

Carbon and Hydrogen Stable Isotope Fractionation during Aerobic Bacterial Degradation of Aromatic Hydrocarbons†

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¹³C/¹²C and D/H stable isotope fractionation during aerobic degradation was determined for *Pseudomonas putida* strain mt-2, *Pseudomonas putida* strain F1, *Ralstonia pickettii* strain PKO1, and *Pseudomonas putida* strain NCIB 9816 grown with toluene, xylenes, and naphthalene. Different types of initial reactions used by the respective bacterial strains could be linked with certain extents of stable isotope fractionation during substrate degradation.

Intrinsic microbial degradation is an important process in elimination of contaminants in polluted aquifers, which can be used for the sustainable cleanup of contaminated sites. However, cost-effective remediation strategies such as natural attenuation require a profound knowledge of the microbial degradation processes in the subsurface. Although biodegradation of aromatic hydrocarbons by aerobic and anaerobic bacteria has been investigated in detail in laboratory systems (11, 32), assessment at field sites remains difficult. Stable carbon isotope analysis is one approach to quantify microbial activities in situ. For laboratory cultures, isotope fractionation has been shown to occur during degradation of aromatic hydrocarbons, such as toluene (1, 19, 20), or chlorinated hydrocarbons, such as trichloroethene (3, 7, 26). In addition, in contaminated field sites, carbon isotope fractionation could be observed and was interpreted to be indicative of microbial degradation in situ (15, 24). For toluene as a model compound, it has been demonstrated that isotope fractionation is caused mainly by the first enzyme reaction in the degradation pathway, whereas transport to and into the cell appears not to be relevant for fractionation. The extent of isotope fractionation is considered to be independent of differences in the growth kinetics of the bacteria (20). Isotope fractionation during anaerobic degradation of toluene was on the same order of magnitude for denitrifying, iron (III)-reducing, sulfate-reducing, and fermenting bacteria (1, 19), probably because, in these cases, degradation was initiated by benzylsuccinate synthase. This finding suggests that, in anoxic environments, isotope fractionation could be applied to assess biological degradation quantitatively, as has been worked out recently for several aquifers (23, 25).

The objective of this study was to examine whether carbon and hydrogen isotope fractionation could be used to quantify intrinsic biodegradation as well in oxic environments. Previous studies with the aerobic bacterium *Pseudomonas putida* strain mt-2 showed an extent of isotope fractionation similar to that of anaerobic toluene-degrading strains (19), whereas isotope fractionation during toluene degradation by undefined aerobic microbial communities was not detected (26). Therefore, we started a systematic investigation of the effects of different oxygenase enzymes and stable isotope fractionation.

P. putida strain mt-2 (20), *Ralstonia pickettii* strain PKO1 (J. J. Kukor, Rutgers University, New Brunswick, N.J.), and *P. putida* strain F1 (A. M. Cook, Konstanz, Germany) were taken as representatives of bacteria by using different toluene degradation pathways in polluted aquifers. Strains were grown in 200-ml batch cultures with 15 μ l of toluene, *m*-xylene, or *p*-xylene (analytical grade quality; Fluka, Buchs, Switzerland) as described previously (20). Cultures for isotope fractionation experiments were inoculated with 5% (vol/vol) of precultures (optical density at 578 nm [OD₅₇₈] = 0.25), and growth was monitored by measuring the OD₅₇₈. Hydrocarbon concentrations were determined by high-performance liquid chromatography (20). During degradation of toluene by *P. putida* strain mt-2 (methyl monooxygenase), the ¹³C/¹²C isotope ratios in the nondegraded toluene were determined by gas chromatography–combustion–isotope ratio monitoring mass spectrometry (GC-C-IRMS) (20) and shifted from $\delta^{13}\text{C} = -29.18\text{‰} \pm 0.35\text{‰}$ to more positive values of $\delta^{13}\text{C} = -23.22\text{‰} \pm 0.32\text{‰}$ after 80% of the substrate was degraded (equation 4) (Fig. 1). Analysis of the carbon isotope ratios $\ln(R_t/R_0)$ and the respective concentrations $\ln(c_t/c_0)$ according to the Rayleigh equation for closed systems (equation 1) (13, 22) resulted in an isotope fractionation factor: $\alpha_C = 0.9967 \pm 0.0003$ (Fig. 2A). R_t and c_t are the ¹³C/¹²C isotope ratio and the residual substrate concentration at time t , and R_{Std} is the isotope ratio of an international standard (Vienna PDB).

