The use of isotopic and lipid analysis techniques linking toluene degradation to specific microorganisms: applications and limitations

Jiasong Fang[a],*, Nanh Lovanh[b], Pedro J.J. Alvarez[b,1]

[a] *Department of Geological and Atmospheric Sciences, Iowa State University, Ames, IA 50011-3212, USA*
[b] *Department of Civil and Environmental Engineering, The University of Iowa, Iowa City, IA 52242-1527, USA*

Abstract

Phospholipid fatty acid (PLFA) analysis combined with ^{13}C-labeled tracers has been used recently as an environmental forensics tool to demonstrate microbial degradation of pollutants. This study investigated the effectiveness and limitations of this approach, applied to the biodegradation of toluene by five reference strains that express different aerobic toluene degradation pathways: *Pseudomonas putida* mt-2, *P. putida* F1, *Burkholderia cepacia* G4, *B. pickettii* PKO1, and *P. mendocina* KR1. The five strains were grown on mineral salts base medium amended with either 10 mM natural or [^{13}C-ring]-labeled toluene. PLFA analysis showed that all five strains incorporated the toluene carbon into membrane fatty acids, as demonstrated by increases in the mass of fatty acids and their mass-spectrometry fragments for cells grown on ^{13}C-labeled toluene. Because of its ubiquitous presence and high abundance in bacteria, C16:0 fatty acid might be a useful biomarker for tracking contaminant degradation and ^{13}C flow. On the other hand, the ^{13}C-label (which was supplied at relatively high concentrations) generally exerted an inhibitory effect on fatty acid biosynthesis. Differences in fatty acid concentrations between cells grown on natural versus ^{13}C-labeled toluene would affect the interpretation of lipid profiles for microbial community analysis as indicated by principal component analysis of fatty acids. Therefore, caution should be exercised in linking lipid data with microbial population shifts in biodegradation experiments with ^{13}C-labeled tracers.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Toluene; ^{13}C-label; Stable isotopes; Fatty acids; GC/MS; *Pseudomonas*; *Burkholderia*

1. Introduction

Aromatic hydrocarbons such as benzene, toluene, ethylbenzene and xylenes (i.e., BTEX) are common ground water pollutants that threaten water supplies and public health [1,2]. BTEX released into the subsurface are subjected to a variety of mass transfer or removal processes. Physical (advection, dispersion), chemical (volatilization, sorption, dissolution), and biological processes (microbial degradation) control the fate and transport of the contaminants in the subsurface. Intrinsic bioremediation refers to the action of microorganisms to degrade contaminants in situ under natural conditions without human intervention to enhance microbial activities. To assess the efficiency of intrinsic bioremediation, it is essential to ensure that changes in concentration of a contaminant are due to biodegradation reactions rather than abiotic weathering processes. Therefore, verifying whether bioremediation candidate bacteria degrade hydrocarbons is a pressing issue in contaminant hydrogeology.

*Corresponding author. Tel.: +1-515-294-6583; fax +1-515-294-6049.

E-mail address: jsfang@iastate.edu (J. Fang).

[1] Current address: Department of Civil and Environmental Engineering, Rice University, Houston, TX 77251-1892, USA.

Among monoaromatic hydrocarbons, toluene is the most studied compound. Biodegradation of toluene has been extensively studied in the laboratory, mostly with *Pseudomonas* species [3–12]. Recently, ^{13}C-labeled tracers have been applied to both laboratory-controlled microcosm experiments and field studies to verify the biodegradation of toluene [13–20] and other pollutants [21,22]. Another promising bioremediation forensics approach is the use of microbial lipids as indicators of toluene degradation and substrate utilization [17,23–25]. Lipids are primary components of bacterial cell membrane, accounting for 40–70% of the membrane mass [26]. Lipid profiling has been used to detect changes in microbial communities in contaminated aquifers [27–30]. The analysis of lipids combined with the use of ^{13}C-labeled tracers allows effective tracking of substrate usage and carbon flow during biodegradation of pollutants. However, the limitations of this approach have not been evaluated.

In this study, we used ^{13}C-labeled toluene as substrate to characterize cell membrane phospholipid profiles for five reference *Pseudomonas* strains and demonstrate the incorporation of substrate carbon into microbial membrane lipids. In doing so, information was obtained about the applicability and limitations of ^{13}C-based tracer approach to assess biodegradation and in situ bioremediation.

