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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE MICROBIAL METABOLISM OF BENZENE AND CRUDE OIL UNDER DIVERSE ANAEROBIC CONDITIONS

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

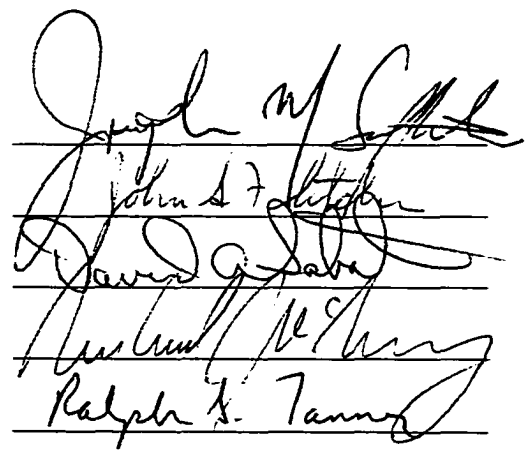
By

Matthew Edward Caldwell
Norman, Oklahoma
2000

THE MICROBIAL METABOLISM OF BENZENE AND CRUDE OIL
UNDER DIVERSE ANAEROBIC CONDITIONS

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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PREFACE

The worldwide demand for petroleum products has resulted in a myriad of ecological problems due to the introduction of hydrocarbons in environments through improper storage or spillage during the exploration, transport, processing and distribution of fuel. This dissertation focuses on the susceptibility of a variety of petroleum-derived compounds to anaerobic biodegradation in diverse environments. The work presented here primarily focuses on the anaerobic biodegradation of benzene under anaerobic conditions, but also touches on the fate of crude oil components in anoxic marine environments. The investigation of benzene degradation was examined using microorganisms from a variety of habitats including anaerobic aquifers from California, North Carolina, Michigan and two in Oklahoma. Each site differed in its predominant terminal electron accepting condition, as well as the length of, exposure to and type of hydrocarbon contamination. Crude oil decay studies were undertaken with samples from a contaminated marine environment from San Diego Bay, CA.

Chapter 1 deals with the anaerobic biodegradation of benzene from a variety of habitats under differing redox conditions. This chapter was written in collaboration with Dr. Junko Kazumi, Dr. Lily Young and Dr. Derek Lovley. The focus of this chapter was to concentrate information on the contentious issues concerning the prospects for anaerobic benzene biodegradation. This strategy was an attempt to marry disparate studies by several research groups into a single article, rather than scatter the information throughout the scientific literature. This article was the first report of anaerobic benzene mineralization under methanogenic conditions by sediment microorganisms. This aspect

of the work was exclusively performed here at the University of Oklahoma. More specifically, all data reported in Chapter 1 concerning sediments from Michigan, Oklahoma, and California were performed by me at the University of Oklahoma. The work presented in this chapter concerning New York/New Jersey Harbor sediments was performed by Dr. Junko Kazumi and Dr. Lily Young. And data presented from the Potomac River, Maryland was performed by Dr. Derek Lovley. This chapter was written in the style of the journal to which the article was ultimately submitted, *Environmental Science and Technology*.

Chapter 2 also examines anaerobic benzene biodegradation, but under the Fe(III)-reducing conditions prevalent in a petroleum-contaminated aquifer. During the course of the study, the anoxic oxidation of Fe(II) to Fe(III) under nitrate-reducing conditions was noted. Though this is not the first report of anoxic Fe(II) oxidation by nitrate-reducing microorganisms, it is the first to suggest that the regeneration of this potential electron acceptor might be used for anaerobic bioremediation purposes. Chapter 2 was written in collaboration with Dr. Ralph Tanner and in the format for the journal *Anaerobe*.

Chapter 3 is an extension of the observation on anaerobic benzene biodegradation. This chapter focuses on the pathway by which benzene is metabolized by anaerobic microorganisms under defined terminal electron accepting conditions. The study uses heavy isotopes to help establish for the first time that benzoate is an intermediate of anoxic benzene metabolism. In addition, the study confirms the finding that phenol is also an oxidation product of benzene. Chapter 3 was written in the format for the journal *Environmental Science and Technology*.

Chapter 4 concerns the anaerobic fate of a complex hydrocarbon mixture; specifically a weathered crude oil in chronically petroleum-contaminated marine sediments. The findings illustrate for the first time that anaerobic degradation of crude oil can be at least as effective as aerobic biodegradation mechanisms. In fact, sulfate-reducing microorganisms from these sediments metabolized straight chain alkanes up to 34 carbons in length. The crude oil analysis presented in this chapter was done in collaboration with Drs. Roger Prince and Robert Garrett of Exxon Research and Engineering Company. Chapter 4 was written in the format for the journal *Environmental Science and Technology*.

ABSTRACT

Benzene is the simplest aromatic hydrocarbon, yet poses one of the greatest environmental health risks due to its high water solubility and the fact that it is a known carcinogen. Often times benzene is introduced into the subsurface due to spillage or the leaking of gasoline from underground storage tanks. The large influx of carbon often results in the depletion of oxygen reserves due to heterotrophic respiration during metabolism of the more labile compounds thereby producing anoxic conditions. Thus, the metabolic fate of benzene in anaerobic environments has come under intense scrutiny.

Using inocula from a variety of anoxic environments that varied in exposure, extent of hydrocarbon contamination and redox potential, studies were undertaken to examine the prospective for the microbial degradation of benzene under strictly anaerobic conditions. Results indicated that when chronically hydrocarbon-contaminated sediments were used as a source of inoculum, the anaerobic degradation of benzene under sulfate-reducing, Fe(III)-reducing and methanogenic conditions was achievable. Confirmation of benzene mineralization was accomplished by the recovery of $^{14}CO_2$ from radiolabeled benzene in sulfate- and Fe(III)-reducing incubations, and the recovery of $^{14}CO_2$ and $^{14}CH_4$ in methanogenic incubations. The latter finding was the first confirmation of benzene degradation with carbon dioxide serving as the terminal electron acceptor.

Further examination of the metabolic fate of benzene in each of these microbial enrichments indicated the appearance of phenol and benzoate as putative intermediates. Using ^{13}C-benzene as a starting material, ^{13}C-phenol and ^{13}C-benzoate were detected during the course of degradation under sulfate-reducing conditions. Further, ^{13}C-phenol

was also detected in Fe(III)-reducing and methanogenic enrichments, however no evidence for labeled benzoate was obtained. Nonetheless, these findings are the first to confirm the importance of benzoate as an intermediate during anaerobic benzene decay.

To further investigate the susceptibility of hydrocarbons to anaerobic decay, experiments were carried out in which chronically petroleum-contaminated marine sediments were amended with an artificially weathered crude oil. Under sulfate-reducing conditions, the results obtained indicated an almost complete loss of *n*-alkanes from the oil within 201 days of incubation. The mineralization of alkanes was confirmed using ^{14}C-14,15-octacosane ($C_{28}H_{58}$) with greater than 97% of the amended radioactivity recovered as $^{14}CO_2$. The degradation of *n*-alkanes from C_{15} to C_{34} in length, extend the range of straight chain aliphatic compounds known to be amenable to anaerobic biodegradation. Further, these results indicate that the extensive alteration of *n*-alkanes can no longer be considered a defining characteristic of aerobic oil biodegradation processes alone.

The research reported here expands the range of knowledge for anaerobic hydrocarbon metabolism, as well as provides a sound foundation for the microbial destruction of compounds once thought to be completely recalcitrant under anaerobic conditions.

Chapter 1.

Anaerobic Degradation of Benzene in Diverse Anoxic Environments

Abstract

Benzene has often been observed to be resistant to microbial degradation under anoxic conditions. A number of recent studies, however, have demonstrated that anaerobic benzene utilization can occur. This study extends the previous reports of anaerobic benzene degradation to sediments that varied with respect to contamination input, predominant redox condition, and salinity. In spite of differences in methodology, microbial degradation of benzene was noted in slurries constructed with sediments from various geographical locations and range from aquifer sands to fine-grained estuarine muds, under methanogenic, sulfate-reducing, and iron-reducing conditions. In aquifer sediments under methanogenic conditions, benzene loss was concomitant with methane production, and microbial utilization of [^{14}C]benzene yielded $^{14}CO_2$ and $^{14}CH_4$. In slurries with estuarine and aquifer sediments under sulfate-reducing conditions, the loss of sulfate in amounts consistent with the stoichiometric degradation of benzene or the conversion of [^{14}C]benzene to $^{14}CO_2$ indicates that benzene was mineralized. Benzene loss also occurred in the presence of Fe(III) in sediments from freshwater environments. Microbial benzene utilization, however, was not observed under denitrifying conditions. These results indicate that the potential for the anaerobic degradation of benzene, which

was once thought to be resistant to non-oxygenase attack, exists in a variety of aquatic sediments from widely distributed locations.

Introduction

Benzene is a naturally occurring aromatic compound present in petroleum fuels and is a frequent contaminant in both surface and subsurface environments. It is also fairly water soluble, toxic, and carcinogenic (*1*), thus its transport and fate in the environment has attracted a considerable amount of regulatory scrutiny. Aerobic microorganisms readily oxidize benzene to carbon dioxide (*2, 3*), thus benzene does not typically persist in oxic environments. Many aquifers and aquatic sediments, however, have extensive anoxic zones in which denitrification, Fe(III) reduction, sulfate reduction, or methanogenesis predominates (*4*). Although microbial utilization of toluene (*5-9*), ethylbenzene (*10*), and xylenes (*6, 11-14*) under various anoxic conditions has been well-documented (as reviewed in ref *15*), biodegradation of benzene has been inconsistently observed in the absence of oxygen. In fact, a number of reports have shown that benzene resists anaerobic metabolism in the field (*16, 17*) and in laboratory enrichments established with sewage sludge, groundwater sediments, and contaminated soils (*15, 17-20*). Furthermore, in several studies, low dissolved oxygen concentrations were often associated with a diminution in benzene utilization (e.g., refs *12*, *17*, and *21*).

Benzene degradation in the absence of oxygen was noted as early as 1980, when Ward et al. (*22*) reported the formation of small amounts (up to 2%) of $^{14}CO_2$ and $^{14}CH_4$ from [^{14}C]-benzene in methanogenic enrichments derived from petroleum-contaminated salt marsh and estuarine sediments. Microbial metabolism of benzene to carbon dioxide and methane has been observed under methanogenic conditions in enrichment cultures from sewage sludge (*23*), and benzene mineralization to CO_2 has been observed in mesocosms containing river sediments (*24*). In enrichment cultures with sewage sludge,

^{18}O from [^{18}O]H_2O was incorporated into benzene as a hydroxyl group with the formation of phenol (*9*). A pathway for anaerobic benzene metabolism was proposed in which the phenol was subsequently converted to cyclohexanone before complete degradation to CO_2 and CH_4 (*23*). Only a minor portion (<6%) of the [^{14}C]benzene added to the cultures, however, was converted to $^{14}CO_2$. Benzene was also consumed in methanogenic aquifer material incubated under anoxic conditions, but it was not determined whether the benzene was mineralized to carbon dioxide and methane (*25*).

More recent studies indicate that, under appropriate conditions, benzene can be oxidized to CO_2 in the absence of oxygen with either sulfate or Fe(III) serving as the electron acceptor. For example, rapid benzene mineralization under sulfate-reducing conditions in marine and freshwater sediments and in aquifer material have been reported (*26-31*). In instances in which the stoichiometry of benzene consumption and sulfate loss has been determined, the results have been consistent with sulfate serving as the electron acceptor for benzene oxidation (*27, 28, 31*). Benzene mineralization was also observed in sediments taken from the Fe(III)-reduction zone of a petroleum-contaminated aquifer and amended with synthetic Fe(III) chelators or humic acids (*32, 33*). The stoichiometry of benzene metabolism and Fe(III) reduction indicated that Fe(III) was the sole electron acceptor for benzene oxidation in these sediments (*32*).

Evidence for the microbial degradation of benzene under denitrifying conditions is less pursuasive. Although nitrate-dependent uptake of benzene was observed in aquifer sands or groundwater incubated under anoxic conditions (*34, 35*), neither of these studies demonstrated the oxidation of benzene to CO_2 or established stoichiometric relationships between benzene metabolism and nitrate loss. Other studies have

investigated the potential for benzene oxidation coupled to nitrate reduction and have found that benzene persists under denitrifying conditions (*11-13, 17*).

In the study reported here, the potential for benzene degradation was examined in a diversity of sedimentary environments obtained from various locations and examined in different laboratories. The fact that anaerobic benzene degradation was observed despite differences in sites, methodologies, and analytical techniques underscores the strength of the observations. The results demonstrate that, for the first time, [^{14}C]benzene can be effectively mineralized to $^{14}CO_2$ and $^{14}CH_4$ under methanogenic conditions and substantiate the potential for anaerobic benzene oxidation under sulfate-reducing and Fe(III)-reducing conditions.

Materials and Methods

Site Descriptions. Sediment samples were obtained from several sites that varied with respect to contamination history, predominant redox condition, and salinity. Complete descriptions of the sites can be found in references cited in Table 1 or are summarized below. Table 1 also includes an indication of the assay conditions under which benzene utilization was evaluated.

Collection and Characterization of Inocula. Sediments used as inocula were collected as previously described from a variety of locations including the following: (i) a shallow, anoxic aquifer polluted by leachate from the municipal landfill in Norman, OK (*36*); petroleum-contaminated aquifers at (ii) the Sleeping Bear Dunes National Lakeshore near Empire, MI (*37*), and (iii) Seal Beach, CA (*26*). Sediments were also collected from (iv) the New York/New Jersey Harbor (NY/NJ) near the Fresh Kills landfill and (v) the Potomac River, MD (*38*). Sediments from NY/NJ Harbor were taken using a gravity corer with a PVC-lined sleeve (6.5 cm i.d.) from the surficial 30-40 cm of sediment. The sediment cores were capped immediately after collection and placed on ice for transport back to the lab. The cores were kept at 4 °C until use in the experiments. All sites were chronically exposed to fuel hydrocarbons except the leachate-contaminated aquifer in Norman, OK, and the Potomac River, MD. The sampling sites were previously characterized as methanogenic (i and ii), sulfidogenic (iii and iv), or Fe(III)-reducing (v).

Anaerobic Biodegradation Assays. The incubations were designed to assess benzene metabolism under methanogenic, sulfate- or Fe(III)-reducing, or denitrifying conditions. Procedures for the biodegradation assays are referenced in Table 1 or are described below. Evidence for microbial utilization of benzene was based on (1) the disappearance

TABLE 1. Characteristics of Samples Assayed for Presence of Microorganisms Capable of Anaerobic Benzene Degradation and Reference to Procedures Used in Assay Protocol

sample type	location	site characteristics	redox condition at site[a]	assay conditions[a]	assay procedure[b]
sediment	OK	aquifer contaminated with landfill leachate	M	M,S	43
sediment	MI	aquifer contaminated with gasoline	M	M, S, N	43
sediment	CA	aquifer contaminated with gasoline	S	S	43
sediment	NY/NJ	estuary contaminated with petroleum	S	M, S, I, N	this study
sediment	MD	river sediments	I	I	38

[a] Redox and assay conditions: M, methanogenic; S, sulfate-reducing; I, iron-reducing; N, denitrifying. [b] Literature citation to detail anaerobic biodegradation assay procedure.

of the substrate and its conversion to metabolic end products including methane and carbon dioxide or (2) the production of Fe^{2+} or the loss of nitrate or sulfate, when ferric iron or the latter anions were included in the sediment slurries as terminal electron acceptors. The resulting information was interpreted relative to both sterile and benzene-unamended controls. The experimental bottles were made in at least triplicate.

Aquifer Sediments from OK, MI, and CA. The sediment slurries were constructed in an anaerobic glovebox under N_2:H_2 (90:10). The slurries consisted of 50 g of sediment and 75 mL of groundwater or media (see below) added to sterile 160-mL serum bottles. Sterile Na_2S (1 mM) and resazurin (0.0001%) were added as a reductant and a redox indicator, respectively. The bottles were closed with a composite stopper made of the top of a butyl rubber stopper fused to the bottom of a Teflon-coated stopper. The bottles were then removed from the glovebox, and the closures were held in place with aluminum crimp seals. The headspace of the vials was exchanged three times with N_2:CO_2 (80:20). Sterile controls were obtained by autoclaving the slurries on three successive days. In experiments where sulfate or nitrate served as the terminal electron acceptor, slurries were amended with sulfate or nitrate from sterile, anoxic stock solutions to a concentration of 20 mM. Due to sample constraints, we substituted a basal medium (*39*) to construct slurries with MI or CA sediments in experiments where sulfate served as the terminal electron acceptor. The basal medium was modified by omitting the cysteine hydrochloride and by adding 5 mL L^{-1} of a vitamin mixture (*40*). Slurries of CA sediments were constructed with either the modified basal medium or a mineral salts medium (*26*). Undiluted benzene was added to achieve concentrations of 1.4-4.3 mM in the experiment with OK sediments. In one study with MI sediments, benzene was

introduced at a concentration of 675 μM. In this experiment, benzoate was added to some bottles to serve as a positive control for methanogenesis. Benzoate was introduced from a sterile, anoxic stock solution to achieve a carbon concentration of 50 ppm, which was equivalent to the amount of carbon in the benzene-amended bottles. For radiolabel studies with MI and CA sediments, [^{14}C-UL]benzene (Sigma Chemical Co., St. Louis, MO; >98% purity; specific activity of 53.4 or 63.2 mCi mmol^{-1}) in an anoxic stock solution of unlabeled benzene (7.5 mM) was added to the bottles. The radioactivity was 3.5-7.0 x 10^6 dpm (7.0-9.3 x 10^4 dpm mL^{-1}), and the total benzene concentration was approximately 50 μM in each bottle. All incubations were carried out at room temperature (22 °C) in the dark.

