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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE ANAEROBIC BIODEGRADATION OF PETROLEUM-RELATED COMPOUNDS

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

By

KATHLEEN LOUISE LONDRY

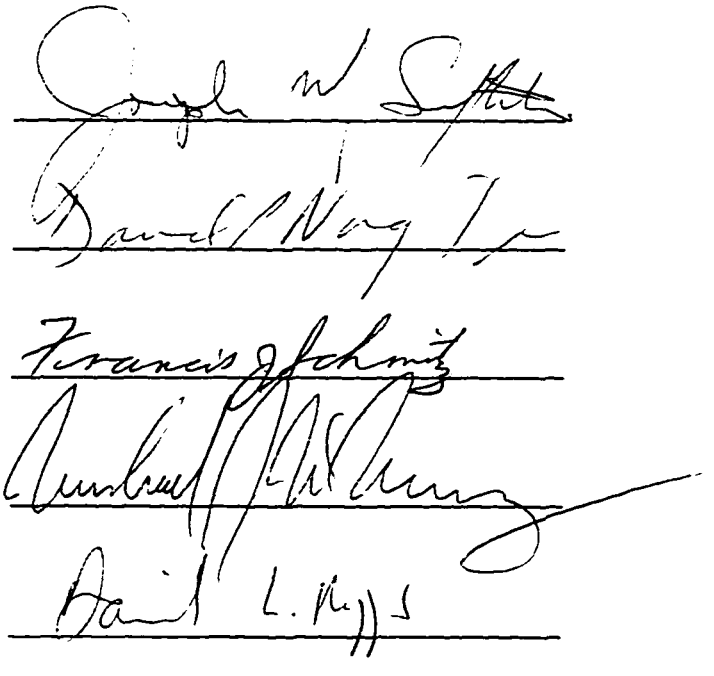
Norman, Oklahoma

1997

THE ANAEROBIC BIODEGRADATION OF PETROLEUM-RELATED COMPOUNDS

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



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This achievement would not have been possible without the love and support of my family. I dedicate this dissertation to my husband, Steve Londry, who has helped me in so many ways that this is as much his accomplishment as mine. I thank my parents, Douglas and Evelyn Wiggins, and my twin Bruce Wiggins for their encouragement, and for making me believe I could achieve anything if I was determined enough. The reassurance and understanding of my entire family has been a source of strength and balance in my life throughout this difficult time of being a graduate student.

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PREFACE

Fossil fuels such as petroleum are used extensively to meet the energy needs of our industrial society, but the environmental impact of their accidental or intentional release is not fully understood. In order to predict the transport and fate of petroleum and oily wastes released into the environment, it is necessary to understand the fate of these compounds under anaerobic conditions, since marine sediments and the terrestrial subsurface are often anoxic, or quickly becomes so following introduction of anthropogenic carbon sources. As an approach to investigating the microbial biodegradation of these complex and highly variable mixtures, I have chosen to systematically examine the metabolic fate of several different classes of oily waste components. By comparing different model compounds within each class, the effects of chemical structure on biodegradation were evaluated in relation to the predominate processes governing the flow of carbon and energy in anaerobic environments.

This dissertation consists of seven chapters dealing with various aspects of the anaerobic biodegradation of compounds associated with oily wastes. The first two chapters contain results from studies of the degradation of cresols, and *m*-cresol in particular, under sulfate-reducing conditions. Cresols are phenolic compounds associated with wastewaters of the petroleum industry. Chapter 1 describes a proposed metabolic pathway for *m*-cresol degradation by the sulfate-reducing bacterium *Desulfotomaculum* strain Groll based on studies of substrate degradation and metabolite formation. Chapter 2 represents a continuation of this work, in which a metabolic pathway is proposed for *p*-cresol degradation, and the metabolism of the two cresol isomers is compared through metabolic and enzymatic studies. Chapters 1 and 2 are written in formats appropriate for the journals Applied and Environmental Microbiology and Archives of Microbiology, respectively.