2. Materials and methods

2.1. Microbial strains

Pseudomonas putida mt-2 (ATCC# 33015), *P. putida* F1 (ATCC# 700007), *Burkholderia cepacia* G4 (ATCC# 10856), *B. pickettii* PKO1 (ATCC# 700590), and *P. mendocina* KR1 (ATCC# 25412), were selected for this study based on the fact that they harbor different toluene degradation pathways (i.e., the TOL, TOD, TOM, TBU, and T4MO pathways [4,5,8]. All seven toluene carbons are incorporated into Krebs cycle in the TOD (PpF1), TOM (G4), TBU (PKO1), and T4MO (KR1) pathways, and six carbons in the TOL (mt-2) pathway [4,5,8].

All cells were grown at 25°C on mineral salts base medium (MSM) [31] in 250-mL bottles amended with 10 mM toluene as sole carbon source. After 5 days of incubation, 1-mL samples were transferred to 500-mL Pyrex media bottles with 250 mL MSM, and the initial optical density was measured spectrophotometrically at 660 nm (OD$_{660}$). Toluene was introduced in the vapor phase by a glass bulb suspended from the flask stopper, as described by Gibson et al. [32]. For each strain, two sets were prepared in duplicate. One set was exposed to natural toluene, and the other to [^{13}C-ring]-labeled-toluene (Cambridge Isotope Laboratories, Andover,

MA). All cultures were incubated for four weeks before harvesting for phospholipid fatty acid analyses. The OD$_{660}$ of each culture was measured again at this time, and the dry cell weight concentration (W, mg L^{-1}) was estimated using the following correlation [33]:

$$W = 364.74(OD_{660}) + 6.7(OD)_{660}^2.$$

2.2. Lipid extraction

Total lipids were extracted with a modified Bligh and Dyer extraction method [26,34]. Approximately 7.5 mL of liquid bacteria culture was added to a test tube filled with 22.5 mL of methanol, dichloromethane (DCM), and phosphate buffer (2:1:0.8) extraction solution. The extraction mixture was allowed to stand overnight in darkness at 4°C. The lipids were then partitioned by adding DCM and water such that the final ratio of DCM-methanol-water was 1:1:0.9. The upper aqueous phase was discarded and the lower organic phase was then decanted through a cellulose #4 filter into a test tube. The solid residue retained on the filter was washed with 3×1 mL dichloromethane. The total lipid extract was dried under a gentle stream of nitrogen and was once again dissolved in methanol. Total lipids were separated into different lipid classes using miniature champagne columns (Supelco Inc., Bellefonte, PA). Neutral lipids, glycolipids, and phospholipids were eluted with 4 mL of chloroform, acetone, and methanol, respectively [34].

2.3. Analysis of fatty acid methyl esters by gas chromatography/ mass spectrometry (GC/MS)

Ester-linked phospholipid fatty acids were subjected to a mild alkaline trans-methylation procedure to produce fatty acid methyl ester [34]. The fatty acid methyl esters were analyzed on an Agilent 6890 GC interfaced with an Agilent 5973N Mass Selective Detector. Analytical separation of the compounds was accomplished using a 30 m × 0.25 mm i.d. DB-5 MS fused-silica capillary column (J&W Scientific, Folsom, CA). The column temperature was programmed from 50°C to 120°C at 10°C/min, then to 280°C at 3°C/min. Individual compounds were identified from their mass spectra. Response factors were obtained for each compound using duplicate injections of quantitative standards at five different concentration levels. Concentrations of individual compounds were obtained based on the GC/MS response relative to that of an internal standard (C22:0 fatty acid ethyl ester), after correction for recovery efficiency using a C18:0 fatty acid ethyl ester.

Method blanks were extracted with each set of samples and were assumed to be free of contamination if chromatograms of the blanks contained no peaks. A

standard containing known concentrations of 11 fatty acids was analyzed daily on the GC/MS to check analytical accuracy (> 90%). Replicate analyses (2 ×) of samples were done to ensure reproducibility (variation ⩽ 10%).

Double-bond position and geometry of monounsaturated fatty acids were determined by using methods described by Dunkleblum et al. [35]. Fatty acids are designated by the total number of carbon atoms to the number of double bonds (i.e., a 16 carbon alkanoic acid is C16:0). The position of the double bond is indicated with a Δ number closest to the carboxyl end of the fatty acid molecule with the geometry of either c (*cis*) or t (*trans*). The cyclopropyl group is indicated with *cyc*.

2.4. Principal component analysis

Principal component analysis (PCA) (JMP, SAS Institute, Inc., Gary, NC) was conducted to compare the lipid profiles of the bacteria grown on natural versus [^{13}C-ring] labeled toluene. Phospholipid fatty acid concentrations were analyzed without any sort of data normalization, and there were no null values in the data set. The original variables (phospholipid fatty acids) were orthogonally transformed and new uncorrelated (or orthogonal) variables called principal components (PCs) were extracted consecutively [36]. The PCs are independent of each other. The first PC accounts for most of the original variability; the second factor contains the second largest variability, etc., as indicated by the eigenvalues. The difference of fatty acid profiles can be evaluated based on principal component loadings plots (e.g., Fig. 1).