For direct comparison between the bacterial pure cultures, isotope fractionation will be referred to as “enrichment factor ϵ ” (equation 2), which is indicated as ϵ_n in experiments with unlabeled toluene. During toluene degradation by *P. putida*

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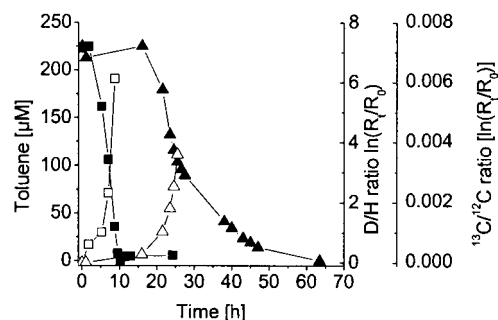


FIG. 1. Isotope shifts during degradation of toluene and a toluene-toluene- d_8 mixture by *P. putida* strain mt-2. The decrease in toluene (■) and concomitant increase in ^{13}C ($R = ^{13}\text{C}/^{12}\text{C}$) in the remaining substrate (□) and the decrease in toluene-toluene- d_8 concentration (▲) and concomitant increase in deuterium $R_t = [\text{toluene-}d_8]/[\text{toluene}]$ in the residual nondegraded substrate fraction (△) were monitored over time. In every case, data are shown for one representative experiment out of three performed.

strain mt-2, the enrichment factor was $\epsilon_n = -3.3 \pm 0.3$ for $^{13}\text{C}/^{12}\text{C}$ isotope fractionation (Table 1).

$$\frac{R_t}{R_0} = \left(\frac{c_t}{c_0}\right)^{(\alpha C - 1)} \quad (1)$$

$$\epsilon = (\alpha - 1) \times 1,000 \quad (2)$$

$$\ln\left(\frac{R_t}{R_0}\right) = \ln\left[\frac{\left(\frac{c_t}{c_0}\right)}{(1 + R_t)/(1 + R_0)}\right] \times (\alpha H - 1) \quad (3)$$

$$\delta^{13}\text{C} = \left(\frac{R_{\text{Sample}}}{R_{\text{Std}} - 1}\right) \times 1,000 \quad (4)$$

P. putida strain mt-2 also degrades *m*- and *p*-xylene by using the same enzyme as toluene (methyl monooxygenase) (29). The enrichment factors for carbon isotope fractionation obtained during degradation of *m*- and *p*-xylene were slightly smaller than those of toluene (Table 1).

Toluene degradation by *R. pickettii* strain PKO1 (ring monooxygenase) resulted in a carbon isotope fractionation that was three times lower than that with strain mt-2. The third toluene-degrading strain, *P. putida* strain F1, hydroxylated the substrate with a toluene-dioxygenase to the corresponding catechol. The concomitant $^{13}\text{C}/^{12}\text{C}$ isotope fractionation was insignificant. The same was observed with *P. putida* strain NCIB 9816 in degradation experiments with naphthalene (DSM 8368; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Strain NCIB 9816 attacks naphthalene by a dioxygenase reaction similar to that of strain F1. It was grown in batch culture with 150 to 200 μM dissolved naphthalene as a carbon source. Naphthalene was transferred into the culture bottles as a solid crystal and autoclaved in 180 ml of H_2O . After cooling, the stock solutions for mineral medium M9 were added. The carbon isotope ratio in the residual naphthalene did not change during degradation over time, and the isotope fractionation was not significant (Fig. 2A and Table 1).

Hydrogen isotope fractionation during degradation of aro-

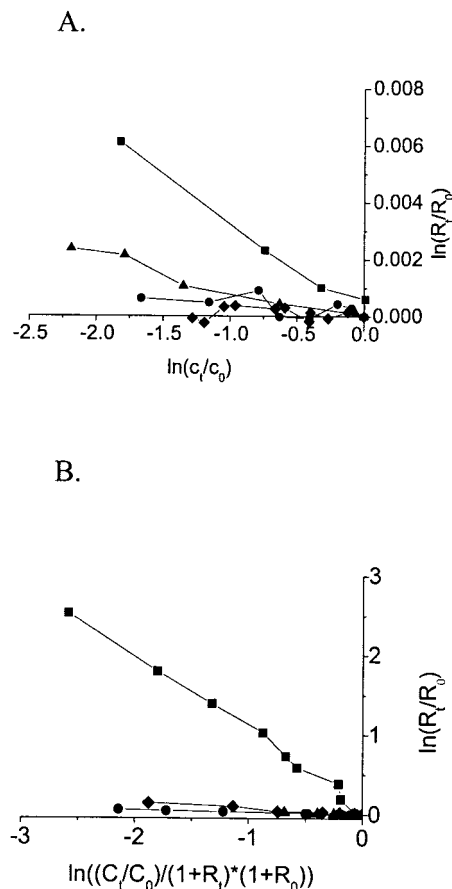


FIG. 2. Carbon (A) and hydrogen (B) stable isotope fractionation during degradation of toluene by *P. putida* mt-2 (■), *R. pickettii* PKO1 (▲), and *P. putida* F1 (●) and isotope fractionation during degradation of naphthalene by *P. putida* NCIB 9816 (◆). $^{13}\text{C}/^{12}\text{C}$ carbon isotope fractionation was plotted according to equation 1. D/H isotope fractionation was determined with equation 3 during growth with equal amounts of per-deuterated toluene- d_8 and toluene and per-deuterated naphthalene- d_8 and naphthalene.