Chapter 3 describes research addressing the fate of *n*-alkanes and partially oxidized products of the aerobic metabolism of these hydrocarbons under anaerobic conditions. *n*-Alkanes are dominant hydrocarbons in petroleum and could represent an abundant source of carbon and energy for organisms able to metabolize them. Comparison of the *n*-alkanes with the partially oxidized *n*-alkanols and *n*-alkanoic acids demonstrated that the introduction of the terminal hydroxyl or carboxyl group renders these compounds more susceptible to anaerobic degradation. Chapter 3 is written in a format suitable for the Canadian Journal of Microbiology.

The degradation of components in petroleum-derived oily wastes can result in the production of large amounts of sulfide, a noxious and corrosive gas. Under the hypothesis that organic constituents from the oily wastes could serve as substrates for sulfate-reducing bacteria that produce sulfide, I have investigated the feasibility of using an ecological approach to control sulfide production associated with oil-water separators and settling tanks on board ships and at treatment facilities. Chapter 4 describes the use of nitrate as both an alternative electron acceptor to sulfate and an electron acceptor for the oxidation of sulfide, for the prevention of sulfide generation in cultures established with oily sludge. This chapter is formatted according to the style of the Journal for Industrial Microbiology and Biotechnology.

To further evaluate the role of sulfur in the environmental impact of petroleum, I have studied the toxicity and degradation of four classes of organo-sulfur compounds under anaerobic conditions. Chapter 5 reports the toxicity effects of thiols, thiophenes, thiophenic acids, and aromatic sulfides on anaerobic microbial activity, and is formatted in accordance with the journal Environmental Toxicology and Chemistry. Chapter 6 describes the degradation of organothiols under nitrate-reducing conditions and is formatted for the journal Biodegradation.

The appendix describes research conducted on the biochemistry of the reduction of nitroaromatic compounds by the sulfate-reducing organism *Desulfomonile tiedjei*.

Nitroaromatic compounds such as di- and trinitrotoluene (TNT) are environmentally significant contaminants associated with the manufacture, distribution, and use of ammunitions. This organism catalyzed the reduction of the nitro groups of a wide variety of nitroaromatic compounds, thereby transforming them to the corresponding amines. This research has been placed in the appendix because it is not directly related to petroleum and the work described in the other chapters.

ABSTRACT

A variety of compounds associated with petroleum and petroleum-derived wastes were examined for their susceptibility to biodegradation under methanogenic, sulfate-reducing, and nitrate-reducing conditions. Inocula from chronically contaminated environments were used to explore the limits of anaerobic degradation potential. In addition, an ecological approach to the prevention of sulfide emissions was evaluated. Nitrate was added to oily sludge incubations to test its efficacy in preventing sulfidogenesis by sulfate-reducing bacteria. Nitrate inhibited sulfate reduction in a concentration-dependent manner, and reduced sulfide concentrations under a variety of salinity conditions.

For biodegradation studies, enrichment cultures were established with target compounds added singly or as mixtures, often in a hydrophobic carrier. The degradation of compounds such as *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids depended on chemical structure as well as redox potential. *n*-Alkanoic acids were degraded faster than *n*-alkanols, whereas *n*-alkanes were not metabolized. The susceptibility of *n*-alkanols and *n*-alkanoic acids to degradation was chain-length dependent. Alkanols were transformed to the corresponding alkanoic acids, and the formation of esters as a minor fate process was also noted.

The toxicity and biodegradation of mixtures of thiols, thiophenes, thiophenic acids and aromatic sulfides were determined under anaerobic conditions. Toxicity effects were dependent on the structure of the organosulfur compounds, the amounts added to cultures, and the available electron acceptor. The anaerobic biodegradation of eight different organothiols was observed under nitrate-reducing conditions but not methanogenic or sulfate-reducing conditions. Degradation of hexanethiol was linked to nitrate reduction and nitrite production.