Estuarine Sediments from NY/NJ Harbor. Sediment slurries (10:90; vol sediment:vol media) were prepared in the same manner as previously described (*41*), except that the 50-mL serum bottles were capped with Teflon-coated, butyl rubber stoppers (Emsco, Philadelphia, PA) and crimp-sealed. Media used to slurry the sediments were prepared using standard anaerobic techniques. Each liter of basic medium contained 1.3 g of KCl, 0.2 g of KH_2PO_4, 23 g of NaCl, 0.5 g of NH_4Cl, 0.1 g of $CaCl_2 \cdot 2H_2O$, 1.0 g of $MgCl_2 \cdot 6H_2O$, 2.5 g of $NaHCO_3$, 0.1 mg of resazurin, 10 mL of vitamin stock, and 15 mL of trace salts solution. Each liter of trace salts stock contained 30 mg of $CoCl_2 \cdot 6H_2O$, 0.15 mg of $CuCl_2$, 5.7 mg of H_3BO_3, 20 mg of $MnCl_2 \cdot 4H_2O$, 2.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 1.5 mg of $NiCl_2 \cdot 2H_2O$, and 2.1 mg of $ZnCl_2$. For each reducing condition, 1 L of basic medium was amended with the following: 0.368 g of $FeCl_2 \cdot 4H_2O$ and 0.5 g of $Na_2S \cdot 9H_2O$ for methanogenic medium; 2.84 g of Na_2SO_4, 1.49 mg of $FeCl_2 \cdot 4H_2O$, and 0.35 g of $Na_2S \cdot 9H_2O$ for sulfidogenic medium; freshly precipitated amorphous Fe floc for

Fe(III)-reducing medium; 3.3 g of KNO_3 and 1.49 mg of $FeCl_2 \cdot 4H_2O$ for denitrifying medium. The initial concentration of electron acceptors was 20 mM sulfate, 200 mM Fe(III), and 30 mM nitrate for sulfidogenic, Fe(III)-reducing, and denitrifying media, respectively. Benzene was added to the incubation mixtures to an initial concentration of 125 μM. All incubations, with autoclaved controls, were incubated without shaking in the dark at 30°C.

Freshwater Sediments from MD. The brown, oxidized layer of sediment from the Potomac River was used to construct slurries (100 mL) and transferred under N_2:CO_2 (93:7) into serum vials, which were sealed with thick butyl rubber stoppers. An anoxic slurry of poorly crystalline iron(III) oxide was added to the sediments to provide approximately 10 mmol L^{-1} additional Fe, as described previously (*38*). Benzene was added from an anoxic aqueous stock solution to provide an initial concentration of ca. 3 μM.

Analytical Techniques. Benzene removal from the various enrichments was monitored by gas (GC) or high-pressure liquid chromatography (HPLC) as indicated below. In experiments with OK or MI aquifer sediments and unlabeled benzene, loss of the compound was monitored by HPLC as previously described (*42*). In addition, methane was determined by GC as previously described (*43*). For [^{14}C]benzene studies with MI and CA sediments, the radiolabel was analyzed with an HPLC system equipped with an UV:in-line radio-isotope detector (Beckman Model LC 1801, 171 radioisotope detector, Fullerton, CA), with the mobile phase (60% acetonitrile,40% sodium acetate of 50 mM) and the scintillation cocktail (Ready Flow-III, Beckman) flow rates at 0.5 mL min^{-1}.

$^{14}CO_2$ was monitored by counting the radioactivity in alkali traps as described previously (*42*), and $^{14}CH_4$ was measured with a GC equipped with a gas proportional counter (*27*). Hydrogen sulfide production was determined spectrophotometrically by the method of Fogo and Popowsky (*44*). In experiments with NY/NJ Harbor sediments, benzene loss was evaluated by GC-FID according to Coschigano et al. (*45*). As described previously, methane production was monitored by GC-TCD, sulfate and nitrate loss were determined by ion chromatography, and Fe^{2+} production was assayed by the ferrozine spectrophotometric method (*41*). Benzene removal from Potomac River sediments was monitored by GC according to Lovley et al. (*27*).

Results and Discussion

This study demonstrates that microorganisms in enrichments established with sediments from a diversity of environments have the potential to degrade benzene under strict anoxic conditions. Despite differences in methodology and initial substrate concentration, anaerobic benzene metabolism was observed in sediments from aquifers, freshwater, and estuarine sources and under methanogenic, sulfate-reducing, and Fe(III)-reducing conditions (Table 2). These results confirm previous reports of anaerobic benzene utilization and extend the observations to other sediments. In addition, evidence for nearly complete conversion of [^{14}C]benzene to $^{14}CH_4$ and $^{14}CO_2$ are presented. Previous studies have shown that benzene is degraded under methanogenic conditions, but the extent of mineralization was minor, with less than 6% of the initial amount of [^{14}C]benzene converted to $^{14}CO_2$ ($^{14}CH_4$ was not monitored) (*23, 24*). In contrast, in the results reported here, over 80% of the [^{14}C]benzene added to MI aquifer sediments was recovered as $^{14}CO_2$ and $^{14}CH_4$ (Table 3). The amount of $^{14}CO_2$ and $^{14}CH_4$ produced was 113% and 64%, respectively, of the theoretically expected values based on the Buswell equation (*46*). In the sterile controls, no $^{14}CH_4$ was noted, and less than 0.5% of the radiolabel added was recovered as $^{14}CO_2$.

Methane production was also observed when MI sediments were incubated with 675 μM unlabeled benzene over 590 d (Figure 1). During this time, no intermediates of benzene decomposition were detected by HPLC. After 600 d, when benzene was no longer detected in the benzene-amended incubations, the amount of methane produced was similar to the amount observed in the positive benzoate control. This is consistent

TABLE 2. Evidence for Anaerobic Biodegradation of Benzene in Samples from Diverse Environments under Different Redox Conditions

sample type	redox condition[a]	benzene concentration (μM) initial	final	corrected[b]	initial lag time (d)	incubation time (d)	detectable endproducts
MI	M	50	16.5	34	420	520	$^{14}CH_4$ & $^{14}CO_2$
	M	675	a[c]		360	590	CH_4
	M control	50	54				
	S	50	0	40	~400	500	$^{14}CO_2$
	S control	50	40				
	N	50	51	NC[e]		530	
	N control	50	53				
CA	S	57	5	22	120	320	$^{14}CO_2$
	S control[d]	68	38				
NY/NJ	M	125	0	55	100	180	
	M control	125	55				
	S	125	0	86	60	100	coupled SO_4^{2-} red.
	S control	125	86				
	I	125	0	52	100	180	
	I control	125	52				
	N	125	40	NC		210	
	N control	125	35				
MD	I	3	0	3	50	60	
	I	3	3				

[a] Redox and assay conditions: M, methanogenic; S, sulfate-reducing; I, iron-reducing; N, denitrifying. [b] Corrected refers to benzene loss in cultures above loss in sterile controls. a[c], bottles monitored for substrate mineralization only. [d] Control refers to sterile control. [e] NC, no change.

TABLE 3. Amount of [^{14}C-UL]Benzene Detected as Gaseous End Products in Incubations of Aquifer Sediments from MI and CA

sample type	Redox condition[a]	[^{14}C]benzene added (dpm)	$^{14}CO_2$ recovered (dpm)	$^{14}CH_4$ recovered (dpm)	^{14}C gas(%)
MI park	M	3.50×10^6	1.48×10^6	1.39×10^6	82
	M control[a]	3.50×10^6	1.50×10^4	0	0.43
	S	9.17×10^6	7.17×10^6	ND[b]	78
	S control	8.20×10^6	9.10×10^3	ND	0.11
Seal Beach. CA	S	1.95×10^7	1.48×10^7	0	76
	S control	1.95×10^7	1.90×10^3	ND	0.01

[a] as in Table 2. [b] ND, not determined.

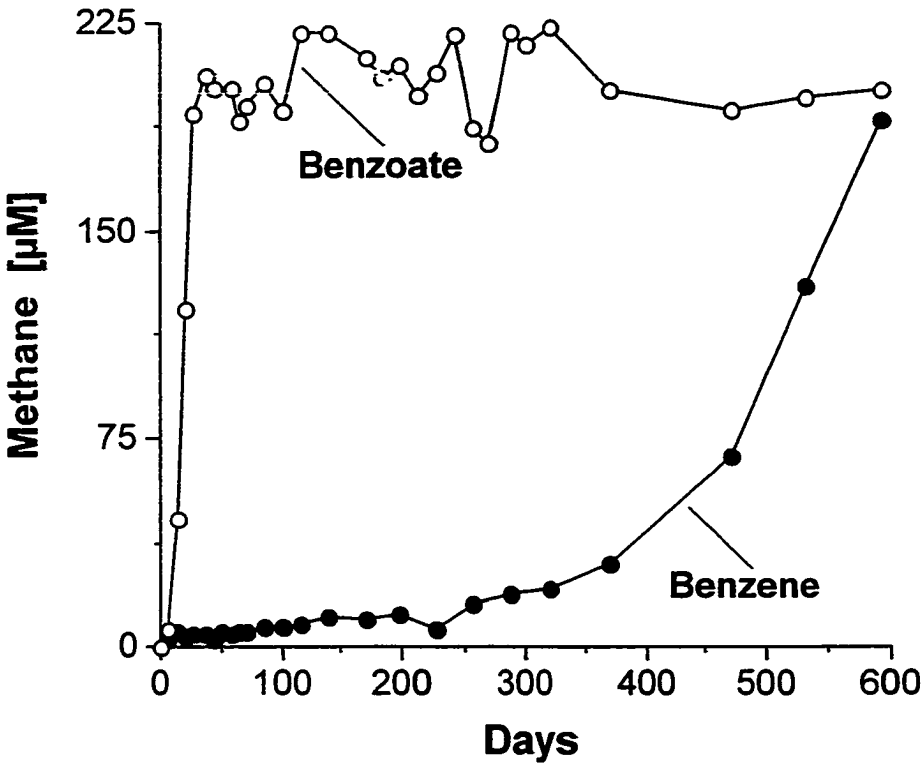

FIGURE 1. Methane production in aquifer sediments from Michigan, incubated under methanogenic conditions. Benzoate and benzene were supplied at a concentration of 50 ppm carbon. The curves are corrected for the amount of methane produced in substrate-unamended controls.

with the Buswell equation (*46*), as both substrates were added at concentrations equivalent to 50 ppm carbon, and similar amounts of methane were expected to be produced in the benzene- and benzoate-amended bottles. Furthermore, the amount of methane produced in the benzene-amended bottles was 73% of that expected, assuming complete metabolism of benzene by the following stoichiometric equation: $C_6H_6 + 4.5H_2O \rightarrow 2.25CO_2 + 3.75CH_4$. The lower than expected values maybe explained by incorporation of substrate into cellular material, adsorption of benzene to sediments or vessels, or loss from the incubations through some other means. These results clearly show, however, that benzene can be metabolized to yield large amounts of gaseous end products under strictly anoxic conditions. To confirm methanogenic benzene utilization in MI sediments, the slurries were reamended with 100 μM of [^{14}C]benzene after unlabeled benzene was no longer detected in the bottles. After 21 d, $^{14}CH_4$ and $^{14}CO_2$ were detected in the headspace, indicating that benzene ring cleavage and mineralization had occurred (data not shown).

Anaerobic benzene degradation was also observed in sulfate-reducing enrichments established with inocula from gasoline-contaminated aquifers in MI and Seal Beach, CA, and from a petroleum-contaminated estuarine site in NY/NJ Harbor (Table 3, Figure 2). Reports of benzene oxidation under sulfate-reducing conditions have been previously noted for slurries with aquifer (*26*), marine (*27-30*), and freshwater (*31*) sediments. The study presented here extends this metabolic potential to estuarine sediments and confirms a previous observation of benzene degradation in aquifer sediments from Seal Beach (*26*). In radiolabel studies with MI sediments, 78% of [^{14}C]benzene added was recovered as $^{14}CO_2$ (Table 3), and a statistically significant

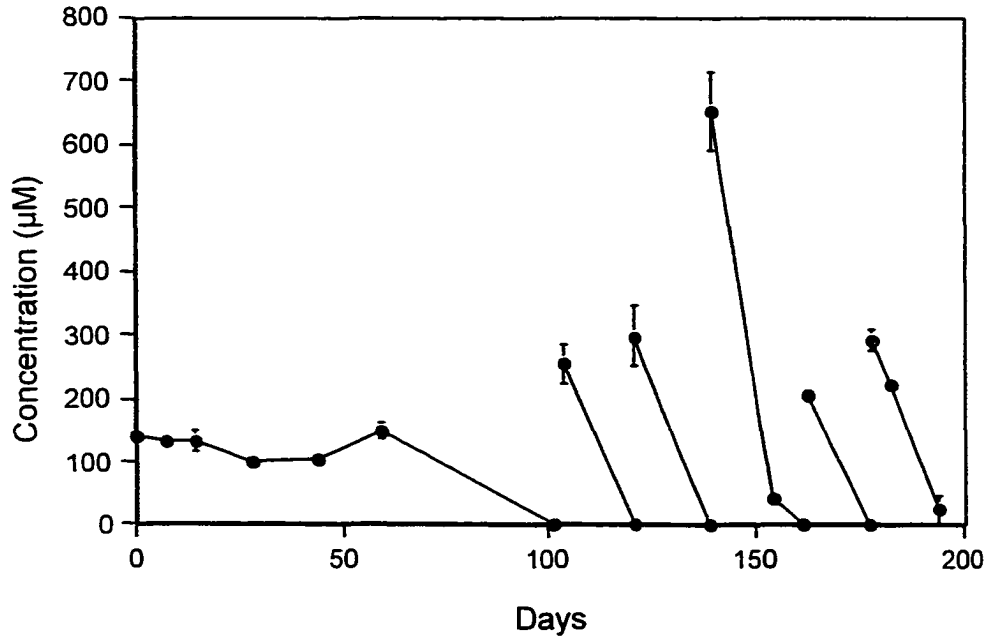

FIGURE 2. Loss of benzene under sulfate-reducing conditions in NY/NJ Harbor sediments. After an initial loss of approximately 125 µM benzene within 100 d, cultures were repeatedly fed with substrate over the next 80 d. Results are means of 3 replicates ± 1 SD and are corrected for abiotic losses in the autoclaved controls.

amount of hydrogen sulfide was produced relative to the benzene-unamended controls (data not shown). Slurries established with CA aquifer sediments also exhibited mineralization of [^{14}C]benzene, with 76% of the label recovered as $^{14}CO_2$ and no $^{14}CH_4$ formation (Table 3). The loss of sulfate or the production of hydrogen sulfide, however, could not be discerned against background levels in substrate-unamended controls (data not shown). Benzene utilization was also observed in slurries with NY/NJ Harbor estuarine sediments, with degradation activity sustained upon numerous re-amendments of the substrate (Figure 2). Furthermore, the loss of sulfate was 85% of that expected for the complete mineralization of benzene to CO_2, indicating that, in these sediments, benzene degradation was coupled to sulfate reduction (Table 4). This stoichiometry is similar to that obtained with benzene-adapted marine sediments (*27*), and comparable stoichiometries have been recently observed in sediments from a hydrocarbon seep (*28*) and benzene-adapted, sulfate-containing freshwater sediments (*31*).