3. Results

3.1. Fatty acid profiles

Major fatty acids identified in the five pseudomonad strains grown on natural toluene include C16:0, the *cis* and *trans* isomers of C16:1Δ9, C18:1Δ9 and two cyclopropane fatty acids, C17:0*cyc* and C19:0*cyc* (Table 1). Small amounts of C14:0 and C18:0 fatty acids were also detected. The fatty acid profiles are similar to those observed in a previous study [12]. Considering cells grown on natural toluene, PpF1 contained the lowest total concentration of fatty acids (4.7 mg g^{-1} cells dry weight), and G4 the highest (10.5 mg g^{-1} cells dry weight). The total concentrations of fatty acids in other three strains (KR1, PKO1, and mt-2) ranged from 6.5 to 9.6 mg g^{-1} cells dry weight (Table 1). For all [^{13}C-ring]-labeled toluene-grown cells, the concentrations of total fatty acids decreased considerably, from 25% to 63% compared to cells grown on natural toluene. The decrease in the total concentration of fatty acids was
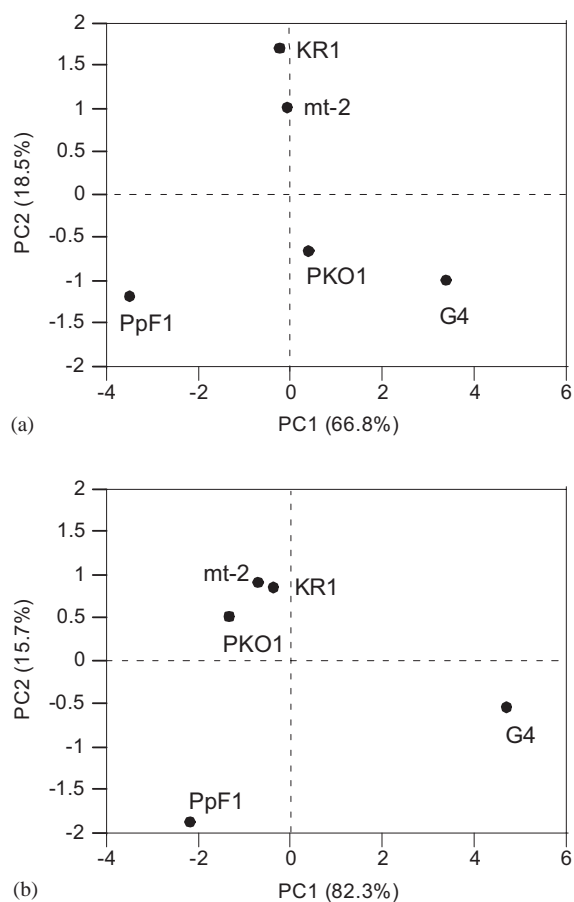

Fig. 1. Principal component analysis of phospholipid fatty acid profiles of the five pseudomonad strains grown on natural toluene (a) and [ring-^{13}C]-labeled toluene (b).

least pronounced for KR1 (25%) and most noticeable for PpF1 (57%), mt-2 (53%), and PKO1 (63%). The only exception was *B. cepacia* G4, which experienced an increase in total fatty acid concentration of 24% following growth on [^{13}C-ring]-labeled toluene. This strain also had significantly higher concentrations of C19:0*cyc* (31- to 81-fold more abundant than in other strains) regardless of the growth substrate (natural or [^{13}C-ring]-labeled toluene). The reason for the response difference by G4 is unclear, although it represents a caveat against generalizations about the effect of stable isotopes on the synthesis of specific cell constituents.

In general, the concentration of C18:0 and C19:0*cyc* fatty acids showed relatively large decreases for cells grown on [ring-^{13}C]-labeled toluene. Except for G4, which responded differently, the concentration of C16:1Δ9c also decreased relative to that of C16:1Δ9t with C16:1Δ9 t/c ratios ranging from 0.0 to 18.5 for natural toluene-grown cells, and from 0.4 to 22.0 for cells grown on [ring-^{13}C]-labeled toluene (Table 1). The

Table 1
Fatty acid (mg g^{-1}, percentages in parenthesis) and cell density measurements of the pseudomonad strains grown on natural and [ring-^{13}C]-labeled toluene