matic hydrocarbons was investigated by cultivating the same four bacterial strains with a mixture of nondeuterated toluene and per-deuterated toluene- d_8 (8 μl each) or with a mixture of nonlabeled naphthalene and per-deuterated naphthalene- d_8 (3 mg each). The ratio of residual labeled to nonlabeled substrate in the culture during growth was analyzed in pentane extracts of liquid samples (2 to 7 ml) by GC (20). Naphthalene and per-deuterated naphthalene- d_8 were separated by GC to the baseline at a constant oven temperature of 140°C. Isotope fractionation factors were calculated according to equation 3 with the isotope ratio $R_t = [\text{deuterated substrate}]/[\text{substrate}]$ (2). For a very high abundance of the heavier isotope, as was used in the experiments for hydrogen isotope fractionation, equation 1 changes to equation 3 (2).

Enrichment factors derived from equation 3 are depicted as ϵ_r . Degradation of half of the substrate mixture by *P. putida* strain mt-2 (methyl monooxygenase) was accompanied by a 40-fold enrichment of per-deuterated toluene- d_8 , correlated with a near-total depletion of nonlabeled toluene (Fig. 1). The

TABLE 1. $^{13}\text{C}/^{12}\text{C}$ and D/H isotope fractionation by different aerobic bacterial strains during growth with aromatic hydrocarbons^a

Strain	Enzyme mechanism	Carbon source	ϵ_{C}	ϵ_{D}
<i>P. putida</i> strain mt-2	Methyl monooxygenase	Toluene	-3.3 ± 0.3	-905 ± 71
	Methyl monooxygenase	<i>m</i> -Xylene	-1.7 ± 0.04	ND ^b
	Methyl monooxygenase	<i>p</i> -Xylene	-2.3 ± 0.3	ND
<i>R. pickettii</i> strain PKO1	Ring monooxygenase	Toluene	-1.1 ± 0.2	-16 ± 5.3
<i>P. putida</i> strain F1	Ring dioxygenase	Toluene	-0.4 ± 0.3	-28 ± 10
<i>P. putida</i> strain NCIB 9816	Ring dioxygenase	Naphthalene	-0.1 ± 0.2	-65 ± 13

^a Stable carbon isotope fractionation is given as the enrichment factor, ϵ_{C} , and was calculated with equations 1 and 2. Hydrogen isotope fractionation with mixtures (50:50) of per-deuterated and nonlabeled substrate was determined with equation 3. The enrichment factor for labeled substances, ϵ_{D} , was determined by equation 2. Mean values \pm standard deviation are given based on three independent growth experiments with fivefold isotope analysis per data point.

^b ND, not determined.

D/H isotope fractionation determined in the first growth phase was $\epsilon_{\text{D}} = -905 \pm 71$. *R. pickettii* strain PKO1 (ring monooxygenase) and *P. putida* strain F1 (ring dioxygenase) degraded the toluene–toluene-*d*₈ mixture, resulting in D/H isotope fractionations more than 10 and 50 times stronger than the carbon isotope fractionation by the same strains, but still 50 and 30 times lower, respectively, than the D/H fractionation by *P. putida* strain mt-2. *P. putida* strain NCIB 9816 (ring dioxygenase) grown with naphthalene–naphthalene-*d*₈ showed a D/H isotope fractionation that was twice as strong as the D/H isotope fractionation by *P. putida* strain F1 (Fig. 2B and Table 1).