In this study, benzene was not always degraded under methanogenic or sulfate-reducing conditions. Even after 3 years, benzene was recalcitrant in aquifer sediments impacted with landfill-leachate from Norman, OK (data not shown). It was initially thought that these results are due to high benzene concentrations (1.4-4.3 mM), and the microorganisms may have been adversely affected. On the other hand, benzene metabolism was not observed subsequently in these sediments even when a lower concentration (1.5 μM) of [^{14}C]-benzene was added (data not shown). A possible explanation is that benzene may not have been an important component of landfill-leachate contamination at the site and that the sediments were not enriched for benzene-degrading microorganisms. Flyvbjerg et al. (*20*) also observed no loss of benzene under

TABLE 4. Consumption of SO_4^{2-} during Degradation of Benzene in New York/New Jersey Harbor Sediments

benzene metabolized (mM)	SO_4^{2-} consumption (mM)		
	predicted[a]	measured[b]	% of expected
0.97 ± 0.02	3.63 ± 0.08	3.09 ± 0.55	85 ± 13

[a] Based on stoichiometry of 1 mol of benzene = 3.75 mol of SO_4^{2-}. The stoichiometric equation for the complete mineralization of benzene is

$$C_6H_6 + 3.75SO_4^{2-} + 3H_2O \rightarrow 6HCO_3^- + 1.875HS^- + 1.875H_2S + 0.375H^+$$

[b] Consumption of SO_4^{2-} in background control cultures (less than 0.1 mM within 195 d) subtracted.

sulfidogenic conditions within 7 months of incubation in mesocosms containing creosote-contaminated groundwater.

The absence of benzene-degrading microorganisms may be another factor affecting the lack of benzene metabolism (*31*). When aquifer sediment with no activity was inoculated with benzene-oxidizing microorganisms derived from other aquatic sediments, then rapid, sulfate-dependent benzene oxidation was noted. Thus, the source of the sediment inoculum and/or history of contamination may be important for microbial benzene degradation to occur.

Benzene loss under Fe(III)-reducing conditions was observed in Potomac River sediments in the absence of added Fe(III) chelators (Figure 3), suggesting that not all sediments require the addition of Fe(III) chelators. This was also shown with sediment from a petroleum-contaminated aquifer (*47*). Anaerobic benzene metabolism at the expense of Fe(III) reduction in aquifer material had been previously reported (*32, 33*). In these reports, benzene loss occurred only when Fe(III) chelators such as EDTA and NTA were added, which makes Fe(III) more available for microbial reduction.

Microbial benzene loss was also noted in slurries established with NY/NJ Harbor estuarine sediments in which Fe(III) was provided as a potential electron acceptor (Table 2). Benzene utilization, however, was not sustained upon re-feeding with the substrate. This may be because the sulfate contained in the original sediment inoculum was the electron acceptor for the initial benzene degradation observed, and once sulfate was depleted, benzene oxidation stopped. The initial sulfate concentration in the sediment slurries established with estuarine sediments was calculated to be approximately 1 mM. In a similar manner, benzene loss was observed in NY/NJ Harbor sediments to which

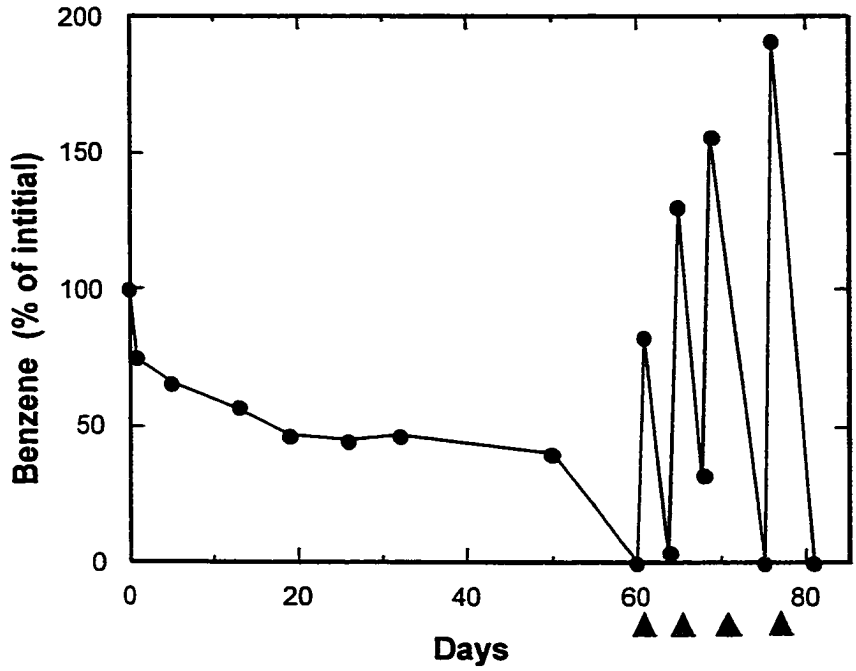

FIGURE 3. Benzene loss in iron(III) oxide-amended cultures established with Potomac River sediments. At day 0, the initial benzene concentration was 3 μM. The arrows indicate when benzene was depleted in the cultures. After an initial loss of 3 μM benzene within 60 d, cultures were re-fed with benzene over the ensuing 20 d.

carbonate was added to promote methanogenic conditions (Table 2), but the activity could not be sustained with re-feeding of the substrate. Methane production was noted in benzene-amended bottles; however, the amount produced was similar to that in the benzene-unamended background controls. These results are also consistent with the likelihood that benzene utilization is coupled to the reduction of sulfate included in the inoculum.

There was no metabolism of benzene under denitrifying conditions in cultures established with sediment from a gasoline-contaminated aquifer (MI) or a petroleum-contaminated estuary (NY) within 530 and 210 d, respectively (Table 2). Nitrate-dependent benzene uptake in anoxic aquifer sediments has been reported (*34, 35*), although in further experiments using the same sediment source, benzene degradation under denitrifying conditions was not observed (*17*). Other field and laboratory studies have also found that benzene was not metabolized under strictly denitrifying conditions (*11-13*).

In this study, benzene utilization proceeded relatively rapidly after an initial lag period of 50-420 d (Table 2). There could be a number of reasons to account for the variable length of time where biodegradation does not occur to an appreciable degree (*48, 49*). These include the time required for an initially small population size to grow sufficiently large to achieve detectable degradation rates, the lack of essential nutrients, and the need for genetic alterations (i.e., mutation, gene exchange, or rearrangement) prior to the onset of metabolism. Although the mechanism(s) involved in each of the cases presented is not clear, it should be noted that benzene metabolism was observed in enrichments established from a variety of sediments, ranging from freshwater aquifer

material to fine-grained, estuarine muds, at different initial benzene starting concentrations, and under a wide variety of electron-accepting conditions.

In summary, the results demonstrate that the potential for microbial benzene degradation exists under a number of different reducing conditions. This is in contrast with many earlier studies which show that benzene degradation is minimal in the absence of oxygen. Although factors that may account for these differences are not clear, possible explanations for our observations include the realization that long incubation times may be necessary for activity to be evident, the addition of benzene as the sole substrate in order to avoid preferential utilization of other aromatic hydrocarbons *(26)*, and the sampling of sediments that are known or suspected to be contaminated with petroleum compounds. Now that the potential for anaerobic benzene degradation has been demonstrated in a diversity of sediment types and is more widespread than previously thought, it will be important to determine the significance of this process in removing benzene from contaminated environments.

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Chapter 2.

Microbial Metabolism of Benzene and the Oxidation of Ferrous Iron under Anaerobic Conditions: Implications for Bioremediation

Abstract

Benzene and toluene were biodegraded when chelated Fe(III) served as the terminal electron acceptor in aquifer sediments contaminated by a petroleum refinery. Benzene biodegradation ceased when Fe(III) was depleted but resumed upon reamendment. Microorganisms from the same sediments degraded toluene, but not benzene, under nitrate reducing conditions. However, the anaerobic oxidation of Fe(II) to Fe(III) was also observed in toluene-degrading incubations. Fe(II) oxidation was dependent on the presence of nitrate and enhanced when organic electron donors were provided. Microbial nitrate-linked Fe(II) oxidation was also documented in other petroleum-contaminated aquifer sediments, sludge from an oil-water separator, a landfill leachate-impacted aquifer and a garden soil. These observations suggest that some of the reported effects of nitrate on hydrocarbon biodegradation may be indirect through the reoxidation of Fe(II).

Introduction

Leaks and spillage during the transportation and storage of petroleum and petroleum products can result in the contamination of surface and subsurface environments. The influx of carbon from gasoline into the subsurface often results in the depletion of oxygen due to aerobic respiratory activity of heterotrophic microorganisms. The ability of microorganisms to degrade anaerobically the gasoline pollutants of major concern (benzene, toluene, ethylbenzene, and the xylene isomers or collectively referred to as BTEX) is well documented [reviewed in 1,2]. With few exceptions, all BTEX compounds have been shown to be degraded by microorganisms that use either nitrate, sulfate, ferric iron, and/or carbon dioxide as electron acceptors. The ability to degrade benzene, the most water soluble, carcinogenic and regulated of the BTEX compounds, is of especial concern. The anaerobic biodegradation of benzene has been conclusively observed under sulfate reducing [3-9], iron reducing [7,8,10-12] and methanogenic conditions [8,13-15]. However the microbial degradation of benzene under nitrate reducing conditions remains somewhat enigmatic. Nitrate amendment is seen as a potential remediation strategy for the cleanup of many gasoline contaminated aquifers since nitrate reducing microorganisms are nutritionally versatile, widely distributed, and the electron acceptor is water soluble and easily transported in the subsurface. The loss of benzene under nitrate reducing conditions has been reported [16-20], but conflicting results, the possible effects of oxygen contamination, and the lack of adequate material balances have undermined several studies. To date, no study definitively links anaerobic benzene degradation to nitrate reduction. Herein, we suggest a possible alternative for the role of nitrate in contaminated aquifers, the nitrate dependent microbial oxidation of

Fe(II) to Fe(III). The ability of microorganisms to oxidize Fe(II) to Fe(III) under anaerobic conditions may provide an alternative sink for nitrate in bioremediation efforts. This process may also explain the results observed in field or laboratory studies which could not conclusively couple hydrocarbon loss with the consumption of nitrate when the latter was provided as a terminal electron acceptor.

Materials and Methods

Sampling

Sediment and pore water were obtained from a shallow refinery-contaminated aquifer near Ponca City, OK at a depth of approximately 2-3 m below the surface. The sediments were generally sandy in nature with some clay aggregates, and smelled of hydrocarbons. Other sediment samples were obtained from a landfill-leachate impacted aquifer located in Norman, OK, as previously described [21]. Microorganisms in sediments from three gasoline-contaminated aquifers were also tested for their ability to oxidize Fe(II) under nitrate reducing conditions. These were obtained from a site previously characterized as sulfate reducing (Seal Beach, CA) [3], iron reducing and methanogenic (Rocky Point, NC) [22] and a methanogenic aquifer located near Empire, MI [8]. Sludge from an oil-water separator tank at the U.S. Navy oily waste repository at Craney Island, VA was also used as inoculum. Lastly, soil a sample was collected from the garden of one of the authors (RST).

Sediment Incubations

Ponca City aquifer slurries were prepared by adding 50 g of sediment and 75 ml of ground water in sterile 160 ml serum bottles as previously described [8]. Resazurin (0.0001 %) and sodium sulfide (0.5 mM) were added as a redox indicator and reductant, respectively. Upon addition of sodium sulfide, a black color developed presumably due to the reaction with Fe(II) and precipitation as iron sulfides. In some experiments, sulfide was not used as the samples reduced resazurin rapidly. All manipulations were carried out in an anaerobic glovebag. Bottles were sealed with composite stoppers [8] and held

in place with aluminum crimp seals. All incubations were placed under an atmosphere of N_2:CO_2(80:20). When used, nitrate was added to an initial concentration of 20mM. Benzene, toluene, ethylbenzene, *m*-, *o*- and *p*-xylene were added by syringe as neat compounds to an initial concentration of 400 μM. All chemicals were of the highest purity available (Aldrich Chemicals, Milwaukee, WI). Benzoate was used as a positive control substrate (1mM). All experiments were conducted in triplicate and incubated at (121°C;20 min) on three successive days.

For iron-reducing experiments, aquifer slurries were constructed similarly but were not amended with sodium sulfide. These incubations received 5 mM of an Fe(III)-NTA solution and either ^{14}C-UL-benzene (Sigma Chemical Co., St. Louis, MO, >98% purity, specific activity 63.22 mCi/mmol or 48.1 mCi/mmol) or ^{14}C-UL-toluene (>98% purity, specific activity 9.7 mCi/mmol). The radiolabeled chemicals were added from anoxic stock solutions of unlabeled benzene (6.6-6.9 x 10^6 dpm/bottle) or toluene (2.5 x10^5 dpm/bottle) to achieve an initial concentration of 50 μM in the slurries. The Fe(III)-NTA solution was prepared by boiling and cooling distilled water under a stream of 100% N_2, then adding per 100 ml: 1.64 g sodium bicarbonate, 2.56 g nitrilotriacetic acid, trisodium salt (NTA), 2.70 g ferric chloride. The solution was filter sterilized (0.2 μm filter) and stored in a sterile serum bottle under a N_2: CO_2 (80:20) headspace until used.

Studies of the ability of sediment microorganisms to oxidize iron anaerobically were initiated as above with the following changes. Five g of sediment from Ponca City, OK and 15 ml of pore water were added to anaerobic culture tubes (Bellco Glass, Vineland, NJ) and incubated with or without sodium sulfide. These incubations were amended from sterile anoxic stock solutions to achieve an initial concentration of nitrate

(20 mM) and/or benzoate (1 mM), lactate (4 mM) or left unamended for controls. No exogenous source of Fe(II) was added to the tubes. All incubations were placed under a N_2: CO_2 (80:20) headspace.

The medium used for screening all other environmental samples for nitrate-dependent Fe(II) oxidizing capabilities contained per liter: 0.5 g NH_4Cl, 2 g NaCl, 0.2 g $MgCl_2 \cdot 6H_2O$, 0.04 g $CaCl_2 \cdot 2H_20$, 2 g TES buffer (N-tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid), 2.4 g sodium lactate, 0.1 g yeast extract (Difco Laboratories, Detroit, MI), and 0.4 g K_2HPO_4. The medium was adjusted to pH 7.5 with KOH, and then 0.2 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added. The medium was degassed, taken into an anaerobic glove box, amended with 0.05 g cysteine, dispensed (9 ml) into anaerobic culture tubes containing a cleaned and degreased iron brad [23], and sealed with a butyl rubber stopper and aluminum crimp. The tubes were then autoclaved for 5 min [24] (121°C) and amended with nitrate or sulfate from sterile anoxic stock solutions of KNO_3 or Na_2SO_4, respectively, to achieve an initial concentration of 10 mM. A starting inoculum from each sediment or soil was achieved by adding 6 g of solid material to 54 mls of the above medium into sterile 120 ml serum bottles. Each sample was serially diluted and incubated in triplicate at 30° C in the dark.

The medium used in the oily sludge incubations was made as described previously [25]. Oily sludge (4 ml) was added to 36 ml of medium in a 60 ml sterile serum bottle, amended with nitrate (20mM), sealed and incubated as the other inocula.

Analytical Techniques

Total and reduced iron was determined as previously reported [26]. Standards where made using ferrous ethylenediamine sulfate (TCI American, Portland, OR). Samples of aquifer slurries (1.5 ml) were periodically collected by syringe, centrifuged to remove particulates, and assayed immediately for total and reduced iron. A portion of the sample was stored frozen for subsequent nitrate and substrate analysis. Nitrate was determined by ion chromatography as previously described [27]. Lactate and acetate were determined by HPLC, using a Dionex AS11 column and a mobile phase of 5 mM NaOH at a flow rate of 2.0 ml per min. Radiolabeled benzene and carbon dioxide were quantified as previously described [8,28].

Results

Microorganisms anaerobically biodegraded benzene with Fe(III) as an electron acceptor (Figure 4). Loss of ^{14}C-UL-benzene depended on the availability of Fe(III) (Figure 4B) and the recovery of $^{14}CO_2$ was essentially complete (90%) after 150 days (not shown). The amount of Fe(III) reduced in these vessels was more than twice that expected even after correcting for ferric iron loss in sterile controls (data not shown). Figure 4B shows benzene degradation in these incubations is continuous, as long as Fe(III) is available. Benzene loss was detected in two out of the three replicates when Fe(III)-NTA was sufficiently supplied to these bottles (Figure 4B, line **b**), but it was not metabolized in the one replicate which was not supplied Fe(III)-NTA (Figure 4B, line **a**). After 40 days, the latter incubation received an Fe(III)-NTA amendment (3.5 mM) and biodegradation resumed (Figure 4B, dashed line). Subsequent benzene amendments were also removed, as long as sufficient Fe(III) was available.

Attempts to demonstrate the biodegradation of benzene in the same sediment under sulfate reducing or methanogenic conditions were unsuccessful. The majority of the radiolabel was recovered as undegraded benzene at the end of the incubation (not shown). A minor amount of radiolabel was recovered as $^{14}CH_4$ and $^{14}CO_2$ in these incubations. However, this recovery was less than the amount of impurity in the starting benzene preparations. Unlike benzene, the transformation of ^{14}C-toluene to $^{14}CO_2$ was relatively easily observed under both sulfate- and Fe(III) reducing conditions with relatively high recovery of the added label as $^{14}CO_2$ (Table 5). Toluene decay was not evaluated under methanogenic conditions.