The anaerobic degradation of a variety of aromatic compounds was investigated with the sulfate-reducing bacterium *Desulfotomaculum* strain Groll. Initial steps of metabolic pathways for the degradation of *m*- and *p*-cresol were proposed. Degradation of both isomers proceeded by hydroxylation of the methyl groups to yield hydroxybenzyl

alcohols which were further oxidized to hydroxybenzoic acids then metabolized as benzoyl-coenzyme A thioesters. These proposed pathways were supported by substrate degradation profiles, detection of metabolites, and biochemical studies. Although strain Groll degraded these cresol isomers by similar reactions, the enzymes catalyzing the oxidation of the *meta* and *para* isomers were biochemically distinct.

Chapter 1.

ANAEROBIC DEGRADATION OF *m*-CRESOL BY A SULFATE-REDUCING BACTERIUM

Abstract

m-Cresol metabolism under sulfate-reducing conditions was studied with a pure culture of *Desulfotomaculum* strain Groll. This organism metabolizes *m*-cresol by methyl group oxidation to 3-hydroxybenzoic acid, which is subsequently converted to benzoic acid. The proposed *m*-cresol metabolite 4-hydroxy-2-methylbenzoic acid was not degraded by this organism, and no evidence was found for a specific ring-carboxylation of the parent substrate. Strain Groll readily degraded the predicted metabolites of a methyl group oxidation pathway, including: 3-hydroxybenzyl alcohol, 3-hydroxybenzaldehyde, 3-hydroxybenzoic acid, and benzoic acid. Degradation of these compounds preceded and inhibited *m*-cresol decay. 3-Hydroxybenzoic acid was detected in cultures that received either *m*-cresol or 3-hydroxybenzyl alcohol, and trace amounts of benzoic acid were detected in *m*-cresol-degrading cultures. Degradation of *m*-cresol by a methyl group oxidation pathway represents an alternative pathway for the metabolism of this compound under sulfate-reducing conditions.

Introduction

Cresols are phenolic contaminants of fuel processing wastewaters that are of environmental concern because of their toxicity and their mobility in subsurface environments. The anaerobic biodegradation of all three cresol isomers has been demonstrated in field and laboratory studies (6). A variety of mechanisms have been reported for the metabolism of cresols under anaerobic conditions. *p*-Cresol is degraded by anaerobic oxidation of the methyl group in methanogenic consortia (17), sulfate-reducing consortia (20), by nitrate-reducing isolates (4, 16), and by an iron-reducing isolate (10). *o*-Cresol is apparently degraded by either *para*-carboxylation (2) or methyl group oxidation (11) under methanogenic conditions, methyl group oxidation under sulfate-reducing conditions (20), and *para*-carboxylation followed by dehydroxylation under nitrate-reducing conditions (16).

The metabolic pathway for *m*-cresol degradation under anaerobic conditions depends on the available electron acceptor. Under nitrate-reducing conditions, studies with the isolate S100 have indicated that *m*-cresol is likely degraded by a methyl group oxidation mechanism (3, 16). In methanogenic consortia, the methyl group is not oxidized (14), *m*-cresol is transformed by an initial *para*-carboxylation (15), followed by an apparent demethylation and dehydroxylation to yield benzoic acid (8). Previous studies have indicated that in sulfate-reducing consortia *m*-cresol is likewise degraded by an initial *para*-carboxylation mechanism (13).

We investigated the metabolic pathway for *m*-cresol degradation using a sulfate-reducing organism previously isolated for its ability to degrade other aromatic compounds. *Desulfotomaculum* strain Groll is a Gram positive, spore-forming, mesophilic, strictly anaerobic rod-shaped bacterium. It is versatile, growing under freshwater or saltwater conditions, and can use a variety of electron acceptors including sulfate, but not nitrate (5). This organism was reported to degrade a wide variety of aromatic compounds including *m*-

cresol. We confirmed that this is the case, and we have elucidated the initial steps in the metabolism of *m*-cresol by this organism. Our findings indicate a second pathway for *m*-cresol degradation under sulfate-reducing conditions that involves methyl group oxidation reactions.