Strain	Culture[a]	Fatty acid									Total lipid[b]	t/c Ratio[c]	R_{OD}[d]
		C14:0	C16:1Δ9c	C16:1Δ9t	C16:0	C17:0cyc	C18:1Δ9c	C18:1Δ9t	C18:0	C19:0cyc			
PpF1	A	0.0(0.0)	0.04(0.9)	0.74(15.7)	1.81(38.5)	0.88(18.7)	0.27(5.8)	0.86(18.4)	0.01(0.2)	0.09(1.9)	4.7	18.5	
	B	0.0(0.0)	0.01(0.3)	0.22(10.8)	0.83(40.8)	0.46(22.7)	0.15(7.5)	0.33(16.2)	0.0(0.0)	0.03(1.7)	2.0	22.0	2.0
G4	A	0.02(0.2)	0.18(1.7)	0.01(0.1)	2.85(27.1)	3.19(30.4)	1.41(13.4)	0.04(0.4)	0.04(0.4)	2.78(26.5)	10.5	0.11	2.1
	B	0.01(0.1)	0.31(2.3)	0.13(1.0)	3.63(27.7)	3.93(30.1)	2.40(18.4)	0.20(1.6)	0.02(0.1)	2.43(18.6)	13.1	04.	
KR1	A	0.01(0.2)	0.13(1.9)	0.77(11.8)	2.48(38.2)	1.36(21.0)	0.86(13.2)	0.73(11.2)	0.02(0.4)	0.13(2.1)	6.5	5.9	1.3
	B	0.0(0.0)	0.04(0.9)	0.50(10.2)	1.83(37.4)	1.26(25.7)	0.69(14.1)	0.50(10.2)	0.01(0.2)	0.06(1.2)	4.9	12.5	
PKO1	A	0.03(0.3)	0.10(1.0)	0.99(10.2)	3.05(31.6)	2.74(28.5)	0.98(10.2)	1.30(13.5)	0.04(0.4)	0.42(4.3)	9.6	9.9	2.6
	B	0.0(0.0)	0.02(0.4)	0.41(11.3)	1.35(37.3)	0.89(24.5)	0.41(11.3)	0.52(14.3)	0.01(0.2)	0.03(0.2)	0.03(0.7)	3.6	20.5
mt-2	A	0.01(0.1)	0.15(1.9)	0.96(10.2)	2.62(33.6)	1.83(23.4)	0.94(12.1)	0.96(12.3)	0.03(0.4)	0.30(3.9)	7.8	6.4	1.4
	B	0.0(0.0)	0.03(1.0)	0.40(11.1)	1.35(36.9)	0.87(23.7)	0.53(14.5)	0.43(11.8)	0.01(0.2)	0.03(0.9)	3.7	13.3	

[a] A = natural toluene-grown cultures; B = [ring-^{13}C]-labeled toluene-grown cultures.
[b] Lipids normalized to microbial biomass (mg g^{-1} dry weight).
[c] Based on C16:1Δ9 fatty acids.
[d] R_{OD} = (OD$_{final}$–OD$_{initial}$)-natural toluene/(OD$_{final}$–OD$_{initial}$)-^{13}C-toluene.

PLFA profiles were analyzed using PCA, and the first two PCs accounted for 85.3 and 98% of the total variance (Fig. 1). Whereas PCA clearly separated the five strains grown on natural toluene (Fig. 1a), growth on [^{13}C-ring] labeled toluene resulted in different groupings that make it difficult if not impossible to discern KR1, mt-2 and PKO1 (Fig. 1b). This suggests that caution should be exercised in linking lipid data with microbial population shifts in biodegradation experiments with ^{13}C-labeled tracers.

3.2. Microbial incorporation of toluene carbons into polar lipid fatty acids

Mass spectrometric analysis revealed assimilation of toluene carbon into membrane fatty acids of all five pseudomonad strains (Figs. 2a, b). This is demonstrated by the detection of a series of isotopomers of the common diagnostic mass fragments and molecular ions of fatty acids isolated from cells grown on [ring-^{13}C]-labeled toluene as the sole carbon source. For example, the methyl ester of C16:0, with a "natural" molecular ion (M$^+$) of 270, exhibited a series of the M + N fragment ions (278–286, Figs. 2 and 3), where N (ranging 8–16) is the number of ^{13}C atoms incorporated into the fatty acid from ^{13}C-labeled toluene.