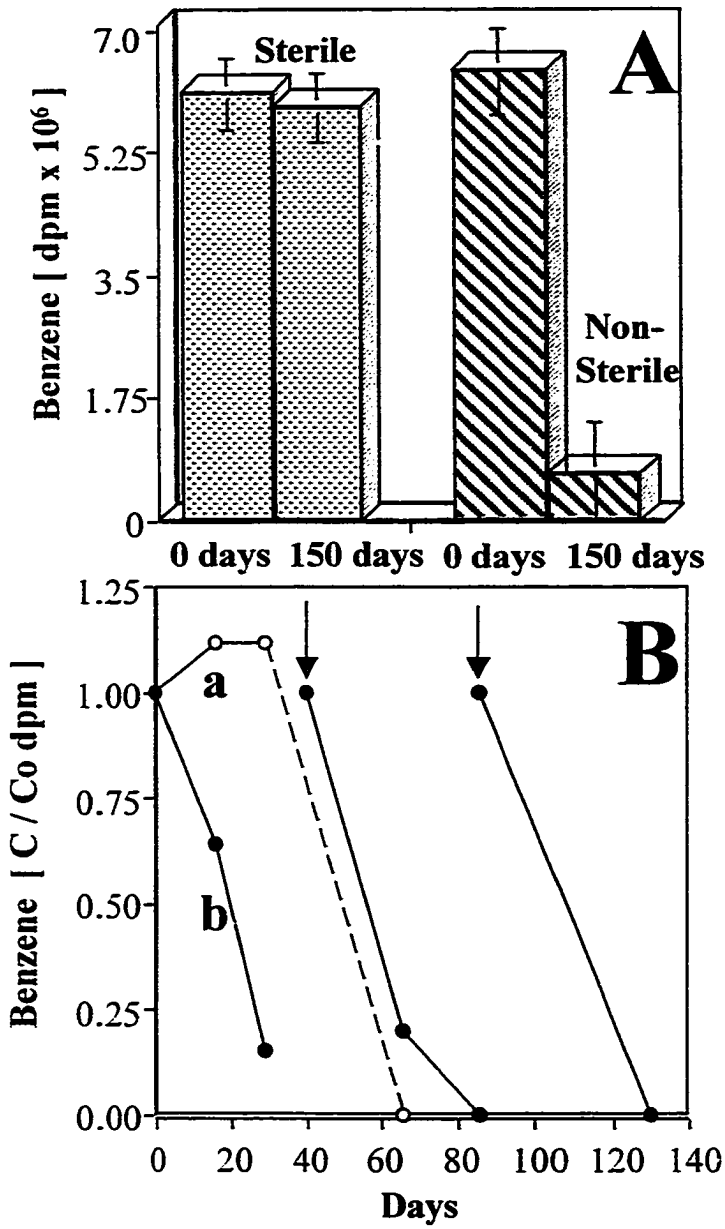

Figure 4. Benzene loss from Ponca City sediment incubations receiving Fe(III)-NTA as an electron acceptor. **A:** Represents the average and standard deviation in benzene recovery from sterile and non-sterile incubations. Starting concentration of benzene was 50 μM. **B:** Line **a** represents Fe(III)-NTA depleted benzene degrading enrichment. Additional electron acceptor was added on day 40, and benzene degradation (dashed line) was concomitant. Line **b** represents an enrichment that was Fe(III)-NTA sufficient. Arrows indicate periodic reamendment of benzene: 50 μM 1st arrow; 100 μM 2nd arrow.

TABLE 5. Recovery of ^{14}C-Labeled Products of ^{14}C-ring-Toluene Biodegradation by Ponca City Sediment Microorganisms under Different Electron Accepting Conditions

Electron Acceptor	Initial Toluene amendment	$^{14}CO_2$ recovered	$^{14}CH_4$ recovered	^{14}C-toluene recovered	Total recovery
Toluene:					
Sulfate	2.5×10^5	2.9×10^5	0	4.1×10^3	117%
Fe(III)	2.5×10^5	2.0×10^5	0	9.7×10^3	84%

All values reported in dpm.

Under nitrate reducing conditions, only limited evidence for BTEX biodegradation was obtained in comparable sediment incubations. After 400 d, only toluene and the positive control substrate, benzoate, exhibited nitrate loss consistent with the mineralization of these substrates (Figure 5). When corrected for substrate-unamended controls, no nitrate reduction was evident in incubations amended with benzene, ethylbenzene or any of the xylene isomers (Figure 5). In fact, these substrates slightly inhibited nitrate removal. There was no loss of nitrate in sterile controls. The toluene incubations were amended again with substrate on day 160 for a total of 800 μM toluene. The loss in nitrate in toluene-amended incubations in excess of substrate-unamended controls, could be correlated with toluene removal (not shown). The benzoate incubations contained 850 μM substrate. Using the stoichiometry below, we calculate that 102% of the expected amount of nitrate was used in both the benzoate and toluene incubations.

$$C_7H_6O_2 + 6NO_3 + 6H^+ \longrightarrow 7CO_2 + 3N_2 + 6H_20 \qquad \text{(eq. 1)}$$

$$C_7H_8 + 7.2NO_3 + 7.2H^+ \longrightarrow 7CO_2 + 3.6N_2 + 7.6H_20. \qquad \text{(eq. 2)}$$

With time, the nitrate reducing incubations exhibited an obvious color change from clear to orange-brown. The latter color was suggestive of oxidized Fe(III). This color development was not evident in other incubations in which no nitrate reduction occurred. In fact, an assay of the liquid phase revealed that Fe(III) was present at 400-500 μM, while dissolved Fe(II) was 10 μM. Investigation of the Fe(III) in the liquid phase indicated that it could not pass through a 0.22 μm filter and was presumed colloidal rather than soluble. After 2 to 3 months, the oxidized iron color gradually diminished and

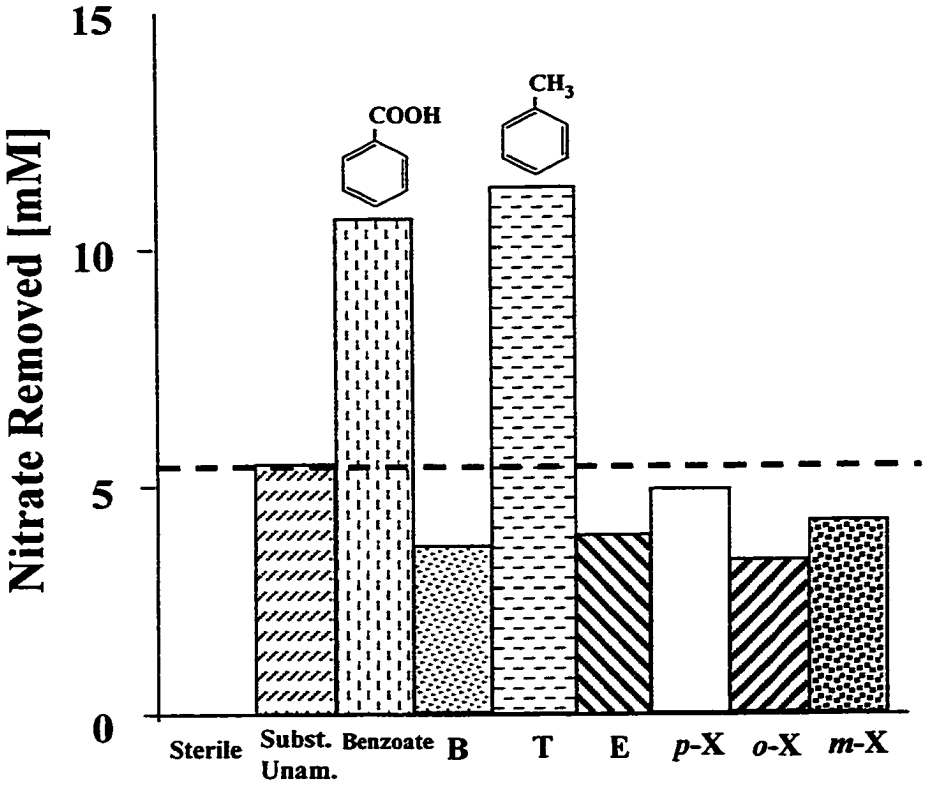

Figure 5. Nitrate lost in Ponca City sediment incubations amended with BTEX. Incubations were initially amended with 20mM nitrate. Benzoate was used as a positive control substrate. Dashed line indicates background level of nitrate reduction. Incubation time was 400 days. Subst. Unam.= substrate unamended, **B**=benzene, **T**=toluene, **E**=ethylbenzene, ***p*-X**=*p*-xylene, ***o*-X**=*o*-xylene, ***m*-X**=*m*-xylene.

the cultures returned to their colorless state. Subsequent experiments were designed to examine further the nitrate-dependent, iron oxidizing capacity of the aquifer sediments.

The ability of microorganisms to anaerobically oxidize endogenous Fe(II) to Fe(III) under nitrate reducing conditions was tested with a variety of electron donors. Using benzoate as a representative compound, we observed the oxidation of Fe(II) to Fe(III) in anaerobic incubations that did not receive sulfide but were reduced (resazurin colorless) (Figure 6A). The amount of colloidal Fe(III) produced (465 μM at 8 d), corresponded with the near stoichiometric loss of 440 μM of Fe(II). This same scenario was apparent whether lactate or benzoate was used as an organic substrate, as well as whether incubations received sulfide or were naturally reduced (data not shown). Loss of Fe(III) observed after 8 days is most probably attributed to its insoluble nature at circumnuetral pH values (Figure 6A), however no loss of Fe(III) was observed in tubes that were reduced with sulfide, where a stable colloidal Fe(III) complex remained in solution for greater than two years (data not shown). The production of Fe(III) and loss of Fe(II) from the incubation corresponded with the degradation of benzoate and reduction of nitrate within the system (Figure 6B). The nitrate depletion ceased when benzoate was exhausted. No loss of nitrate or substrate was noted in any of the sterile control incubations, nor was the production of Fe(III), although there was a slight increase in the amount of Fe(II) (data not shown). In substrate-unamended controls that contained nitrate a slight increase of Fe(III), a loss of Fe(II), and a slight decrease in the nitrate control was noted (data not shown). The production of Fe(III) was much slower and far less pronounced (250 μM) in these incubations and the total amount of Fe(II) was never completely removed from the system.

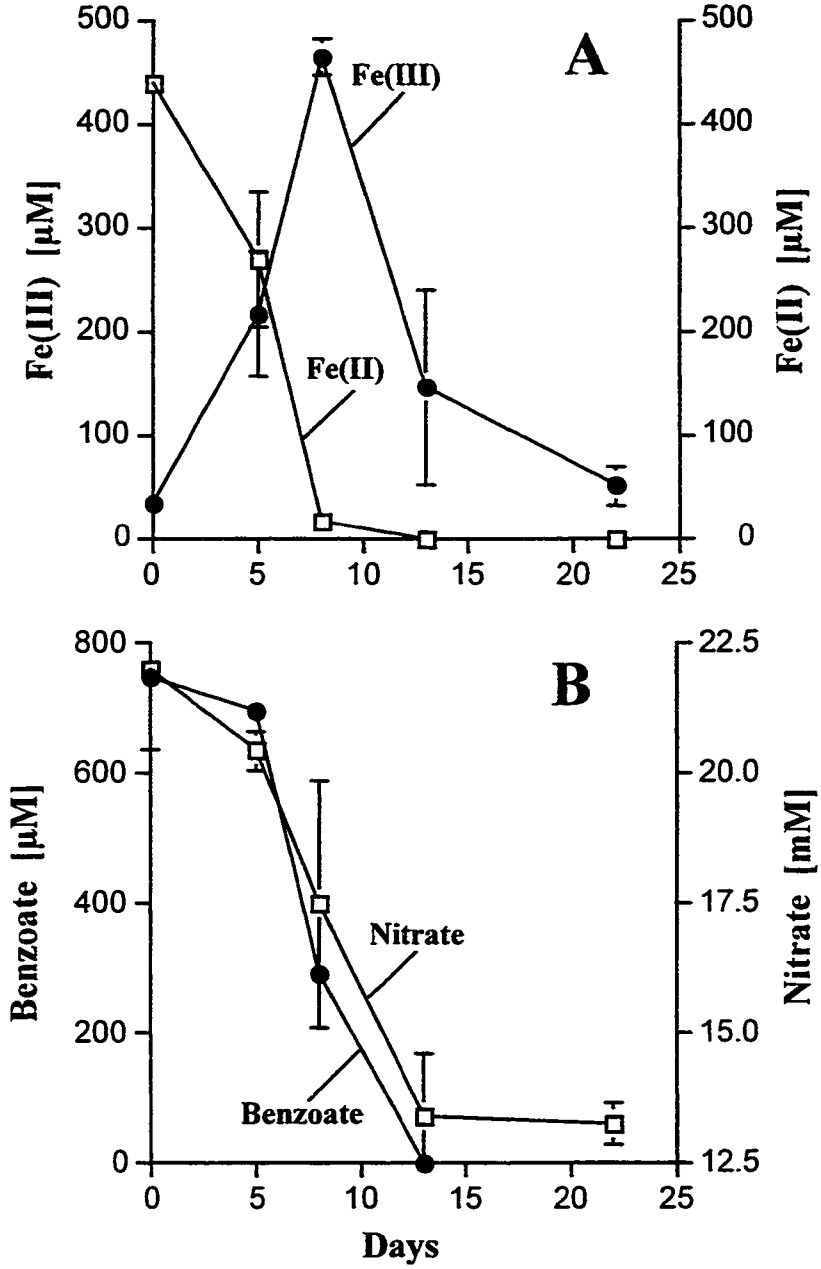

Figure 6. The depletion of nitrate, benzoate and Fe(II), as well as Fe(III) production, in Ponca City aquifer sediment incubations. **A:** The corresponding loss of Fe(II) and with the transient accumulation of Fe(III). **B:** Benzoate depletion coupled with nitrate loss.

To test the ubiquity of the iron oxidation process, samples were taken from a variety of locations including three petroleum-contaminated aquifers, a landfill-leachate impacted aquifer, an oil-water separator sludge, and a garden soil. All inocula were incubated with nitrate and analyzed for Fe(III) production. Using a dilution series to test the extent of the activity, we found that all samples showed some capacity for the transformation of ferrous to ferric iron (Table 6). Samples exhibited a differential capacity to oxidize iron at higher dilutions, but all samples gave a positive response when an easily degradable substrate (lactate) was also present. Without the latter amendment, the ability to oxidize iron was less pronounced in all samples (Table 6). Only a single substrate-unamended incubation, obtained from a site previously characterized as sulfate reducing, was incapable of anaerobic iron oxidation (Table 6). However, the metabolic potential in this sample was evident when lactate was also added.

Table 6. **Fe(II) Oxidizing Capacities of Various Samples Assayed under Nitrate Reducing Conditions**

Petroleum-Contaminated Aquifers:	**No Substrate**	**Lactate**
Rocky Point, NC	++	+++
Empire, MI	+	++
Seal Beach, CA	-	+
Various Environments:		
Naval Oily Sludge (brackish)	++	+++
Leachate-Impacted Aquifer	++	++
Garden Soil	+	+++

+++ : positive for biological activity $\geq 10^{-3}$ dilution of original sample;

++ : positive $\leq 10^{-2}$; + : positive @ 10^{-1}; - : negative for activity

Discussion

Fe(III) reduction is quantitatively the most important terminal electron acceptor for the reduction of organic matter in the subsurface [29], and iron reducing microorganisms have been readily isolated from environmentally diverse habitats [30]. Several observations on anaerobic benzene biodegradation linked to Fe(III) reduction have been reported in the past few years [as reviewed in 1,2;7,8,10-12]. Using a chelated form of Fe(III), we could confirm the findings of anaerobic benzene oxidation coupled with this electron acceptor (Figure 4). The biodegradation of benzene did not occur when Fe(III) was not available or in sterile controls that did contain Fe(III). Repeated additions of benzene showed an increased rate in benzene decay when Fe(III) was kept sufficient. This is indicative of the enrichment of benzene-degrading microorganisms in these sediments.

The biodegradation of benzene under sulfate-reducing and methanogenic conditions was negligible in comparable sediment incubations. Recovery of the majority of the label as undegraded hydrocarbon indicated little transformation capacity by the resident microflora under the latter two conditions. The small amount of labeled end products recovered in our methanogenic incubations ($^{14}CH_4$ (49%) and $^{14}CO_2$ (51%)) exhibited a different ratio than that expected based on previously reported stoichiometry (62.5%CH_4: 37.5%CO_2) [31]. This aspect of our work contrasts with Lovley and colleagues who found that benzene could be transformed without lag in the same sediments when methanogenesis was the predominant terminal electron accepting process [32,33]. However, toluene mineralization was confirmed under sulfate- and Fe(III)-reducing conditions (Table 5). Toluene has been shown to be the easiest of the BTEX

hydrocarbons to anaerobically biodegrade [1,2], so it's not unexpected to see the utilization of this hydrocarbon under varying electron accepting conditions.