MATERIALS AND METHODS

Growth of strain Groll. *Desulfotomaculum* strain Groll was obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG (#7213). The organism was cultivated in anaerobic medium as previously described (5) but with only 1 mM sulfide as reductant. The medium was dispensed in an anaerobic chamber to incubation vessels which were sealed with butyl rubber stoppers and aluminum crimp seals. The headspace of the vessels was then exchanged to N_2:CO_2 (80:20) and the media amended with substrates from neutralized, sterile, anoxic stock solutions. Strain Groll was incubated at 32°C in the dark without shaking. Cultures were maintained by periodic additions of substrate to 250 μM from stock solutions and by repeated transfers. Samples of culture fluid were withdrawn using strict anaerobic, aseptic technique and stored frozen until analyzed. Prior to high-performance liquid chromatography (HPLC) analysis, samples were thawed and centrifuged at 10 000 × g for 5 min.

Metabolite degradation studies. To test the degradation of putative metabolites, medium was prepared as described above and inoculated (10% vol/vol) with a culture of strain Groll which was substrate depleted. All test compounds were added to an initial concentration of 500 μM unless otherwise indicated, and sterile controls were autoclaved. Degradation was monitored by the depletion of test compounds over time. Sulfate was added in non-limiting concentrations (5-20 mM) and reduction of sulfate was confirmed at the end of the incubations. For dual-substrate studies, *m*-cresol and a test compound were added at equimolar concentrations and the degradation of both was monitored.

To test the effects of carbon dioxide availability on degradation of compounds, medium was prepared as above but with addition of 50 mM TES buffer, (pH 7.3). This medium was prepared two ways, by adding bicarbonate and providing a N_2:CO_2 headspace as above (+CO_2), or else bicarbonate was excluded and a 100% N_2 headspace was provided (-CO_2). Cultures were inoculated from cell suspensions washed three times with carbonate-free medium.

Analytical techniques. Concentrations of aromatic compounds were determined by HPLC (Beckman Instruments, Inc., Berkeley, Cal.) with an Econosphere reverse phase C_{18} column (250 mm by 4.6 mm, 5 μm; Alltech Associates, Inc., Deerfield, Ill.). The isocratic mobile phase consisted of a mixture of 50 mM sodium acetate buffer (pH 4.5) and 20% to 40% acetonitrile at 1 ml/min. A variable-wavelength UV absorbance detector (model 165, Beckman Instruments) was used to detect substrates and metabolites at 254 nm or 280 nm. Compounds were identified by comparison of retention times to authentic standards, and were quantified by comparison to standard curves prepared the same day.

Butyrate was analyzed using an Aminex HPX-57H organic acid column (Bio-Rad Laboratories, Richmond, Cal.) with a 0.016N H_2SO_4 mobile phase at 0.9 ml/min and UV detection (214 nm), with a Beckman HPLC system. This system was also used to detect and identify metabolites in culture fluids. Sulfate concentrations were determined using a Dionex IC system with an AS4A-SC 4 mm column, a CD20 conductivity detector, and a mobile phase of 1.8 mM sodium carbonate + 1.7 mM sodium bicarbonate at 2 ml/min (Dionex, Sunnyvale, Cal.). Toluene and *m*-xylene were determined by gas chromatography using a Hewlett Packard (Palo Alto, Cal.) 5890 series II GC with a flame ionization detector and a 30 m Carbograph VOC capillary column (Alltech Associates), isothermally at 70°C.