All five strains showed very similar patterns of carbon assimilation (Fig. 3). For the C16:0 fatty acid, the number of carbons incorporated from [ring-^{13}C]-labeled toluene ranged from 8 to 16. No C16:0 fatty acids with less than eight ^{13}C atoms were detected (Fig. 3). The
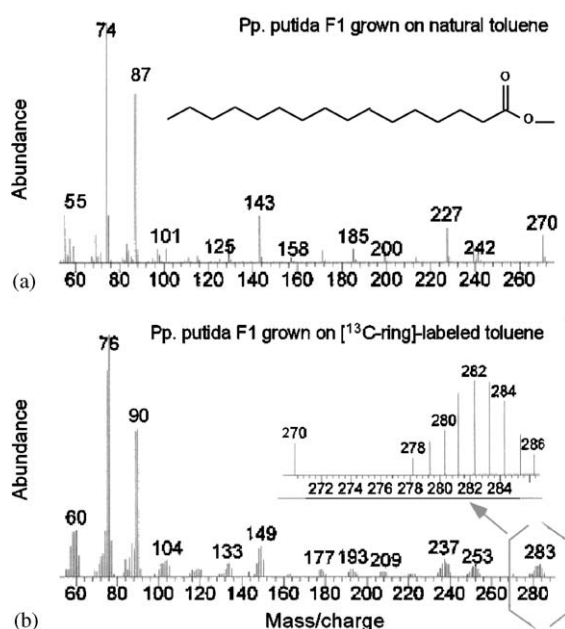

Fig. 2. Mass spectra of the C16:0 fatty acid isolated from *Pseudomonas putida* F1 grown on natural toluene (a) and on [ring-^{13}C]-labeled toluene (b).

most abundant C16:0 fatty acids were those that incorporated 12 (strains PpF1, G4, KR1 and PKO1) or 13 (strain mt-2) ^{13}C atoms, irrespective of the toluene biodegradation pathway. The other mass fragments of this fatty acid (e.g., m/z 74, 87) also displayed mass
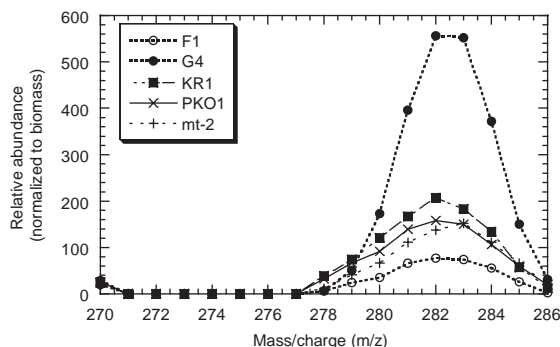
Fig. 3. Patterns and the number of [ring-^{13}C]-labeled toluene carbons incorporated into C16:0 fatty acid ($m/z = 270$) by the five pseudomonad strains. The GC/MS response was based on the integration area of the mass fragmentograms of m/z (mass/charge) 270–286, which was normalized to microbial biomass (dry weight).

increases of 2 and 3, respectively, to m/z 76 ($[^{13}CH_2 = {}^{13}C(OH)-O-CH_3]^+$) and m/z 90 ($[^{13}CH_2 = {}^{13}CH_2({}^{13}CO)-O-CH_3]^+$). In fact, cluster ions were observed in all major mass fragments of the fatty acids (data not shown).

4. Discussion

4.1. Effect of ^{13}C on microbial degradation of toluene

An appropriate monitoring protocol is essential to demonstrate the efficacy of intrinsic bioremediation, by showing that changes in contaminant concentrations are due to biodegradation rather than to abiotic phase-transfer and dilution processes that do not destroy the pollutant [27]. The use of lipids and stable isotopes to trace biodegradation of contaminants has proven to be useful in this regard (e.g., [14]). However, except for isotope fractionation kinetic studies [37], the effects of ^{13}C labeling on lipid biosynthesis and bacterial growth have not been evaluated. Thus, experiments were conducted to characterize lipid profile changes associated with growth on a ^{13}C labeled substrate as sole carbon and energy source. A high degree of ^{13}C labeling (i.e., 86% labeled toluene, with all six C atoms labeled out of 7 C atoms in toluene) was used to enhance method sensitivity to evaluate response variability among different strains that express different degradation pathways, and to delineate the applicability and limitations of ^{13}C-based tracers to assess bioremediation. Our results demonstrate that growth on ^{13}C-labeled toluene could affect bacterial metabolism. All strains except G4 exhibited decreases in fatty acid concentrations, suggesting that the ^{13}C-labeled substrate

may have exerted inhibitory effects on fatty acids biosynthesis.

Growth occurred on both natural and ^{13}C-labeled toluene, as indicated by the observed increases of OD_{660}. Cells fed natural toluene grew to a higher extent than cells grown on [ring-^{13}C]-labeled toluene. This is reflected in the R_{OD} values, defined as the ratio of the increase in OD_{660} for cells grown on natural toluene ($\Delta OD_{(12)}$) to the corresponding increase for cells grown on ^{13}C-labeled toluene ($\Delta OD_{(13)}$) (Table 1). The R_{OD} ranged from 1.3 to 2.6 for the five strains, suggesting that high relative amounts of ^{13}C-label can inhibit bacterial metabolism.