Of the four strains examined, *P. putida* strain mt-2 showed by far the highest $^{13}\text{C}/^{12}\text{C}$ and D/H isotope fractionation during aerobic toluene degradation. The methyl monooxygenase of this bacterium exhibits similarities in its amino acid sequence to the methane monooxygenase of *Methylosinus trichosporium* OB3b (12, 33). Oxygen bound as an oxene group to the catalytic iron of methane monooxygenase is proposed to lead to a homolytic C-H bond cleavage of the substrate. The reaction is associated with strong isotope effects during methane conversion and minor effects during ethane and propane conversion (4). The authors assume that the reaction mechanism with methane differs slightly from that with other substrates. Similarly, in our study, the xylene monooxygenase of *P. putida* strain mt-2 caused strong hydrogen and carbon isotope fractionation. Minor differences in the enzymatic reactions with toluene and *m*- and *p*-xylene might be the reason for the variations in the respective $^{13}\text{C}/^{12}\text{C}$ isotope fractionation factors. Cytochrome P450 is another well-known example of an enzyme that dissociates a C-H bond and inserts oxygen into benzylic or aliphatic substrates (35). In an experimental study, Manchester et al. (18) found the strongest isotope effects with *p*-xylene and toluene, which showed six times higher rate constants than deuterated *p*-xylene and toluene.

Toluene degradation by *R. pickettii* strain PKO1 is catalyzed by a monooxygenase reaction hydroxylating carbon 3 of the aromatic ring (21). The amino acid sequence of this toluene-3-monooxygenase is remarkably similar to that of toluene-4-monooxygenase of *Pseudomonas mendocina* strain KR1, an enzyme studied in great detail (31). The poor hydrogen isotope fractionation of strain PKO1 is probably due to an enzymatic reaction with the π -electron system of the aromatic substrate. The hydroxylation presumably is a two-step process starting with an electrophilic attack of iron-bound oxygen on the aro-

matic ring and the formation of a C-O σ -bond. In a faster second step, the hydrogen atom bound to this carbon atom is released as a proton, and the electrons are used to reconstitute the aromaticity of the carbon ring skeleton (5). The weak carbon and hydrogen isotope fractionation observed here might be due to secondary isotope effects caused by atoms of the substrate molecule not directly contributing to the reaction.

P. putida strains F1 and NCIB 9816 both attack toluene through an initial dioxygenase reaction on the aromatic ring (6, 16, 34). Initially, Π -electrons of the aromatic substrate are attracted by activated oxygen, which is bound to a catalytic iron center (28). Primary isotope effects do not accompany this reaction, because there is no distinct bond cleavage involved. The first reaction product of toluene and naphthalene oxidation is a *cis*-dihydrodiol, which is dehydrogenated in a subsequent step to form a catechol derivative (9). The terminal oxygenase units of toluene and naphthalene dioxygenase of strains F1 and NCIB 9816 are both hexamers ($\alpha_3\beta_3$) with catalytic mononuclear ferrous iron centers (8, 17, 30). The amino acid sequences of their subunits suggest that they derived from a common ancestor (12) and share the same reaction mechanism (10). Accordingly, $^{13}\text{C}/^{12}\text{C}$ isotope fractionation was very weak during toluene degradation by *P. putida* strain F1 and was not significant during naphthalene degradation by *P. putida* strain NCIB 9816. The more pronounced secondary D/H isotope fractionation effects of *P. putida* strain NCIB 9816 might be caused by individual features of the naphthalene degradation mechanism.

Recently, Hunkeler et al. (14) found relatively small amounts of $^{13}\text{C}/^{12}\text{C}$ and D/H isotope fractionation during benzene degradation by *Acinetobacter* sp. and *Burkholderia* sp. Others observed a small amount of $^{13}\text{C}/^{12}\text{C}$ isotope fractionation during benzene degradation by an aerobic mixed culture that was enriched from groundwater of a petrochemical site (27). The small isotope fractionation factors and the increase in $\delta^{13}\text{C}$ for the initial mono- or dioxygenase reactions at the benzene ring that were documented in these papers are in agreement with our observations that oxygenases acting on Π -electron system of the aromatic ring produce only minor isotope effects.

The present study and previous investigations allow some preliminary conclusions to be drawn about when stable isotope fractionation could be used to assess biodegradative activities in contaminated groundwater. Consistent carbon isotope frac-

tionation during toluene degradation by anaerobic microorganisms allows a quantitative assessment of bacterial degradation under anoxic conditions. Hydrogen isotope effects are stronger than carbon isotope effects by 2 or 3 orders of magnitude, but are also more variable between different types of anaerobic bacteria. Small extents of degradation are detectable, but quantification *in situ* might be difficult. However, D/H and $^{13}\text{C}/^{12}\text{C}$ isotope fractionation in oxic zones of contaminated aquifers could be used only as a qualitative indicator of biodegradation. The differences in isotope fractionation between bacterial strains with different oxygenase reactions preclude a quantitative assessment of *in situ* biodegradation based on isotope fractionation in oxic zones. Nevertheless, combined measurement of carbon isotope fractionation for *in situ* quantification and hydrogen isotope fractionation as a positive indicator of bacterial activities could be a valuable tool to assess biodegradation in the environment.

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