To identify metabolites, culture fluids were first alkali-treated (pH 12 for 20 min) to cleave thioester bonds, acidified to <pH 2 with 10 M H_2SO_4, and extracted three times with

diethyl ether. The ether extracts were filtered through anhydrous sodium sulfate then pooled and concentrated. Trimethylsilyl (TMS) derivatives of compounds in culture extracts were prepared with N,O-bis(trimethylsilyl)acetamide in acetonitrile by using the manufacturer's instruction (method 5; Pierce Chemicals, Rockford, Ill.). Aqueous solutions of authentic standards were extracted and derivatized the same way. The ether extracts and the derivatized extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) with a 5890 series II GC with a 5970 MS detector (Hewlett Packard) and a 30 m DB-5 fused silica capillary column (J&W Scientific, Folsom, Cal.). The oven temperature was initially held at 50°C for 1 min then raised at 6°/min to 170°C.

Chemicals. 4-Hydroxy-2-methylbenzoic acid and 6-fluoro-3-methylphenol were synthesized as described in Londry and Fedorak (8). Sodium benzoate, *m*-cresol, *p*-cresol were obtained from Sigma Chemical Co. (St. Louis, Mo); all other compounds were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). The chemicals obtained were of the highest purity available (>96%) and were used without further purification.

RESULTS

Degradation of *m*-cresol linked to sulfate reduction. Strain Groll was initially propagated on benzoate which was previously reported as a preferred substrate (5). After an initial lag period of 8 weeks, strain Groll was also able to metabolize *m*-cresol when transferred to fresh medium with this substrate (250 μM). The organism subsequently mineralized repeated additions of *m*-cresol (250 - 500 μM), resulting in increased culture turbidity and sulfate reduction. The stoichiometry of sulfate depletion coupled with *m*- or *p*-cresol metabolism was 110% of the theoretically expected amount based on equation 1 which is not corrected for incorporation into biomass:

$$C_7H_8O + 4.25SO_4^{2-} \rightarrow 7CO_2 + 4.25S^{2-} + 4H_2O \quad (1)$$

Strain Groll could metabolize *m*-cresol up to a concentration of 2 mM. There was always a lag period prior to *m*-cresol degradation regardless of whether the substrate was added to an pre-grown culture, or if the organism was inoculated into fresh medium. When sodium dithionite was used to reduce the medium, the lag time for degradation of many substrates was shortened, but dithionite was not required for metabolism. Strain Groll could metabolize aromatic substrates in media with sulfide concentrations of up to 15 mM. Thus, strain Groll has a relatively high tolerance for toxic substrates as well as products.

Tests for *m*-cresol degradation by a *para*-carboxylation pathway. Previous studies with a sulfate-reducing consortium (13) indicated that *m*-cresol was initially *para*-carboxylated to 4-hydroxy-2-methylbenzoic acid prior to mineralization of the substrate, with formation of trace amounts of the apparent dead-end metabolite 2-methylbenzoic acid. Using the same consortium, we also found trace amounts of 4-hydroxybenzoic and benzoic acids in *m*-cresol-degrading cultures by HPLC and GC-MS techniques (9). Therefore, we tested 4-hydroxy-2-methylbenzoate, 4-hydroxybenzoate, benzoate, and 2-methylbenzoate as substrates for the sulfate-reducing isolate. Strain Groll did not metabolize 4-hydroxy-2-methylbenzoate or 2-methylbenzoate, even in dual substrate experiments in which *m*-cresol was degraded (Table 1). 4-Hydroxybenzoate and benzoate were readily degraded, but since these are central metabolites for the degradation of many aromatic compounds, their depletion may not be related to *m*-cresol metabolism specifically. In dual substrate studies, 4-hydroxybenzoate had no effect on *m*-cresol degradation by the isolate whereas benzoate inhibited *m*-cresol decay (Table 1). This suggested that only the latter compound was involved in the parent substrate metabolism, and was the first indication that *m*-cresol was not transformed by *para*-carboxylation in strain Groll.