Further evidence on physiological stress comes from the ratios of the *trans* and *cis* isomers of the C16:1Δ9 fatty acid. The ratio of *trans/cis* C16:1Δ9 has been used as an indicator of stress due to toxicity, contamination, or oxygen tension on microbial communities [38]. Microorganisms synthesize more *trans*-monounsaturated acids under stress conditions [38]. The increased biosynthesis of *trans*-monounsaturated fatty acids has been suggested to be a mechanism by which microorganisms maintain their membrane fluidity [38]. The *trans/cis* ratios increased markedly (1–2 fold) when the five pseudomonad strains grew on [ring-^{13}C]-labeled toluene (Table 1). Furthermore, the absolute value of the *trans/cis* ratios, ranging from 0.4 to 22.0, is much greater than those observed in laboratory starvation and nutrient deprivation experiments [38]. These results suggest that all strains were under physiological stress, which may be due to toxicity of toluene (a solvent) and the "mass" effects of [13]C.

4.2. Applications and limitations of ^{13}C-labeled tracers in studying biodegradation

The fatty acid profiles were typical for bacteria of the genus *Pseudomonas* [12,39]. Growth on [ring-^{13}C]-labeled toluene resulted in the incorporation of ^{13}C into membrane fatty acids. A simple GC/MS analysis of microbial membrane fatty acids showed unequivocally the biodegradation and assimilation of toluene carbon into membrane lipids. Examining the ^{13}C distribution of the most abundant C16:0 fatty acid suggests that its relative abundance was not significantly affected by growth on ^{13}C-toluene (Table 1), and that the patterns of ^{13}C incorporation in C16:0 were very similar in all five strains (Fig. 3). In all cases, at least eight and up to 16 ^{13}C atoms were incorporated into C16:0 (i.e., m/z 278–286, Fig. 3). These results show that most of the carbons of the C16:0 fatty acids were derived from the toluene ring, regardless of the degradation pathway. Therefore, C16:0 (which is ubiquitous in bacteria and is often used to estimate microbial biomass [40]) might be a useful biomarker to trace the biodegradation of ^{13}C-labeled contaminants. On the other hand, several fatty acids of

relatively low abundance were greatly affected by growing on [ring-^{13}C]-labeled toluene, such as C16:1Δ9c, C18:0, C19:0cyc. These fatty acids carry useful information for microbial identification and scored high in our PCA (e.g., C16:1Δ9c = 0.36 on the first PC, C18:0 = 0.83 on the second PC for the five pseudomonad strains grown on [^{13}-C]-labeled toluene).

The total amount of lipids varied among the five strains. Interestingly, the traditional cell optical density measurement (OD$_{600}$) suggests that bacterial numbers increased due to growth on [ring-^{13}C]-labeled toluene (Table 1), whereas the lipid content decreased in four out of the five strains (G4 was the exception). It is possible that this difference reflects microbial tolerance to ^{13}C-labeled substrates. Therefore, interpretation of lipid profiles in biodegradation experiments using ^{13}C-labeled substrates may be affected by metabolic diversity, which could result in large interspecies variations in fatty acid concentrations. For example, growth of the five strains on ^{13}C-labeled toluene confounded their differentiation by PCA (Fig. 1).

4.3. Biogeochemical implications

Recently, ^{13}C-labeled lipid biomarkers or macromolecules (DNA) have been used to link biogeochemical processes to specific individual or groups of microorganisms [14,17,24,41]. This approach of combining lipid biochemistry and carbon isotope geochemistry provides greater analytical capabilities for biogeochemical systems than the sum of the two approaches applied separately. However, the potential inhibitory effects of ^{13}C to microbial biosynthesis have apparently been overlooked. Results from this study demonstrate that microbial lipid profiles could change as a result of growth on a ^{13}C-labeled substrate (toluene). These changes include (a) the total lipid content of four out of the five strains grown on [ring-^{13}C]-labeled toluene decreased significantly (25%–63% compared to unlabeled toluene-grown cells), (b) the concentration of C18:0 decreased drastically ($\sim$50%); (c) the concentration of C19:0cyc reduced substantially and almost disappeared completely ($\sim$1% of total fatty acids), and (d) the C16:1Δ9 *trans/cis* ratio increased markedly. These changes in fatty acid profiles could complicate the identification of bacteria based on fatty acid profiles (Fig. 1). Thus, interpretation of lipid data from such labeling studies on microbial biomass and community structure must be exercised with caution.