Though the ability of nitrate reducing microorganisms to metabolize toluene, ethylbenzene, and the xylene isomers has been conclusively demonstrated [1,2, 34-40], the biodegradation of benzene under nitrate reducing conditions remains somewhat contentious. The ability of these organisms to utilize a diverse array of substrates such as polyaromatic hydrocarbons [41-46], straight chain alkanes [40,47,48] and branched alkanes [49] suggests the possibility that the simplest aromatic compound, benzene, could be metabolized under nitrate-reducing conditions. However, in our hands, there was only a limited ability of the resident aquifer microflora to utilize BTEX hydrocarbons (Figure 5). Differences in the ability to anaerobically degrade benzene under nitrate reducing conditions versus Fe(III)-NTA conditions (Figure 4) might be attributed to toxicity effects owning to an eight fold increase in benzene used in the nitrate reducing bottles (400 μM) versus the Fe(III) reducing bottles (50μM).

During the course of these incubations, a strong color change indicative of oxidized iron was noted in the liquid phase of these cultures. The reoxidation of ferrous to ferric iron has been generally considered an oxygen-dependent reaction under both biotic and abiotic conditions [50,51]. The abiotic anaerobic oxidation of Fe(II) to Fe(III) has been observed with nitrate, nitrite or nitrous oxide as the oxidizing compounds [52-58], but in most cases the pH dependence and/or the requirement for specific catalysts implied relatively modest environmental significance. Recently, the microbially mediated anaerobic oxidation of Fe(II) to Fe(III) by photosynthetic bacteria was recognized as a new type of anaerobic respiration [59,60]. Documentation of anaerobic

microbial oxidation of ferrous iron by nitrate reducing eubacteria and a hyperthermophilic archaeum has been reported [61-64]. Further studies to test whether the oxidation we observed was a biologically catalyzed reaction were performed.

In separate experiments, the anaerobic oxidation of Fe(II) to Fe(III) was clearly evident when readily degradable substrates such as lactate or benzoate were added to the nitrate containing incubations (Figure 6). No Fe(III) production was evident in sterile controls or incubations that did not contain nitrate, indicating the process was biologically catalyzed. Nitrate loss in our experiments exceeded the amount necessary for complete benzoate mineralization (167%). This explanation suggested that an additional sink for this electron acceptor existed, presumably Fe(II). However, the stoichiometry first suggested by Sørensen [65] and later confirmed by Straub et al. [61] suggest a molar ratio of Fe(III) to nitrate of at least 5 to 1.

$$10Fe^{2+} + 2NO_3^- + 24H_2O \longrightarrow 10Fe(OH)_3 + N_2 + 18H^+ \qquad \text{(eq. 3)}$$

$$8Fe^{2+} + NO_3^- + 21H_2O \longrightarrow 8Fe(OH)_3 + NH_4^+ + 14H^+ \qquad \text{(eq. 4).}$$

However, much more nitrate was consumed than could be accounted for by the Fe(III) measured in our experiments. This disparity can likely be partially explained by our measurement of only the soluble/colloidal-ferric iron. Presumably, the remainder is insoluble ferric iron which is largely insoluble at circumnuetral pH values and thus escaped our detection. Similarly, a pure culture capable of ferric iron oxidation coupled with nitrate reduction (strain BrG2) did not exhibit a clear stoichiometric balance [61]. That is, more nitrate was reduced than could be accounted for by the formation of nitrite and Fe(III). The authors proposed the formation of an unknown oxidized nitrogen species which could possibly complex with iron in the culture [66].

Further explanation for the disproportionate loss of nitrate observed in our incubations could be the reduction of nitrate to nitrite coupled to Fe(II) oxidation as proposed by Stetter and colleagues for the thermophilic archaeum *Ferroglobus placidus* according to equation 5 [62].

$$2FeCO_3 + NO_3^- + 6H_2O \longrightarrow 2Fe(OH)_3 + NO_2^- + 2HCO_3 + 2H^+ + H_2O \quad \text{(eq. 5)}$$

The ratio of Fe(III) oxidized to nitrate reduced was close to the theoretically expected amount for their isolate. However, nitrite was only transiently observed in our incubations and the excess loss of nitrate observed in our incubations would still not account for the low Fe(III) concentrations observed.

All tested inocula harbored cells capable of nitrate dependent Fe(II) oxidation, thereby attesting to the potential for the wide occurrence of this process in nature (Table 6). Though not all inocula showed the same capacity for this ability, the presence of an easily degradable substrate and high levels of reduced iron allowed all samples to give a positive response. The anaerobic microbial oxidation of Fe(II) represents a relatively novel microbial process and further studies on the prevalence of this activity are needed. Straub et al. [61] showed that several previously isolated nitrate reducing organisms were able to couple nitrate reduction with Fe(II) oxidation. Two previous denitrifying strains capable of using aromatic compounds could oxidize greater than 50% of the added iron when acetate was provided as a substrate. In addition, strain ToN1, which can degrade toluene under nitrate reducing conditions, as well as *Thiobacillus denitrificans* and *Pseudomonas stutzeri* could autotrophically oxidize Fe(II). But two other organisms tested, *Thiomicrospira denitrificans* and *Paracoccus denitrificans*, did not oxidize Fe(II) [61].

The capacity for microorganisms to oxidize ferrous to ferric iron under nitrate reducing conditions has only recently been recognized as an important component for the cycling of iron in natural systems [61-64]. Our study indicates that Fe(II) may serve as an alternate sink for nitrate in contaminated environments as well. For example, in a field study where nitrate was employed as a bioremediation strategy for the clean up of jet fuel, approximately 10 times more nitrate was consumed in the aquifer than could be accounted for by the loss of BTX biodegradation alone [67]. Speculation that oxidized intermediates or other components of the jet fuel served as substrates for nitrate reduction was the only explanation offered. The process of ferrous iron oxidation was not considered.

The fact that the Fe(III) produced in our incubations was likely colloidal is also of importance to bioremediation efforts. Colloids have garnered much attention recently since they are known to enhance the transport of chemical contaminants in the subsurface [68]. In fact, the transport of colloids through porous media can actually occur at a velocity greater than conservative solute tracers like tritiated water and nitrate [69-71]. The prevalence of natural organic matter (eg.humic acids) increases the mobility and stability of Fe(III) oxyhydroxide colloids. Consequently, colloidal transport mechanisms may effectively exacerbate subsurface contamination problems.

On the other hand, the availability of electron acceptors in an aquifer is often a factor limiting the microbial metabolism of contaminants. An Fe(III) resupply and transport mechanism may also serve to overcome such limitations. In our case, we observed the anaerobic degradation of benzene when chelated Fe(III) was available as an electron acceptor (Figure 4) and described a potential resupply mechanism. Colloidal

iron is likely to be important for bioremediation purposes since Lovley and colleagues observed that the biodegradation of toluene and benzene is more rapid when Fe(III) is supplied in a more soluble form including Fe(III) complexed with naturally occurring or synthetic ligands [7,10,11]. Future studies on the relationship of anaerobic iron oxidation processes linked with nitrate reduction may provide important insights for a more accurate evaluation of the transport and fate of contaminants in the terrestrial subsurface.

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Chapter 3.

Detection of Phenol and Benzoate as Intermediates of Anaerobic Benzene Biodegradation under Different Terminal Electron Accepting Conditions

Abstract

A sulfate-reducing bacterial enrichment that anaerobically metabolized benzene was obtained from a petroleum-contaminated aquifer. During biodegradation, we observed the transient accumulation of phenol and benzoate as putative benzene intermediates. As these compounds are intermediates in many anaerobic metabolic pathways, we investigated their relation to anaerobic benzene decay with ^{13}C-labeled starting material. We were able to confirm the presence of ^{13}C-phenol and ^{13}C-benzoate as intermediates of anaerobic ^{13}C-UL-benzene decay. Mass spectral evidence indicated that the carboxyl group of benzoate also originated from ^{13}C-labeled benzene. Benzoate was also found as a putative benzene intermediate when inoculum from the same site was incubated under methanogenic conditions or when organisms enriched from a different petroleum-contaminated location were incubated with chelated Fe(III) as an electron acceptor. These findings are the first to confirm the importance of benzoate during anaerobic benzene metabolism and suggests that concerns over the accumulation of potentially recalcitrant intermediates in anaerobic environments contaminated with this substrate are unwarranted.

Introduction:

The environmental fate of gasoline in anoxic aquifers has attracted a great deal of regulatory attention. Benzene has merited particular scrutiny as it is the most water soluble and carcinogenic of all gasoline hydrocarbons (*1*). Many studies have indicated that benzene is recalcitrant under anaerobic conditions (as reviewed in *2, 3*). However, recent findings have conclusively shown that benzene is susceptible to biodegradation under a variety of terminal electron accepting conditions including nitrate- (*4*), sulfate- (*5-11*), and Fe(III)-reducing conditions (*12-15*), as well as methanogenic conditions (*6, 10, 16-18*). Despite these findings, relatively little is known of the pathways associated with anaerobic benzene decay. Such information is needed for more than just esoteric scientific reason. The literature is replete with examples of substrates that undergo primary degradative events to form persistent intermediates that are associated with their own environmental concerns (*19*). As benzene has no substituents to destabilize the aromatic ring, any transformation would likely result in a more polar and water soluble intermediate with a greater potential for migration in the environment. Thus knowledge of the identity and the fate of benzene intermediates formed under anaerobic conditions is as important as the destruction of parent substrate.

Only three studies, all under methanogenic conditions, have detected any metabolites of benzene decay (*16-18*). In each case, the activation of benzene was observed to proceed through the oxidation of the aromatic ring to phenol. Further metabolism of phenol to cyclohexanone (*17*) was hypothesized as a bioconversion product preceding ring cleavage to form the fatty acids propionate and/or acetate (*17, 18*). With respect to phenol, several degradative routes have been proposed which include

direct ring reduction (*16, 17, 20, 21*) and the carboxylation of parent substrate to form *para*-hydroxybenzoate. The latter compound is then dehydroxylated to form benzoate which is subsequently metabolized to a variety of aliphatic acids (as reviewed in *22, 23*). To examine the fate of benzene more closely, we obtained enrichments from two sites harboring bacteria that exhibited anaerobic benzene decay (*10, 15*). Using ^{13}C-labeled benzene, we confirmed the importance of phenol as an oxidized intermediate of benzene metabolism under sulfate- and Fe(III)-reducing conditions. We also observed ^{13}C-benzoate from labeled benzene in sulfate-reducing enrichments. This is the first report of benzoate as an intermediate of benzene decay under any anaerobic condition, and the first report of the detection of phenol as an intermediate of benzene decomposition under conditions other than methanogenic. Further, our finding of benzoate and phenol as benzene intermediates suggests that the accumulation of recalcitrant intermediates during anaerobic benzene biodegradation is unlikely.

Material and Methods:

Enrichments. Sulfate-reducing and methanogenic enrichments were obtained from a petroleum-contaminated aquifer located in the Sleeping Bear Dunes National Lakeshore near Empire, MI as previously described (*10*). An Fe(III)-reducing enrichment was obtained from a petroleum-contaminated aquifer in Ponca City, OK as described previously (*15*). Unlabeled benzene was added to the incubations as a neat compound (Aldrich Chemical Co., Milwaukee, WI; 99.8% purity), while radiolabeled benzene (^{14}C-UL-benzene) amendments were as previously described (*10*). ^{13}C-UL-benzene (Cambridge Isotope Laboratories, Andover, MA; 99% purity) was added to achieve an approximate starting concentration of 750 μM. In each enrichment, aquifer sediment was allowed to settle and culture fluids were removed during the course of the incubation for metabolite analysis (below) and replaced with a basal microbiological medium buffered with $NaHCO_3$ (*24*). All enrichments were placed under a headspace of N_2:CO_2 (80:20) and incubated at room temperature in the dark. Sulfate was amended as necessary from a sterile, anoxic stock solution. Ferric iron was supplied from an sterile, anoxic stock solution of ferric chloride chelated with nitrilotriacetic acid (Fe(III)-NTA) as described previously (*15*).

Analytical techniques. Radiolabeled benzene was measured as previously described (*10*). Unlabeled benzene was measured by gas chromatography (GC) following the same procedures used with toluene (*25*), except an isothermal oven temperature of 100°C was used. To identify possible intermediates, culture fluid (75-90 ml) was periodically removed from the incubations while inside an anoxic glovebag and placed in acid-washed glassware. Sample preparation, trimethylsilyl derivatization

(TMS) and GC-mass spectrometry (GC-MS) were accomplished as described previously (*25*), except the GC oven temperature was held at 40°C for 2 min and then raised at a rate of 10°C/min to 220°C. Authentic standards of suspected benzene intermediates were derivatized and analyzed in the same fashion. Sulfate concentrations were measured by ion chromatography as previously described (*26*). No ^{13}C-labeled phenol or benzoate were detected in sterile controls or substrate-unamended incubations analyzed in an identical fashion. Further, GC-MS analysis of the ^{13}C –benzene revealed that no ^{13}C-phenol or ^{13}C-benzoate could be detected in sterile preparations, even when the labeled starting material was four times the concentration normally added to the incubations.

Results:

Anaerobic degradation of benzene. A microbial enrichment was obtained that could biodegrade benzene using sulfate as a terminal electron acceptor. The degradation of ^{14}C-UL-benzene coupled with the loss of sulfate is shown in Figure 7A. The loss of sulfate for the three amendments of 50 μM, 50 μM and 100 μM benzene was 170 μM, 200 μM and 380 μM, respectively. This is approximately 91%, 107% and 101%, respectively, of the amount theoretically expected based on the following stoichiometry for complete benzene mineralization:

$$C_6H_6 + 3.75\ SO_4 + 3\ H_2O \longrightarrow 6HCO_3^- + 1.875\ HS^- + 1.875\ H_2S + 0.375\ H^+.$$

Transformation of radiolabeled benzene to radiolabeled carbon dioxide was easily observed and documented elsewhere (*10*). The bacterial enrichment was able to metabolize repeated amendments of unlabeled benzene and sulfate (Figure 7B), and this metabolic stability facilitated the search for benzene metabolites.

Metabolite Detection. During benzene decay, culture fluids were periodically removed, derivatized and analyzed by GC-MS for possible intermediates. The analysis of samples from the sulfate-reducing enrichment amended with unlabeled benzene showed the presence of GC peaks which co-chromatographed with, and had the same spectral characteristics as, authentic phenol and benzoate when the latter were similarly analyzed (data not shown). In all experiments except one, the concentration of phenol and benzoate detected by GC-MS was ≤ 1 μM. In one experiment, the highest concentration of benzoate was only about 4 μM. However, benzoate and phenol are common to a wide variety of anaerobic metabolic pathways, and further evidence of their status as putative

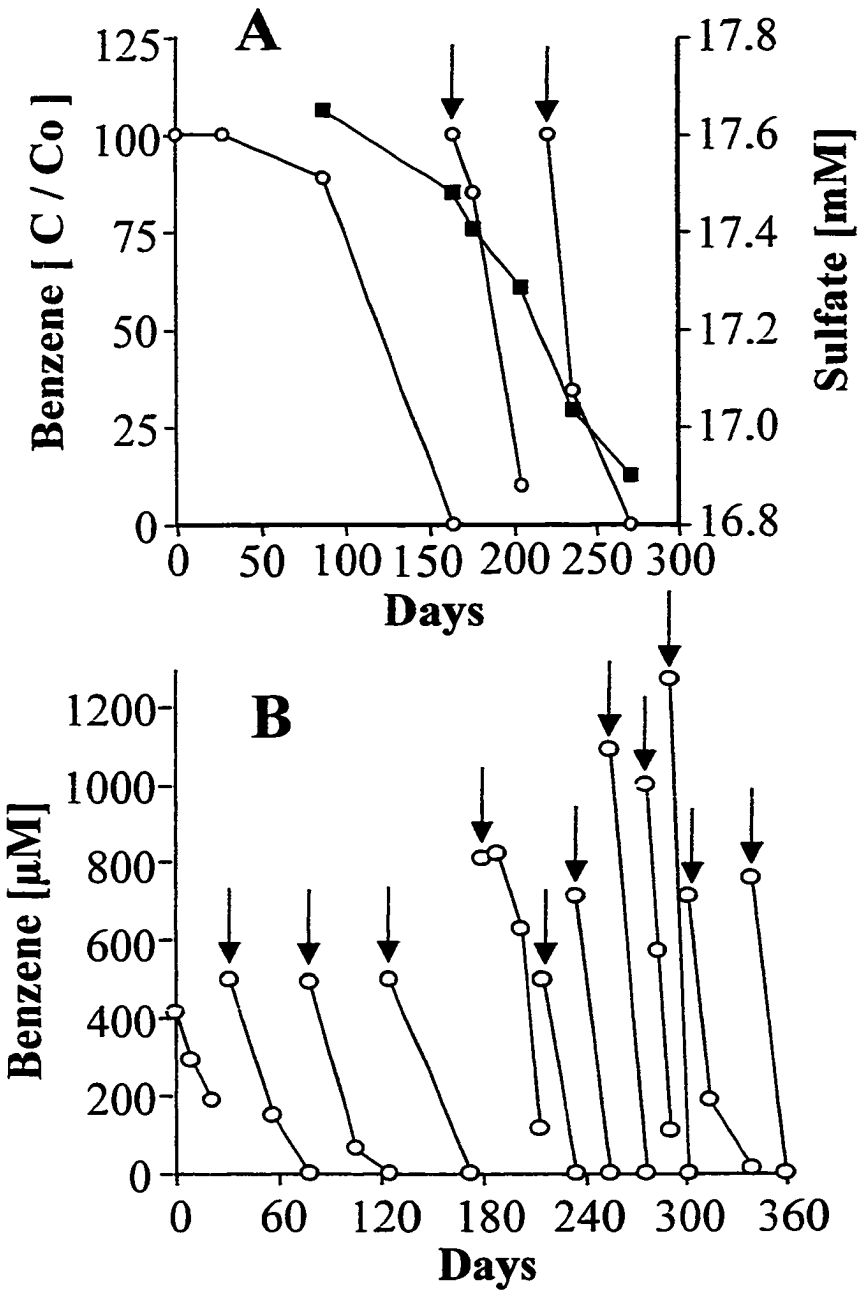

Figure 7. (A) Loss of ^{14}C-benzene (50µM) coupled with the depletion of sulfate over time in sediment slurries. Arrows represent reamendments of ^{14}C-benzene: 50µM for second amendment, 100µM for third amendment. Symbols: benzene, ○; sulfate, ■. (B) Enhanced rate of benzene decay over time in an enrichment bottle under sulfate-reducing conditions. Arrows indicate reamendments of benzene.

benzene intermediates was sought using ^{13}C-benzene as a starting substrate. Clearly, if benzoate and phenol were closely associated with the anaerobic biodegradation of benzene the mass spectral profile of these compounds should exhibit an increase of at least six mass units.