TABLE 1: Aromatic compounds (500 μM) tested as potential substrates for strain Groll. The lag period indicates the time prior to degradation of >10% available substrate; the rate refers to substrate depletion following the lag period. Effects on *m*-cresol degradation were determined by monitoring degradation of both compounds in dual substrate experiments.

compound added	degraded	lag period	rate (μM/day)	effects on *m*-cresol
hydrocarbons				
toluene	no	-	-	none
m-xylene	no	-	-	n.d.[1]
cresols				
o-cresol	no	-	-	none
m-cresol	yes	3 wk	28	
p-cresol	yes	2 wk	≥30	none
phenolic compounds				
phenol	yes	6 wk	32	n.d.
methyl hydroquinone	no[2]	-	-	none
4-methylcatechol	yes[3]	4 wk	16	none
hydroxylated benzoates				
3-methylsalicylate	no	-	-	none
4-methylsalicylate	no	-	-	n.d.
5-methylsalicylate	no	-	-	none
4-hydroxy-2-methylbenzoate	no	-	-	none
4-hydroxyphthalate	no	-	-	none
4-hydroxybenzoate	yes	2 wk	≥25	none
methyl group oxidation				
3-hydroxybenzyl alcohol	yes	7 d	48	inhibited
3-hydroxybenzaldehyde	yes	1 d	≥36	inhibited
3-hydroxybenzoate	yes	11 d	32	inhibited
benzoates				
benzoate	yes	7 d	27	inhibited
2-methylbenzoate	no	-	-	none
3-methylbenzoate	yes	3 wk	11	none
4-methylbenzoate	no	-	-	none
2-fluorobenzoate	yes	1 wk	10	inhibited
3-fluorobenzoate	no	-	-	inhibited
4-fluorobenzoate	yes	1 wk	7	inhibited

[1] not determined

[2] abiotic transformation but products not further degraded

[3] abiotic transformation followed by degradation of products

Previous studies with this organism indicated a potential for carboxylation of aromatic compounds, as catechol was apparently degraded by an initial *para*-carboxylation, forming protocatechuate (5). We tested the dependence of *m*-cresol degradation on carbon dioxide and found that *m*-cresol was not degraded in the absence of CO_2 (not shown). However, CO_2 was also required for the degradation of benzoate, 3-hydroxybenzoate, and 4-hydroxybenzoate, indicating a more general requirement for CO_2 for the degradation of carboxylated aromatic compounds. However, degradation of a non-aromatic carboxylated substrate, butyrate, was independent of CO_2 availability.

Substrate utilization studies. Since the evidence indicated that *m*-cresol was not *para*-carboxylated, we investigated other possible routes for its degradation. Hypothetical metabolites were tested as substrates for strain Groll (Table 1). Carboxylation of *m*-cresol *ortho* to the hydroxyl group would yield 4-methylsalicylate, but this compound was not degraded by strain Groll. In fact, none of the methylsalicylate isomers were transformed by this organism (Table 1). When methyl hydroquinone and 4-methylcatechol were tested as substrates, both were abiotically transformed; the products of the former accumulated but the products of the latter were degraded after a six week lag period. This degradation occurred long after *m*-cresol was depleted in dual substrate cultures, and 4-methylcatechol had no effect on *m*-cresol degradation (Table 1). Such findings suggested that it was unlikely that the initial attack on *m*-cresol was by ring hydroxylation. Methylbenzoates were included in the study because 2-methylbenzoate has been proposed as a dead-end metabolite of *m*-cresol (15). Only the 3-methylbenzoate isomer was degraded by this organism (Table 1). This metabolism occurred after a longer lag period than for *m*-cresol and had no effect on the degradation of the latter. In all of the dual substrate studies, the addition of *m*-cresol neither stimulated nor inhibited the degradation of any of the test compounds. *m*-Cresol was degraded in all cultures, indicating that the test compounds were not inhibitory at the concentrations added.