5. Summary and conclusions

Bacterial membrane lipid analysis can provide unequivocal evidence of microbial metabolism of toluene. Five reference strains grown on ^{13}C ring-labeled toluene as sole carbon source assimilated toluene-derived carbon into membrane fatty acids. The number of ^{13}C atoms incorporated into fatty acids was determined by a shift in the mass spectra of the molecular and fragment ions of the fatty acids relative to controls from bacteria grown on natural toluene. Growth on ^{13}C-labeled toluene exerted inhibitory effects on four out of the five pseudomonad strains tested, as demonstrated by the decreases in the total concentration and relative abundance of individual fatty acids. A high degree of labeling was used in this study to enhance the detection of the ^{13}C-label in fatty acids and identify possible changes in membrane lipids associated with bacterial growth on isotope-labeled tracers. Thus, it should be pointed out that the inhibitory effect of ^{13}C may not be as pronounced in studies with relatively low ^{13}C tracer concentrations. Nevertheless, the potential (concentration-dependent) effects of isotope-labeled tracers on phospholipid biosynthesis suggest that lipid profiles data linking contaminant degradation with microbial population shifts should be interpreted with caution.

References

[1] Alvarez PJJ, Vogel TM. Substrate interactions of benzene, toluene, and *para*-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. Appl Environ Microbiol 1991;57:2981–5.

[2] Tursman JF, Cork DJ. Subsurface contaminant bioremediation engineering. Crit Rev Environ Control 1992;22:1–26.

[3] Worsey MJ, Williams PA. Metabolism of toluene and xylenes by *Pseudomonas putida* (*arvilla*) mt-2: evidence for a new function of the TOL plasmid. J Bacteriol 1975;124:7–13.

[4] Zylstra GJ, Gibson DT. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2-BADE* genes and their expression in *Escherichia coli*. J Biol Chem 1989;264:14940–6.

[5] Shields MS, Montgomery SO, Chapman PJ, Cuskey SM, Pritchard PH. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl and Environ Eng 1989;55:1624–9.

[6] Smith MR. The biodegradation of aromatic hydrocarbons by bacteria. Biodegradation 1990;1:191–206.

[7] Whited GM, Gibson D. Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. J Bacteriol 1991;173:3017–30.

[8] Olsen RH, Kukor JJ, Kappammer B. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J Bacteriology 1994;176:3749–56.

[9] Newman LM, Wackett LP. Purification and characterization of toluene-2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry 1995;34:14066–76.

[10] Lang E. Diversity of bacterial capabilities in utilizing alkylated benzenes and other aromatic compounds. Lett Appl Microbiol 1996;23:257–60.

[11] Shim H, Yang S. Biodegradation of benzene, toluene, ethylbenzene, and *o*-xylene by a coculture of *Pesudomonas putida* and *Pseudomonas fluorescene* immobilized in a fibrous-bed reactor. J Biotechnol 1999;67:99–112.

[12] Fang J, Barcelona MJ, Alvarez PJ. Phospholipid compositional changes of five Pseudomonad archetypes grown with and without toluene. Appl Microbiol Biotechnol, 2000;54:382–9.

[13] Migaud ME, Chee-Sanford JC, Tiedje JM, Frost JW. Benzylfumaric, benzylmaleic, and Z- and E-phenyltaconic acids: characterization and correlation with a metabolite generated by *Azoarcus tolulyticus* Tol-4 during anaerobic toluene degradation. Appl Environ Microbiol 1996;62:974–8.

[14] Hanson JR, Macalady JL, Harris D, Scow KM. Linking toluene degradation with specific microbial populations in soil. Appl Environ Microbiol 1999;65:5403–8.

[15] Meckenstock RU, Annweiler E, Warthmann R, Schink B, Michaelis W, Richnow HH. $^{13}C/^{13}C$ stable isotope fractionation of toluene by anaerobic degradation. Environ Microbiol 1999;1:409–14.

[16] Pelz O, Tesar M, Wittich R-M, Moore ERB, Timmis KN, Abraham W-R. Towards elucidation of microbial community metabolic pathways: unreveling the network of carbon sharing in a pollutant-degrading bacterial consortium by inmunocapture and isotopic ratio mass spectrometry. Environ Microbiol 1999;1:167–74.

[17] Pelz O, Chatzinotas A, Andersen N, Bernasconi SM, Hesse C, Abraham W-R, Zeyer J. Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microorganisms to specific microbial populations. Arch Microbiol 2001;175:270–81.

[18] Sherwood-Loller B, Slater GF, Ahad J, Sleep B, Spivack J, Brennan M, MacKenzie P. Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: implications for intrinsic bioremediation. Org Geochem 1999;30:813–20.

[19] Wilks H, Boreham C, Harms G, Zengler K, Rabus R. Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. Org Geochem 2000;31:101–15.

[20] Morasch B, Richnow HH, Schink B, Meckenstock RU. Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: mechanistic and environ aspects. Appl Environ Microbiol 2001;67:4842–9.

[21] Richnow HH, Eschenbach A, Seifert R, Wehrung P, Albrecht P, Michaelis W. The use of ^{13}C-labeled polycyclic aromatic hydrocarbons for the analysis of their transformation in soils. Chemosphere 1998;36:2211–24.