When the enrichment was amended with ^{13}C-benzene and similarly analyzed, a derivatized metabolite was observed that co-chromatographed with the TMS-derivative of phenol (RT=6.9 min). The metabolite also exhibited mass spectral features that were six mass units more than that associated with a phenol standard (Figure 8). That is, fragments corresponding to a mass/charge ratio of 166, 151, 135 and 77 in authentic phenol (Figure 8A) were observed at 172, 157, 141 and 83, respectively with the metabolite. Therefore, phenol was conclusively identified and this finding strongly implicates phenol as an intermediate of anaerobic benzene decay.

Similarly, a second intermediate was also detected during the degradation of ^{13}C-benzene by the enrichment (Figure 9). The second intermediate co-chromatographed with a standard of derivatized benzoate (RT=9.9 min). The mass spectral profile of this intermediate indicated a mixture of ^{12}C- and ^{13}C-labeled benzoate (Figure 9B). With increasing incubation time, the ^{12}C-benzoate signal diminished while the ^{13}C-benzoate was much more apparent. The mass spectral features associated with the benzoate formation were increased by either six or seven mass units (Figure 9B and 3C). The latter finding confirms that another atom of ^{13}C was incorporated into the metabolite, presumably during the carboxylation of the ring by a ^{13}C-labeled fragment liberated during the metabolism of starting substrate. The finding of benzoate also clearly implicates this compound as a benzene intermediate.

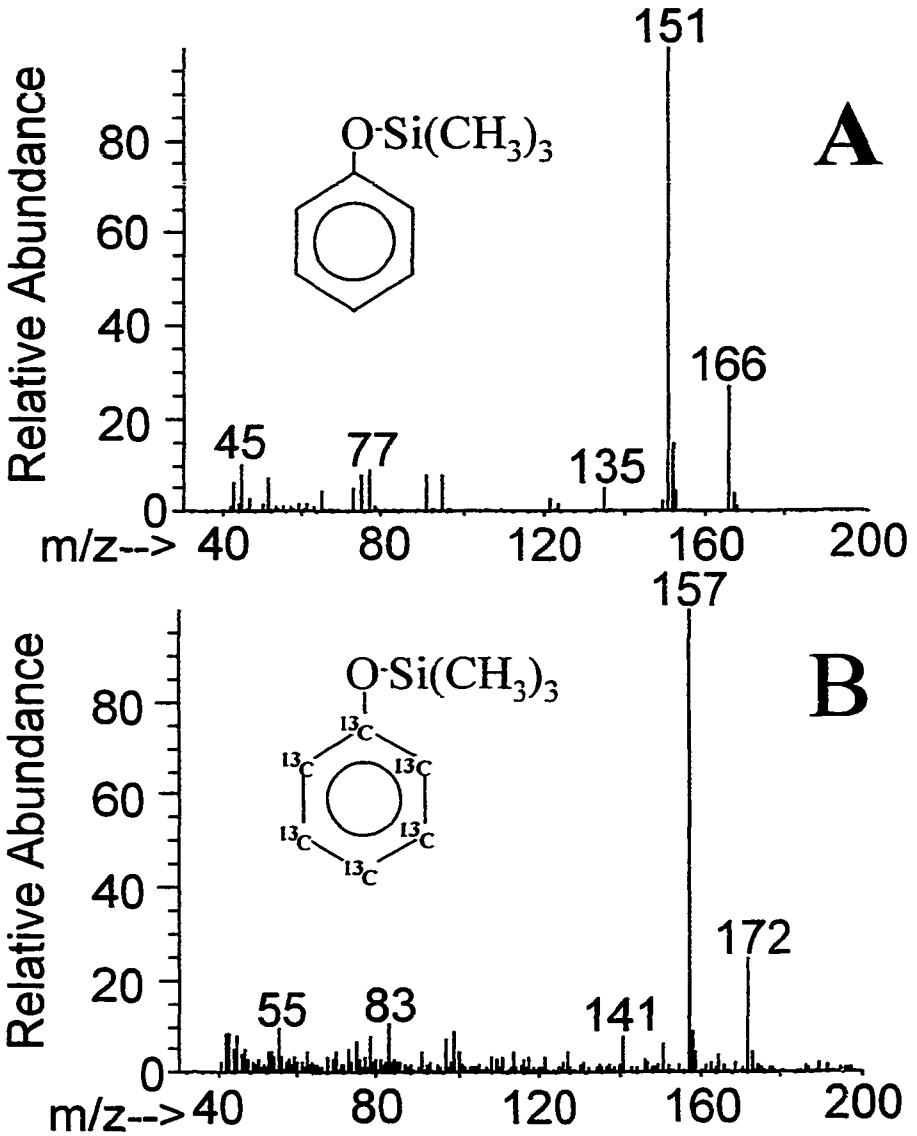

Figure 8. Mass spectra of a TMS-derivatized authentic standard of phenol (A) and a TMS-derivatized metabolite (B) detected in the culture fluid of benzene degrading, sulfate-reducing enrichment amended with ^{13}C-benzene ($^{13}C_6$).

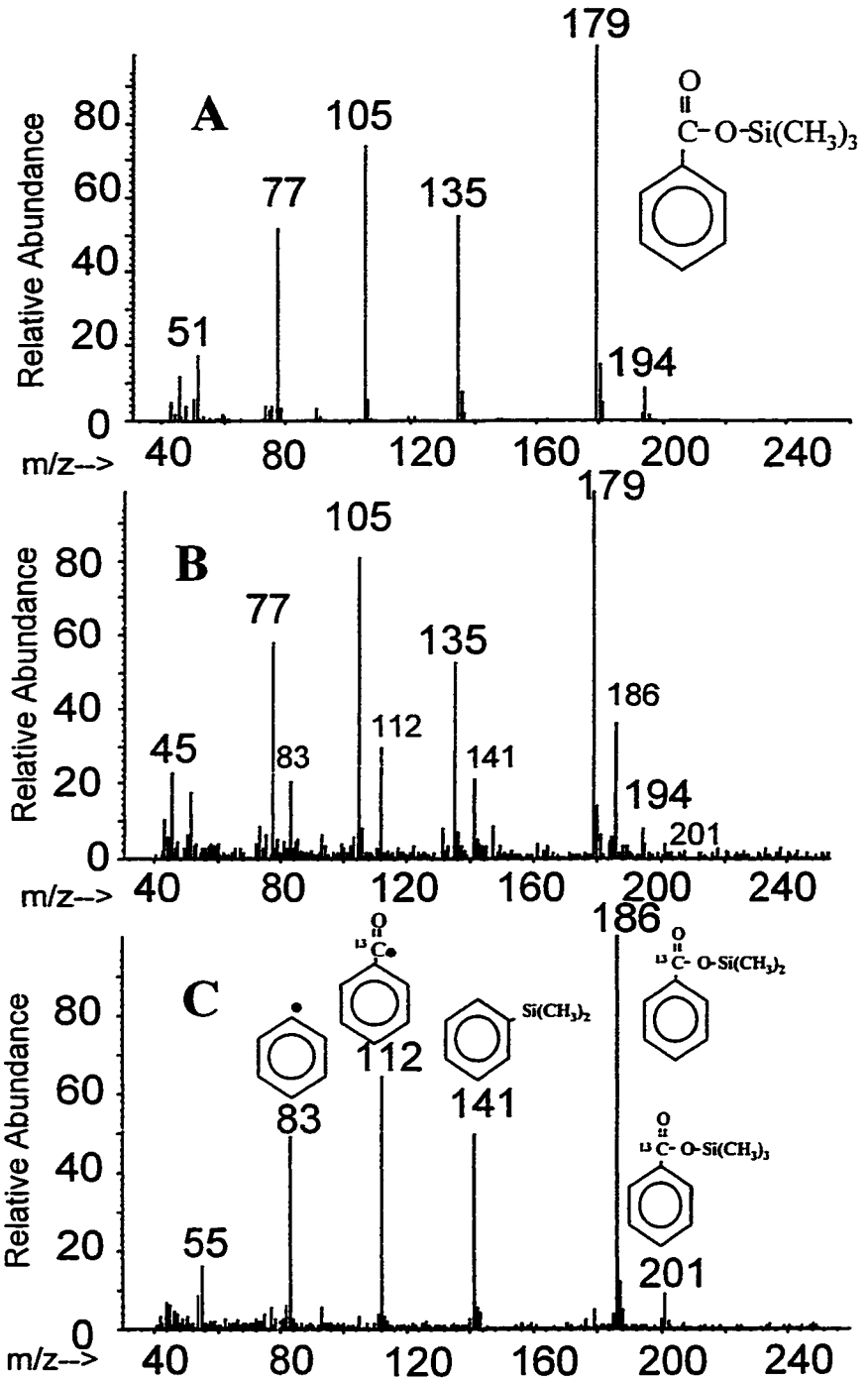

Figure 9. Mass spectra of a TMS-derivatized authentic benzoate standard (A), the TMS-derivatized metabolite detected in a sulfate-reducing bacterial community enriched with ^{12}C-benzene and subsequently amended with ^{13}C-benzene (B), and the derivatized metabolite observed in the same enrichment after repeated additions of ^{13}C-benzene (C). Structures in C help illustrate why some mass spectral features are increased by 6 mass units relative to the benzoate standard (A), while other fragments are increased by 7 mass units, as explained in the text.

Benzoate was also detected as a putative benzene intermediate in methanogenic incubations established with inoculum from the same site (data not shown) and an Fe(III)-reducing enrichment (*15*) (data not shown). In each of these enrichments, ^{13}C-labeled evidence for benzoate formation from benzene has not yet been detected. However, a mixed $^{12}C/^{13}C$ signal associated with derivatized phenol (Figure 10B) and eventually a clear ^{13}C-phenol profile (Figure 10C) was detected in the Fe(III)-reducing enrichment.

In all of the underivatized or derivatized extracts analyzed, no detection of the proposed ring reduction metabolites of benzene or phenol were observed using the GC-MS protocols employed. Our assay included attempts to detect cyclohexanol, cyclohexanone, cyclohexane, adipic acid or *n*-caproic acid, as well as possible benzoate metabolites cyclohexanecarboxylate, cyclohex-1-enecarboxylate, cyclohex-3-enecarboxylate, cyclohex-4-enecarboxylate and 1-hydroxycylohexane carboxylate (data not shown). In addition, *para*-hydroxyphenylacetate was not detected. No detection of ^{13}C-labeled fatty acids such as acetate, propionate, butyrate, succinate, hexanoic acid, heptanoic acid or pamelic acid were observed in any of the extracts. 2-Hydroxycyclohexanone, dihydroxycyclohexane carboxylic acid and phenylphosphate were not tested.

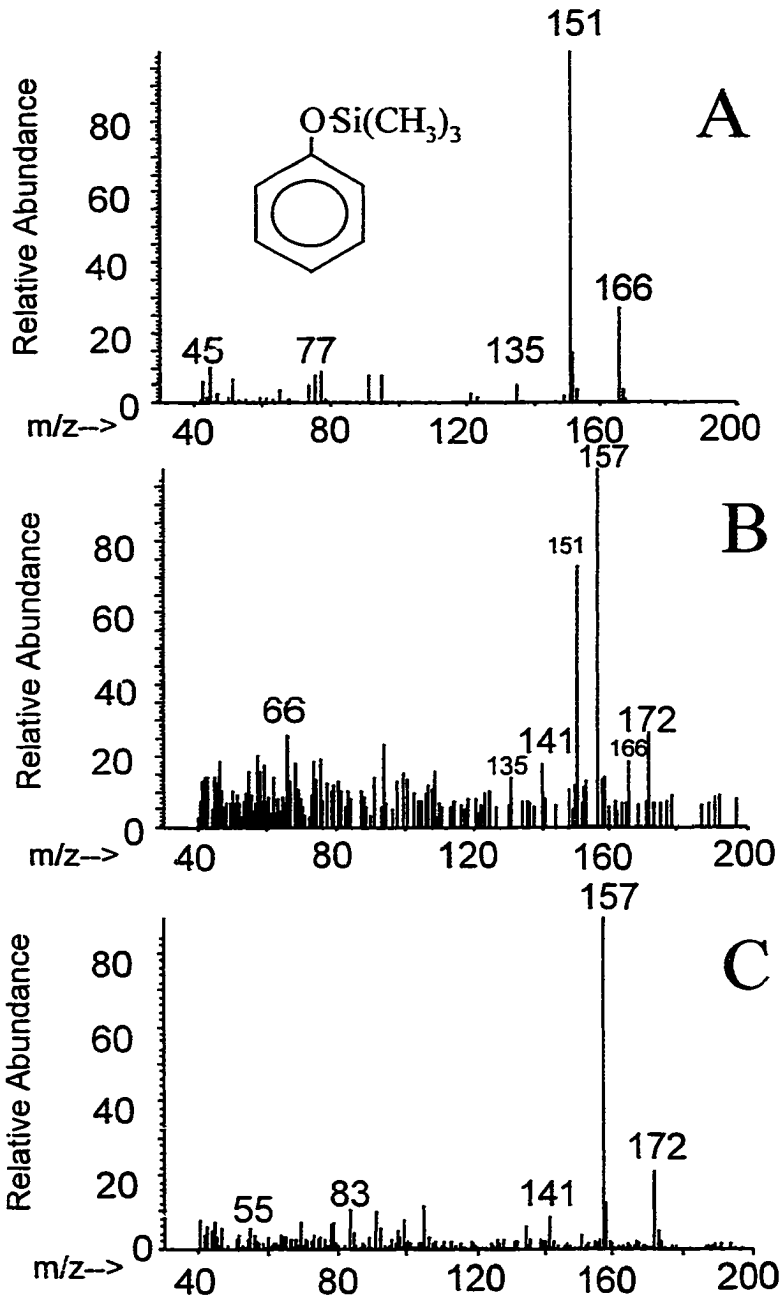

Figure 10. Mass spectra of a TMS-derivatized authentic standard of phenol (A) and a TMS-derivatized metabolite detected in the culture fluid of benzene degrading, Fe(III)-reducing enrichment amended with ^{13}C-benzene after previously fed with ^{12}C-benzene (B) and the metabolite observed in an enrichment adapted solely to ^{13}C-benzene amendments (C).

Discussion:

A stable benzene-degrading sulfate-reducing bacterial enrichment was obtained from a petroleum-contaminated aquifer (Figure 7). Previous indications (*10*), as well as our current experiments, demonstrated that this enrichment could completely mineralize the parent substrate. However, the mechanism(s) employed by anaerobes to metabolize benzene have remained somewhat enigmatic.