The degradation of putative metabolites predicted by a methyl group oxidation pathway is illustrated in Fig. 1. All four of these predicted metabolites were readily degraded by strain Groll, and they were degraded faster than *m*-cresol (Fig. 1). 3-Hydroxybenzaldehyde was only degraded at concentrations ≤100 μM; at higher concentrations it was only partially metabolized. In dual substrate studies, 3-hydroxybenzyl alcohol, 3-hydroxybenzaldehyde, 3-hydroxybenzoic acid, and benzoic acid all caused an increase in the lag period prior to the onset of *m*-cresol degradation (Fig. 2). With the exception of the fluorinated analogs, the other test substrates, whether they were metabolized or not, did not affect the lag period or the rate of *m*-cresol degradation. When added in combination with *m*-cresol, the methyl oxidation metabolites were also degraded faster than *m*-cresol. In cultures containing 3-hydroxybenzaldehyde at >100 μM, *m*-cresol depletion was completely inhibited, indicating that this aldehyde is particularly toxic to this organism.

Addition of analogs to *m*-cresol-degrading cultures. A variety of analogs were tested as inhibitors of *m*-cresol degradation and for their potential to cause accumulation of metabolites. Molybdate (0.5 mM), a sulfate analog used to inhibit sulfate-reducing bacteria, and the common metabolic inhibitor 2-fluoroacetate (10 mM) completely prevented *m*-cresol depletion by strain Groll, but no compounds accumulated. The other analogs tested were all fluorinated derivatives of *m*-cresol, phenol, or benzoic acid. The effects of these analogs on *m*-cresol degradation was tested at three concentrations because in previous studies the accumulation of intermediates was dependent on inhibitor concentration (3, 8). The results are summarized in Fig. 3 which shows the average rate of *m*-cresol degradation in cultures with each analog at all concentrations. The fluorinated cresols (Fig. 3C) caused minimal inhibition of *m*-cresol degradation regardless of concentration. The fluorinated phenols caused moderate inhibition (Fig. 3B) that was concentration dependent, with 2-fluorophenol more inhibitory than 3- or 4-fluorophenol.