[22] Rodgers RP, Blumer EN, Emmett MR, Marshall AG. Efficacy of bacterial bioremediation: demonstration of complete incorporation of hydrocarbons into membrane phospholipids into *Rhodococcus* hydrocarbon degrading bacteria by electrospray ionization fourier transform ion cyclotron resonance mass spectrometry. Environ Sci Technol 2000;34:535–40.

[23] Abraham W-R, Hesse C, Pelz O. Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Appl Environ Microbiol 1998;64:4202–9.

[24] Boschker HTS, Nold SC, Wellsbury P, Bos D, Graaf W de, Pel R, Parkes RJ, Cappenberg TE. Direct linking of microbial populations to specific biogeochemical processes by 13C-labeling of biomarkers. Nature 1998;392:801–5.

[25] Pombo SA, Pelz O, Schroth MH, Zeyer J. Field-scale ^{13}C-labeling of phospholipid fatty acids (PLFA) and dissolved inorganic carbon: tracing acetate assimilation and mineralization in a petroleum hydrocarbon-contaminated aquifer. FEMS Microbiol Ecol 2002;41:259–67.

[26] White DC, Bobbie RJ, King JD, Nickels JS, Amoe P. Lipid analysis of sediments for microbial biomass and community structure. In: Litchfield CD, Seyfried PL, editors. Methodology for biomass determination and microbial activities in sediments. ASTM STP 673. Philadelphia, PA: American Society for Testing and Materials; 1979. p. 87–103.

[27] Fang J, Barcelona MJ, West C. The use of aromatic acids and phospholipid ester-linked fatty acids for delineation of processes affecting an aquifer contaminated with JP-4 fuel. In: Eganhouse RP, editor. Molecular markers in Environ geochemistry. Washington, DC: American Chemical Society; 1997. p. 65–76.

[28] Fang J, Barcelona MJ. Structural determination and quantitative analysis of phospholipids using liquid chromatography/electrospray ionization/mass spectrometry. J Microbiol Methods 1998;33:23–35.

[29] MacNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang Y-J, White DC. Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 1999;65:3566–74.

[30] Rooney-Varga JN, Anderson RT, Fraga JL, Ringelberg D, Lovley DR. Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. Appl Environ Microbiol 1999;65:3056–63.

[31] Stanier RY, Palleroni NJ, Duodorff M. The aerobic pseudomonads: a taxonomic study. J Gen Microbiol 1966;43:159–271.

[32] Gibson DT, Gschwendt B, Yeh WK, Kobal YM. Initial reactions in the oxidation of ethylbenzene by *Pseudomonas putida*. Biochemistry 1973;12:1520–8.

[33] Koch A. Growth Measurement. In: Murray RGE, Wood WA, Krieg NR, editors. Methods for general and molecular bacteriology. Washington DC: ASM; 1994. p. 249–76.

[34] Fang J, Findlay RH. The use of a classic lipid extraction method for simultaneous recovery of organic pollutants and microbial lipids from sediments. J Microbiol Methods 1996;27:63–71.

[35] Dunkleblum E, Tan SE, Silk PJ. Double-bond location in monounsaturated fatty acids by dimethyl disulfide derivatization and mass spectrometry: application to analysis of fatty acids in pheromone glands of four lepidotera. J Chem Ecol 1985;11:265–77.

[36] Jolliffe IT. Principal component analysis. New York: Springer; 1986 p. 502.

[37] Monson KD, Hayes JM. Biosynthetic control of the natural abundance of carbon at specific positions within fatty acids in *Saccharomeces cerevisiae*. J Biol Chem 1982;257:5568–75.

[38] Guckert JB, Antworth CP, Nichols PD, White DC. Phospholipid ester-linked fatty acid profile as reproducible assays for changes in prokaryotic community structure of estuarine marine sediments. FEMS Microbiol Ecol 1985;31:147–58.

[39] Vancanneyt M, Witt S, Abraham W-R, Kersters K, Fredrickson HL. Fatty acid content in whole cell hydrolysates and phospholipid fractions of pseudomonads: a taxonomic evaluation. Syst Appl Microbiol 1996;19: 528–40.

[40] White DC, Pinkart HC, Ringelberg AB. Biomass measurements: biochemical approaches. In: Hurst CJ, Knudson GR, McInerney MJ, Stetzenbach LD, Walter MV, editors. Manual of environmental microbiology. Washington, DC: ASM Press; 1991. p. 91–101.

[41] Boschker HTS, Graaf W de, Koster M, Meyer-Reil TE, Cappenberg TE. Bacterial populations and processes involved in acetate and propionate consumption in anoxic brackish sediment. FEMS Microbiol Ecol 2001; 35:97–103.