Using GC-MS, Vogel and Grbic´-Galic´ observed the oxidation of benzene to phenol in a methanogenic culture of bacteria enriched from sewage sludge for its ability to degrade ferulic acid (*16, 17*). The source of the phenol oxygen was confirmed through the incorporation of ^{18}O-H_2O. Further transformation of phenol was speculated to occur via ring reduction to form cyclohexanone followed by ring cleavage to form aliphatic acids, and eventually carbon dioxide and methane (Figure 11). The identification of cyclohexanone as a putative benzene intermediate was based on studies with unlabeled benzene.

More recently, suggestive evidence for the involvement of phenol in benzene metabolism was proposed by Weiner and Lovley using methanogenic enrichments from sediments (*18*). Isotope trapping studies revealed that the release of $^{14}CO_2$ and $^{14}CH_4$ from ^{14}C-benzene diminished when unlabeled phenol, acetate or propionate were added as potential intermediates in these cultures. Stronger evidence for the involvement of these intermediates was achieved by the detection of radioactive signals in appropriate fractions collected during the chromatographic analysis of incubations which received ^{14}C-labeled benzene. Such findings are consistent with benzene oxidation to phenol, but the pathway associated with the metabolism of the latter was not considered.

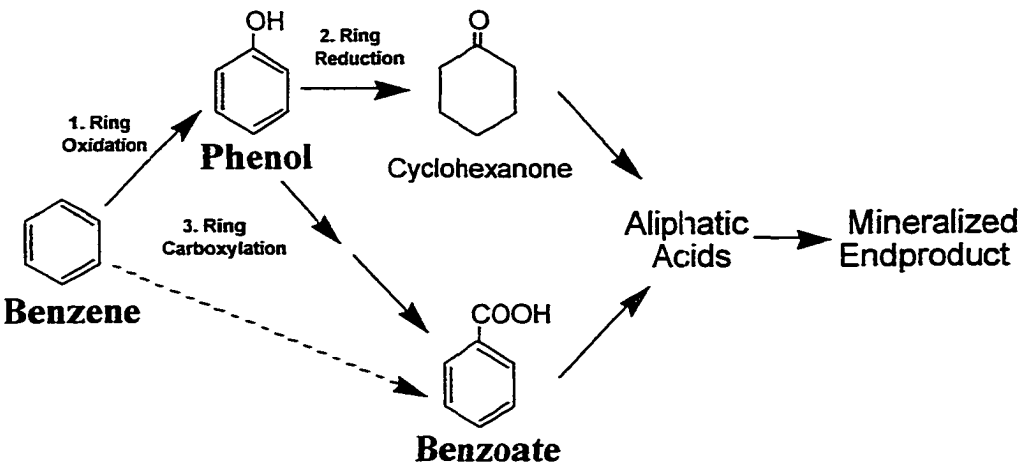

Figure 11. Proposed pathway for the anaerobic biodegradation of benzene which proceeds through benzoate.

The anaerobic decomposition of phenol has been observed to proceed by at least two routes; either a ring reductive pathway (*20, 21*) or through aromatic ring carboxylation to yield benzoate (as reviewed in *22, 23*). In their study, Weiner and Lovley suggest that benzoate, *para*-hydroxybenzoate and butyrate do not appear to be involved in the anaerobic pathway for benzene mineralization (*18*). This implies a ring reduction pathway or an unknown route for benzene metabolism. Support for the ring reduction pathway for phenol conversion was provided by Bakker (*20*). Using a nitrate-reducing consortium, the anaerobic decomposition of phenol to *n*-caproic acid was observed with cyclohexanone as a proposed intermediate (*20*). Further evidence for the reductive pathway was provided by Balba and Evans who detected phenol during the anaerobic biodegradation of catechol (*21*). Labeled cyclohexanone, 2-hydroxycyclohexanone, adipate, succinate, propionate and acetate were detected in culture fluids from a methanogenic consortium growing on ^{14}C-UL-phenol. In the same study, cyclohexanol was implicated as a intermediate formed in cultures growing on unlabeled phenol.

Our studies provide evidence for an alternate pathway for the anaerobic destruction of benzene. Using GC-MS, we observed the ring oxidation of ^{13}C-benzene to ^{13}C-phenol in extracts of culture fluids from sediment enrichments held under sulfate- and Fe(III)-reducing conditions (Figure 8 and Figure 10). This is the first report of phenol as an oxidation product of benzene under an anoxic condition other than methanogenic conditions (*16-18*).

Although the initial ring oxidation of benzene to phenol has been observed, little evidence for subsequent metabolic steps exist. We observed a second ^{13}C-labeled

benzene intermediate in our sulfate-reducing cultures that was conclusively identified as benzoate (Figure 9). This finding is consistent with the detection of benzoate in methanogenic and Fe(III)-reducing enrichments amended with benzene. In all enrichments, evidence for the reductive pathway for benzene transformation was sought, but not obtained. In addition, we did not observe *para*-hydroxyphenylacetic acid in any of the extracts as well. This intermediate was detected in anaerobic cultures of *Rhodopseudomonas palustris* growing on phenol in the presence of light and a second carbon source, acetate (*27*).

Though evidence for ^{13}C-labeled benzoate is apparent in our cultures (Figure 9), the mass spectrum indicates a shift of seven units in several m/z fragments associated with the derivatized ring carboxyl group (eg. 112, 186, and 201; Figure 9B and 9C). This shift must be due to the incorporation of a ^{13}C-labeled fragment formed during the metabolism of the starting substrate. The possible incorporation of $^{13}CO_2$ onto the benzene ring would seem unlikely since the quantity of this reactant would be miniscule in the ^{12}C-bicarbonate-buffered incubation system. It may be that the mineralized product is not free to exchange with the pool of $^{12}CO_2$ or that an alternate mechanism of carboxylation exists.

However, not all spectral fragments exhibited an increase of seven mass units. For instance, a mass spectral shift from 135 m/z in the benzoate standard corresponded to an increase of only six mass units to 141 in the ^{13}C-metabolite profile. The feature at m/z 135 represents the fragment associated with the well known MacLafferty rearrangement product ($[C_6H_5Si(CH_3)_2]^+$) known to occur with trimethylsilylated benzoic acid derivatives (*28*). This rearrangement fragment does not contain the carboxyl carbon and

thus the corresponding metabolic fragment exhibits a shift of only six mass units. A six mass unit increase was also observed in other spectral features when the standard and ^{13}C-metabolite were compared. The fragment at m/z 77 was increased to 83 in the derivatized product when the carboxyl moiety of benzoate was lost.

The detection of benzoate as a benzene intermediate is consistent with the current understanding of anaerobic phenol metabolism. The carboxylation of phenol to *para*-hydroxybenzoate and the subsequent dehydroxylation of the latter to benzoate is a well established route for anaerobic cultures (as reviewed in *22*, *23*; *29-33*). The anaerobic carboxylation of phenol has been shown most often in complex methanogenic consortia. However, a nitrate-reducing isolate *Thauera aromatica* (formerly *Pseudomonas* sp. K172) is known to catalyze the carboxylation of phenol via a phenylphosphate intermediate (*30, 32*), though phenol phosphorylation is not a requirement for uptake, carboxylation or phenol degradation under other anaerobic conditions (*31*).

There are few studies that show the carboxylation of phenol under sulfate-reducing conditions, but evidence for the carboxylation of a variety of substituted phenolic compounds is known (*34-36*). Two sulfate-reducing isolates, *Desulfobacterium* sp. strain Cat2 and *Desulfococcus* sp. strain Hy5, have been shown to carboxylate catechol to protocatechuate and hydroquinone to gentisate, respectively (*37, 38*). *Desulfobacterium phenolicum* was isolated for its ability to degrade phenol using sulfate as the terminal electron acceptor (*39*). Though the pathway for phenol metabolism by this organism is not known with certainty, the cell could not utilize cyclohexanol, cyclohexanone, adipate or *n*-caproic acid as growth substrates, but 4-hydoxybenzoate and benzoate could be metabolized. Such findings would be consistent with a carboxylation

mechanism for phenol metabolism. Similarly, Schnell and Schink observed that aniline was *para*-carboxylated to a 4-aminobenzoate intermediate by *Desulfobacterium anilini* when grown in the presence of CO_2 and phenol biodegradation by this organism was significantly enhanced in the presence of CO_2 (*40*). Further, evidence for anaerobic carboxylation was observed by Lovley and Lonergan when 4-hydroxybenzoate was detected as a transitory intermediate during the degradation of phenol by the Fe(III)-reducing organism *Geobacter metallireducens* (formerly GS-15) (*41*).

The detection of ^{13}C-labeled phenol and benzoate in enrichments amended with ^{13}C-benzene provides strong evidence for an alternate pathway for the anaerobic decomposition of benzene (Figure 11). While we observed both phenol and benzoate in extracts of the sulfate-reducing enrichment, we cannot rule out the prospect of direct benzene carboxylation (Figure 11, dashed line) as the initial degradative event since our studies were performed using a mixed microbial community. Similarly, we cannot rule out alternative routes of benzene decay. However, we did not detect any of the intermediates expected for the ring reduction pathway, or any ^{13}C-labeled hydroxybenzoic acids in extracts from any of the benzene degrading enrichments. It should be noted that several studies observed that *p*-hydroxybenzoate is not a free intermediate during the anaerobic conversion of phenol to benzoate (*42-45*). Additionally, when ^{14}C-benzene was the starting substrate, we could recover stoichiometric amounts of $^{14}CO_2$ (*10*). More importantly, the findings presented are the first to establish the importance of benzoate during anaerobic benzene metabolism and indicate that concerns over the accumulation of potentially recalcitrant intermediates in anaerobic environments contaminated with benzene seem unwarranted.

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Chapter 4.

Anaerobic Biodegradation of Long Chain *n*-Alkanes under Sulfate-Reducing Conditions

Abstract

The ability of anaerobic microorganisms to degrade a wide variety of crude oil components was investigated using chronically hydrocarbon-contaminated marine sediments as the source of inoculum. When sulfate reduction was the predominant electron accepting process, gas chromatographic analysis revealed almost complete *n*-alkane removal (C_{15} to C_{34}) from a weathered oil within 201 d of incubation. No alteration of the oil was detected in sterile control incubations or when nitrate served as an alternate electron acceptor. The amount of sulfate reduced in the oil-amended nonsterile incubations was more than enough to account for the complete mineralization of the *n*-alkane fraction of the oil; no loss of this anion was observed in sterile control incubations. The mineralization of the alkanes was confirmed using ^{14}C-14,15-octacosane ($C_{28}H_{58}$), with 97% of the added radioactivity recovered as $^{14}CO_2$. These findings extend the range of hydrocarbons known to be amenable to anaerobic biodegradation. Moreover, the rapid and extensive alteration in the *n*-alkanes can no longer be considered a defining characteristic of aerobic oil biodegradation processes alone.

Introduction

Fossil fuels are the foundation of many world economies and reliance on this energy source is unlikely to wane in future decades. Such dependency is associated with a myriad of ecological consequences; most noticeably the contamination of marine environments with crude oil or distillate fractions. Of the many responses to oil pollution, the microbial destruction of hydrocarbons is increasingly relied on to convert the contaminants to innocuous end products. In this regard, the patterns of hydrocarbon decay and overall metabolic versatility of aerobic microorganisms have been well documented (as reviewed in *1-4*). The selective utilization of alkanes over other hydrocarbon fractions is frequently observed. The preferential degradation of some hydrocarbons relative to more recalcitrant molecules represents the basis for the use of biomarkers to assess the degree of aerobic biodegradation activities (*5-7*). However, comparable measures for the anaerobic destruction of hydrocarbons do not exist. In fact, relatively little is known about the prospects for microbial metabolism of petroleum hydrocarbons in the absence of oxygen.

Reports on the latter topic have regularly appeared since the 1940's (as reviewed in *8*), though lack of stringent anaerobic technique, appropriate controls, or non-reproducibility tended to undermine the conclusions of early studies. More recently, numerous studies on the anaerobic metabolism of monoaromatic hydrocarbons linked to the consumption of a variety of potential electron acceptors have appeared (as reviewed in *9,10*). However, the fate of hydrocarbons in marine ecosystems deserves particular attention since estimates suggest 1.7 to 8.8 million tons of petroleum hydrocarbon enter marine environments and estuaries each year (*11*). As these environments experience the

influx of labile organic matter, microbial respiratory activity frequently depletes available oxygen reserves, particularly in sediments. As marine and estuarine waters are rich in sulfate, this mobile anion is rarely limiting and most often available as a potential terminal electron acceptor. It is quite likely, therefore, that the microbial destruction of contaminating hydrocarbons might be coupled to the reduction of sulfate in these anaerobic environments.

In this regard, the ability of sulfate reducing bacteria to degrade a variety of pollutants is beginning to be appreciated (*12*). The degradation of mono- and polyaromatic hydrocarbons under sulfate reducing conditions has recently been demonstrated (*9,10,13-18*). In addition, several pure cultures of hydrocarbonoclastic sulfate reducing bacteria are known to metabolize *n*-alkanes. *Desulfobacterium oleovorans* (strain Hxd3) is a marine isolate that can metabolize C_{12}-C_{20} *n*-alkanes (*19*). Strain TD3 can grow with individual *n*-alkanes from C_6-C_{16} coupled to sulfate reduction but could only selectively remove certain *n*-alkanes when challenged to grow on crude oil (*14*). A third marine isolate can couple C_{14}-C_{17} alkane metabolism to the reduction of sulfate and hydrogen sulfide production (*20*). The anaerobic microbial destruction of *n*-alkanes (from C_{15} to C_{23}) was also noted in contaminated marine sediments amended with either diesel or jet fuel (*17*). Production of $^{14}CO_2$ from 1-^{14}C-hexadecane was inhibited by molybdate, thus implicating sulfate reduction as the predominant electron accepting process.

We wanted to further explore the limits of anaerobic hydrocarbon metabolism and help lay the foundation for future biomarker studies to more easily evaluate the ecological significance of such transformations. We used a marine sediment known to harbor a

metabolically diverse microflora capable of hydrocarbon metabolism as an inoculum (*15,17,21*) and a highly characterized but weathered crude oil (*7,22*) as a carbon source. The latter more closely resembles hydrocarbons that typically get deposited on beaches and in sediments following the abiotic removal of the more volatile and water soluble components (*23*). Thus, the oil was essentially devoid of lower molecular weight *n*-alkanes and monoaromatic hydrocarbons. Herein, we report the biodegradation of C_{15} to C_{34} *n*-alkanes under sulfate reducing conditions; thus extending the upper range associated with the anaerobic biodegradation of these hydrocarbons. In addition, we note that the extent of this metabolism is comparable to what has previously only been observed in studies of aerobic hydrocarbon decay.

Materials and Methods

Marine sediment from San Diego Bay, CA was slurried (1:1 g/vol) with an artificial sea water as previously described (*21*), with the following modifications: 0.001% resazurin, 1 mM sodium sulfide, and 3 mM bicarbonate. The latter were added to the slurries from sterile anoxic stock solutions. Approximately 60 ml of slurry were placed in sterile 120 ml serum bottles and sealed with a composite rubber stopper as previously described (*24*). Slurry construction and amendments were done in an anaerobic glove bag containing a headspace of 90% N_2:10% H_2. However, the slurries were placed under a 80% N_2: 20% CO_2 headspace immediately after they were removed from the glove bag. Sodium sulfate or nitrate was added to the slurries from sterile anoxic stock solutions to an initial concentration of 20 mM. Autoclaved slurries (121°C; 30 min; three consecutive days) served as sterile controls and benzoate (1mM) was used as a positive control. Experiments were stored at room temperature in the dark and conducted in triplicate.

The oil was a previously characterized Alaskan North Slope crude oil that had been distilled under reduced pressure until it had lost 30% of its weight (*7,22*). This is the maximum weight loss seen with extensive abiotic weathering (*25*). The oil was added by syringe (600 µl) to the serum bottles under anaerobic conditions using the Hungate technique (*26*) to maintain anaerobic conditions.

Sulfate and nitrate were periodically monitored by ion chromatography using a Dionex DX500 system, an AS4A column and an ion suppressed CD20 conductivity detector with an eluent of 1.7 mM sodium bicarbonate - 1.8 mM sodium carbonate at a flow rate of 2.0 ml per minute (Dionex Corp., Sunnyvale, California). Analysis of the oil

by gas chromatography/mass spectroscopy (GC/MS) followed published procedures (*27*). Separation was performed on a Hewlett Packard HP 5890 gas chromatograph fitted with a 30 m x 0.25 mm fused silica capillary column with 5% crosslinked phenyl methyl silicone as the stationary phase. Helium was used as the carrier gas at a flow rate of 1 ml/min. Samples of 1 µl were injected automatically by a HP 6890 Injector. The column temperature was set to 45 °C for the first 4 min, increased 8 °C/min to a temperature of 270 °C then increased 5 °C/min to 310 °C and maintained at 310 °C for 5 min. Mass spectral data were obtained with a Hewlett Packard 5972 mass selective detector at an electron energy of 70 eV over a mass range of 35-500 atomic mass units in the total ion mode. Spectral tuning with Perfluorotributylamine followed USEPA method 8270C.

