carry out studies on a variety of different biodegradation systems before the controls on the extent of ^{2}H enrichment during biodegradation are well understood. Similarly, although the results of this study indicate that dissolution and volatilization do not change the δ^2H value of toluene within error (±5‰) of the value for the free product, additional physical factors such as sorption that could cause fractionation have not been studied directly. The magnitude of any such fractionation would need to be very large to mask the pronounced >60‰ fractionation associated with biodegradation. For stable carbon isotope analysis, the total amount of fractionation associated with biodegradation of aromatic hydrocarbons has been shown to be relatively small (*6, 8, 10, 11*). This is particularly true for mixed consortia typical of field situations. Total stable carbon isotope fractionation of only a few ‰ will likely be difficult to use as a definitive indicator of the effects of biodegradation. In contrast, the dramatic isotopic fractionation in the hydrogen isotopic value of residual toluene during anaerobic biodegradation indicates that hydrogen isotope analysis has the potential to be a powerful diagnostic tool for identifying and monitoring intrinsic biodegradation in the field. In fact, if the relatively insensitive behavior of stable carbon isotopes with respect to biodegradation by mixed consortia reported in the recent literature is upheld, combined stable carbon and hydrogen isotopic analysis offers a unique opportunity for field investigations. Studies have shown that aromatic hydrocarbons derived from different sources can have isotopically distinct $\delta^{13}C$ values (*14, 15*). Combined analysis of both $\delta^{13}C$ and δ^2H values for dissolved toluene in an anaerobic groundwater have the potential to provide both information about different sources of contamination at the site (from the relatively conservative $\delta^{13}C$ signatures) as well as information about the degree of contaminant attenuation due to biodegradation (from the strongly fractionating δ^2H values).

Acknowledgments

The authors wish to thank Emmanuel Francois of the Department of Chemical Engineering and Applied Chemistry, University of Toronto, and Dr. Scott Smith of the Department of Geology, University of Toronto for assistance with maintenance of the microbial cultures. Funding for this project was made available through the Natural Sciences and Engineering Research Council of Canada E.W.R. Steacie Supplement Grant to B.S.L.

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Received for review March 24, 2000. Revised manuscript received August 3, 2000. Accepted August 11, 2000.

ES001128J