To confirm anaerobic alkane metabolism and to probe the fate of the substrates, a radiolabeled C_{28} *n*-alkane (^{14}C-14,15-octacosane: ≥98 %;specific activity 21.8 mCi/mmol; Sigma Chemical Co, St. Louis, MO) was added to sterile and nonsterile sediment slurries using the artificially weathered crude oil as a carrier (0.1 ml). Slurries were incubated for an additional 90 days at room temperature in the dark, and analyzed for the production of radiolabeled carbon dioxide and methane, as previously described (*28*). Residual oil was extracted from the incubations with methylene chloride and the resulting aqueous and solvent fractions assayed by liquid scintillation counting (*24*).

Results and Discussion

Sulfate reduction in the sediment slurries was monitored in both the presence and absence of the oil (Fig. 1). Sterile controls showed no significant loss of sulfate over the course of the incubation, while little sulfate reduction (~3 mM, 0.18 mmoles) was noted in oil-unamended controls. Nonsterile incubations amended with oil, or oil plus benzoate, exhausted the available sulfate reserves (~25 mM, 1.5 mmoles) within 125 days. The addition of benzoate as a positive control did not significantly influence the rate of sulfate reduction in the slurries (Figure 12) and HPLC analysis confirmed that this compound was depleted within 30 d (data not shown). A subsequent amendment of sulfate on day 125 (20 or 10 mM for slurries receiving oil or oil plus benzoate, respectively) was also reduced, but the rate of sulfate removal decreased after 160 days (Figure 12).

For slurries amended with nitrate, 90% of the electron acceptor was depleted within the first 60 days in all nonsterile incubations, regardless of whether they were also amended with oil or with the positive control benzoate (data not shown). Nitrate depletion could not be correlated with either benzoate or oil removal. No loss of nitrate was noted in the sterile incubations. However, the loss of nitrate could be correlated with an increase in sulfate. That is, sulfate accumulated from ~6 mM to 11-15 mM in nonsterile incubations, whereas no such increase was detected in sterile controls. The microbial oxidation of reduced sulfur species coupled with the reduction of nitrate is known (*29*), and the incubations were visibly less colored, presumably due to this process.

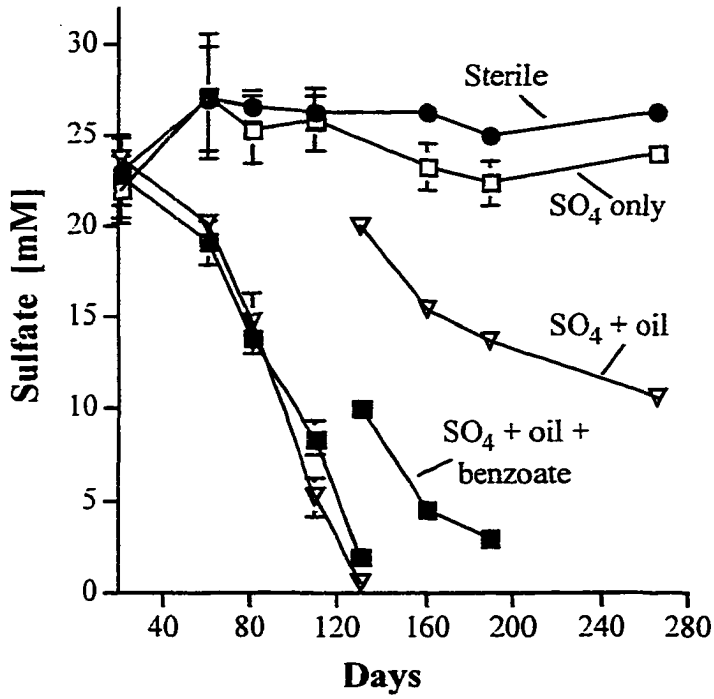

FIGURE 12. Sulfate loss in marine sediment incubations. On day 125, reamendments of 20 mM and 10 mM sulfate was made to incubations containing oil alone (▽) or oil plus the positive control substrate benzoate(■), respectively. Standard deviations are indicated.

The physical appearance of the sulfate and oil-amended slurries changed as the incubation proceeded. Even though the oil was applied as an immiscible layer, small pockets of sediment blackening were easily observed in nonsterile incubations. Apparently, the sediment blackening was due to the production of iron sulfide minerals. However, it is unclear whether the spatially discreet sulfate reduction zones were due to the patchy distribution of the requisite bacteria or if droplets of oil were trapped within the sediment matrix and stimulated sulfate reduction activity where deposited. During incubation it became increasingly more difficult to discern the oil immiscible layer, due to both the apparent break up of the oil layer and the overall blackening of sediment slurries. Comparable physical changes were not evident in either the sterile or oil-unamended controls.

After 201 d of incubation, slurries were extracted and analyzed by gas chromatography to characterize residual oil constituents. No qualitative changes in oil composition could be detected in sterile control incubations (Figure 13A) relative to the starting oil (not shown). Additionally, no changes in oil composition were observed in nonsterile nitrate-amended incubations over this same time period (data not shown). In contrast, the *n*-alkanes were almost completely removed in nonsterile oil-amended incubations (with or without benzoate) when sulfate served as the terminal electron acceptor (Figure 13B). The apparent loss of these hydrocarbons coupled with the depletion of sulfate is consistent with the anaerobic biodegradation of these constituents.

To test this hypothesis, radiolabeled octacosane (^{14}C-14,15-$C_{28}H_{58}$) was added to the slurries and assays for radiolabeled hydrocarbon and mineralized end products (CO_2 and CH_4) were conducted after an additional 90 day incubation period. No

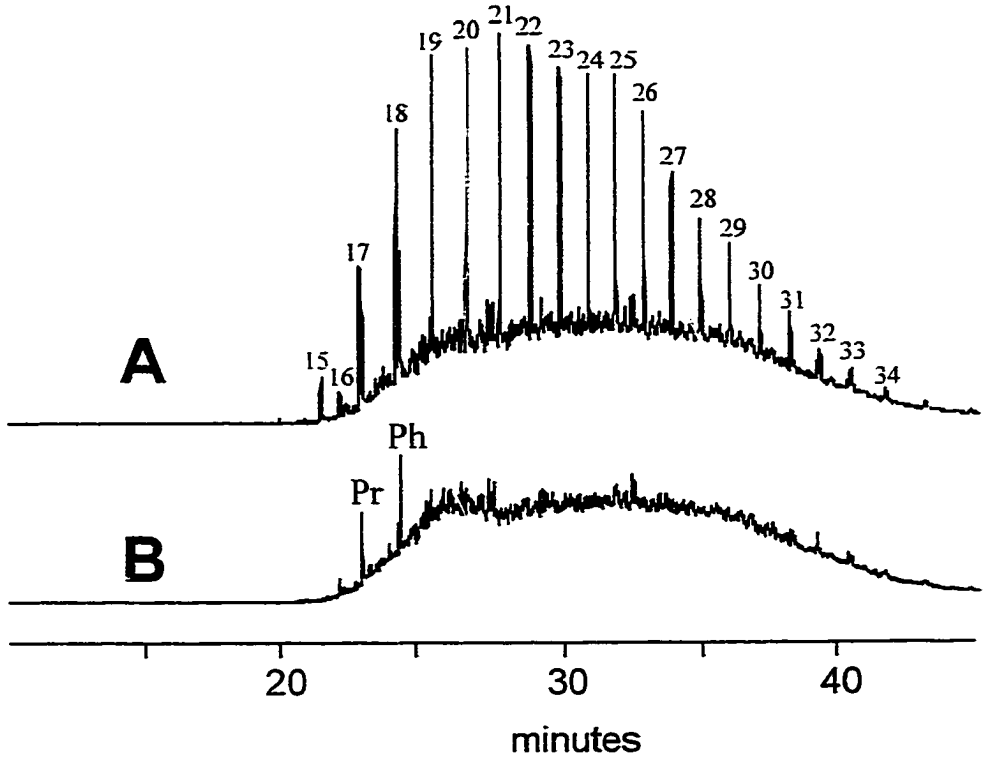

FIGURE 13. Gas chromatographic profiles of the saturated hydrocarbon fraction of weathered Alaska North Slope crude oil extracted from sterile (A) and nonsterile (B) marine sediments following a 201 d incubation. Numbers on the peaks represent chain lengths of *n*-alkanes; Pr, pristane; Ph, phytane.

transformation of octacosane to radiolabeled CO_2 or CH_4 could be detected in the sterile controls (Table 7). In fact, 95% of the applied radiolabel was recovered in the solvent used to extract residual hydrocarbon components. In contrast, approximately 97% of the applied radioactivity could be recovered as $^{14}CO_2$ in nonsterile incubations with only traces in the solvent extract (Table 7). No recovery of $^{14}CH_4$ was obtained from any incubation.

The gas chromatographic profiles (Figure 13) clearly indicate that a diverse range of *n*-alkanes, up to 34 carbons in chain length, could be biologically removed from a weathered crude oil upon incubation with sediment associated marine anaerobes under sulfate reducing conditions. These transformations did not occur when nitrate was provided as a potential terminal electron acceptor. Although nitrate has shown promise as a potential tool in the anaerobic bioremediation of monoaromatic hydrocarbons (*9, 10, 30, 31*), the effectiveness of nitrate is almost exclusively observed in freshwater or aquifer material. Additionally, the lack of monoaromatics in our weathered oil would preclude their biodegradation in our incubations. Zeyer and colleagues (*32, 33*) have indicated that nitrate reducing microorganisms associated with sediment from a freshwater aquifer were able to effectively degrade 50% of a weathered diesel oil and the isoprenoid molecule, pristane. Though these reports open the possibility for nitrate coupled biodegradation of heavier oil components, no loss of pristane or other compounds were indicated in extracts of our live incubations. This is not entirely surprising since marine sediments are considered low nitrate environments.

The amount of sulfate reduced in our incubations helps suggest the ultimate fate of these hydrocarbons. At the time the residual oil was extracted, approximately 30 mM

TABLE 7. Recovery of $^{14}C^{a}$ Labeled Components Incubated with ^{14}C-14,15-octacosane from San Diego Bay sediments after 90 days

Incubation	Starting amendment	$^{14}CO_2$ recovered	Solvent recovery	Aqueous recovery	Total recovery
Sterile	5.5×10^6	BDL[b]	5.2×10^6	1.6×10^4	5.2×10^6 (95%)
Live	5.5×10^6	5.6×10^6	2.4×10^5	9.0×10^3	5.8×10^6 (105%)

[a] all values reported in dpm. [b] BDL, below detection limit.

sulfate (1.8 mmoles) was removed in nonsterile incubations relative to the oil-unamended controls (Fig. 1). The total amount of *n*-alkanes in this crude oil is known (*22*), as was the quantity of oil added to the incubation mixtures. Based on the theoretically expected stoichiometry (comparable to the estimates made in *19*), we calculate that only 24 mM sulfate (1.44 mmoles) would be required for the complete conversion of all the *n*-alkanes to carbon dioxide. Thus, more than enough sulfate was reduced to account for the overall mineralization of the alkanes. The amount of electron acceptor depleted in excess of that theoretically expected probably indicates that other hydrocarbon components in the oil can also be anaerobically metabolized.

Our suggestion of alkane mineralization linked to sulfate reduction is consistent with the findings of Widdel and colleagues (*14,19*). They measured close to theoretically expected amounts of sulfide produced in cultures of *Desulfobacterium oleovorans* (strain Hxd3) metabolizing hexadecane and strain TD3 growing on decane. Similarly, So and Young (*20*) also obtained a marine bacterium capable of coupling the degradation of hexadecane to sulfate reduction. Such comparisons of the observed versus the expected amounts of sulfate required for alkane decay provide strong suggestive evidence for the mineralization of the oil associated *n*-alkanes. However, this contention was confirmed by our recovery of the vast majority (97%) of the radioactivity originally associated with ^{14}C-octacosane as $^{14}CO_2$ in nonsterile incubations, whereas no significant amount of label was found in the carbon dioxide traps for sterile controls (Table 7).

Sulfate containing samples incubated for 201 days exhibited a 40% loss of the isoprenoid molecules pristane and phytane relative to sterile controls. The addition of benzoate reduced pristane and phytane degradation by 50%. No significant degradation of

polycyclic aromatic hydrocarbons was noted at 201 days. Samples incubated for 365 days exhibited a 70% loss of pristane and phytane in incubations without benzoate, approximately a 50% loss in its presence. In addition dimethylnaphthalene and phenanthrene were degraded 25% in the 365 day samples suggesting that while the preference of the inoculum is for straight chain saturates, the anaerobic degradation of aromatic compounds will proceed in the absence of preferred substrates. While degradation of more complex aromatic compounds (dibenzothiophene and chrysene, for example) was not observed at 365 days it is possible that these substrates would be utilized given additional time and sufficient electron acceptor. In this regard it should be noted that Coates and Lovely found that polynuclear hydrocarbons were amenable to anaerobic biodegradation using the same inoculum (*15,17*). However, when exposed to a complex crude oil, the degradation of polynuclear hydrocarbons was clearly less important than the degradation of alkanes in terms of the overall mass loss of the hydrocarbons.

Several investigators have relied on the production of $^{14}CO_2$ from radiolabeled substrates to assess mineralization of *n*-alkanes under anaerobic conditions. Davis and Yarbrough (*34*) observed small amounts of $^{14}CO_2$ from radiolabeled octadecane after 22 days of incubation with *Desulfovibrio desulfuricans*. However, biodegradation was not coupled to sulfate reduction and the amounts of radioactivity recovered were small enough to raise questions about the radio-purity of the starting substrate. Similarly, Ward and Brock (*35*) observed 5.6% recovery of $^{14}CO_2$ from 1-^{14}C-hexadecane incubated with anoxic sediments from Lake Mendota, WI. Formaldehyde inhibited controls exhibited a similar recovery of radioactivity and the process was not stimulated by the addition of

nitrate or sulfate. In a more recent study, Coates et al. (*17*) observed substantial recovery (>70%) of $^{14}CO_2$ from radiolabeled hexadecane in marine sediments from San Diego Bay, CA. Inhibition of this metabolism by molybdate (20mM) implicated sulfate reduction as the major terminal electron acceptor process in these incubations.

The use of ^{14}C-14,15-octacosane allowed us to address two important points. First we could show that even a higher molecular weight alkane was amenable to anaerobic attack. Prior to our study, the highest molecular weight alkane known to biodegrade under anaerobic conditions was C_{23} (*17*). Secondly, with the radiocarbon on the interior of the molecule and its eventual recovery as $^{14}CO_2$, we confirmed that the alkane must have been substantially mineralized as opposed to its incorporation into cell biomass.

The total recovery of radiolabel in the incubations was generally very high (Table 7) with disparities explained by the difficulties in accurately dispensing 100 µl of the weathered Alaska North Slope crude oil used a carrier. For instance, in one replicate we recovered 99% of the label as $^{14}CO_2$, but showed a total recovery of 165% of the expected amount of applied radiolabel (data no shown). However, even with this replicate, almost all radioactivity was recovered as carbon dioxide and confirmed that the octacosane was mineralized under sulfate reducing conditions.

The importance of these findings is worth noting. When assessing the biodegradation of petroleum hydrocarbons in the environment, it is generally presumed that the aerobic microbial metabolism of these substrates is the predominant removal mechanism (*1-4*). In fact, it is commonly observed that alkanes are preferentially utilized by aerobic microorganisms relative to other components when oily mixtures of hydrocarbons are available to the resident microflora (*7,22,36-38*), although exceptions

have been seen (*39,40*). The former is the case in experiments using the same weathered oil where aerobic alkane biodegradation is essentially complete within 14 days of incubation (*7*). Our observations call such generalizations into question. That is, there apparently are environmental compartments where the anaerobic microbial metabolism of hydrocarbons may represent an equally important fate process for these materials. Moreover, if the rather dramatic preferential utilization of the alkanes observed in our experiments proves general, conclusions on the importance of aerobic biodegradation based on the patterns of hydrocarbon decay may at least be called into question.

As noted above, the utilization of *n*-alkanes under sulfate reducing conditions has been observed before, but the diversity and extent of this metabolism documented herein has not been previously recognized. It thus appears that the capacity of anaerobes to metabolize hydrocarbons must be reevaluated when considering the transport and fate of oily materials in the environment. Moreover, a reexamination of the role of these organisms in such diverse areas as the souring of oil reservoirs (*41,42*), the fouling of petroliferous formations (*43,44*), the corrosion of oil field equipment (*45,46*), and their potential to remediate oily waste components would seem appropriate.

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