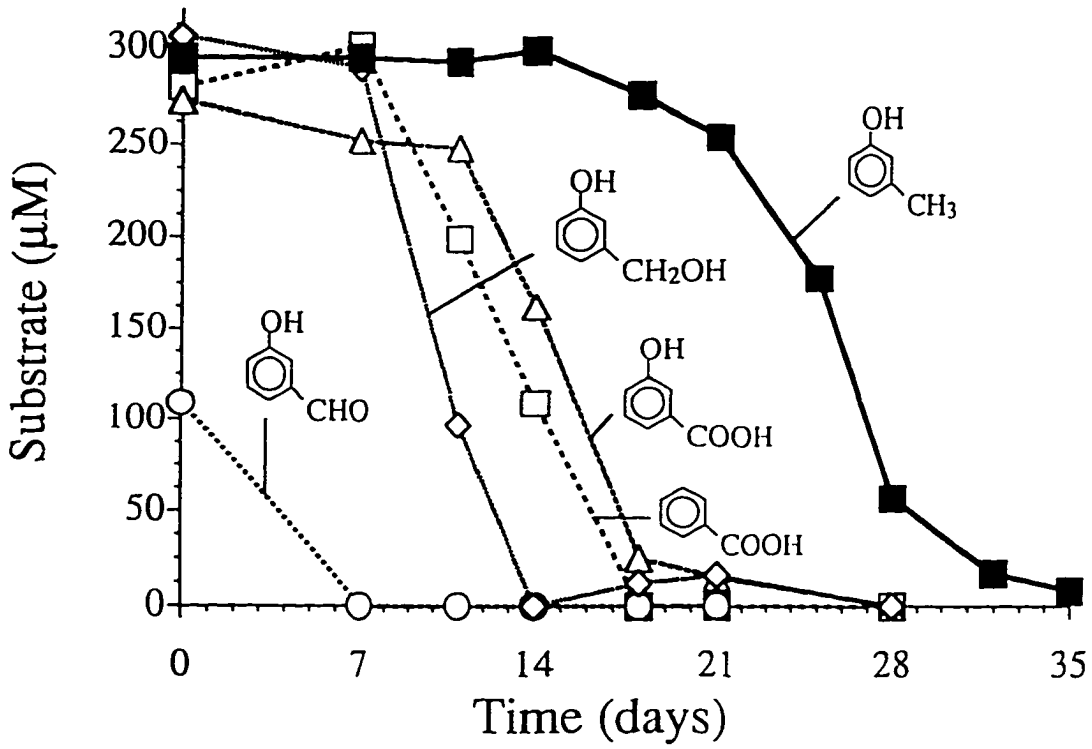

FIG. 1. Degradation of individual proposed *m*-cresol metabolites by strain Groll. *m*-Cresol (■), 3-hydroxybenzyl alcohol (◊), 3-hydroxybenzaldehyde (○), 3-hydroxybenzoate (Δ), and benzoate (□). No transformation of these compounds was observed in autoclaved sterile controls.

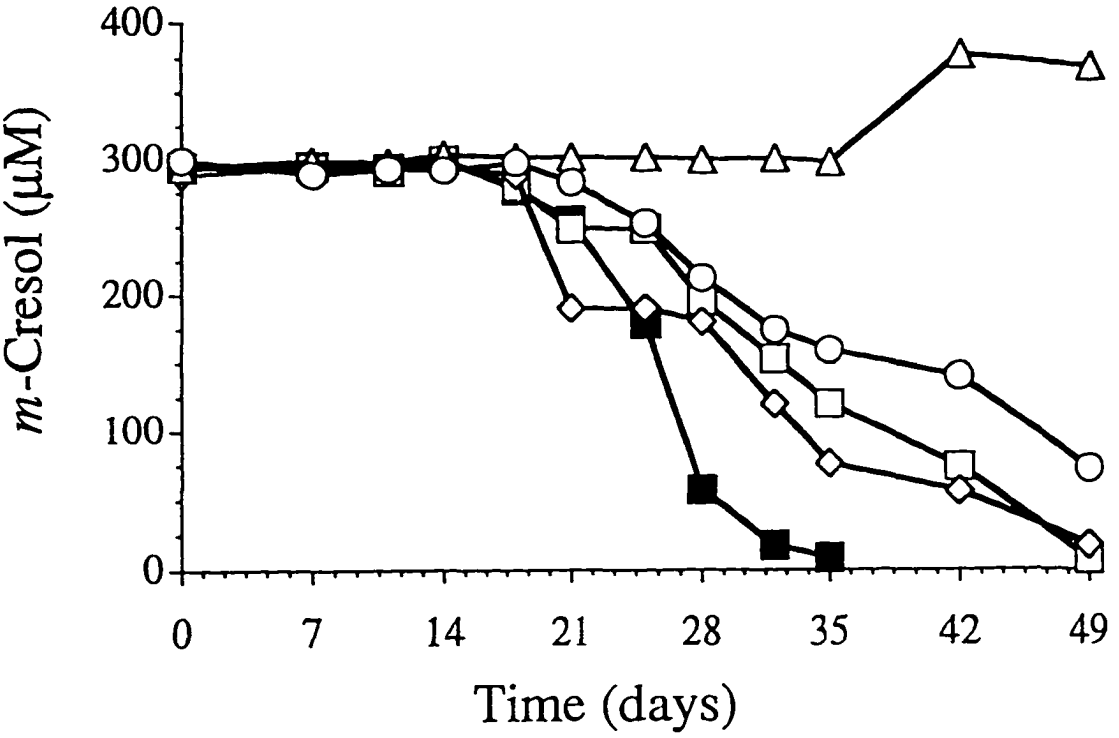

FIG. 2. Inhibition of *m*-cresol degradation by the addition of equimolar amounts of proposed *m*-cresol metabolites. *m*-Cresol only (■), with 3-hydroxybenzyl alcohol (○), with 3-hydroxybenzaldehyde (Δ), with 3-hydroxybenzoate (◊), and with benzoate (□).

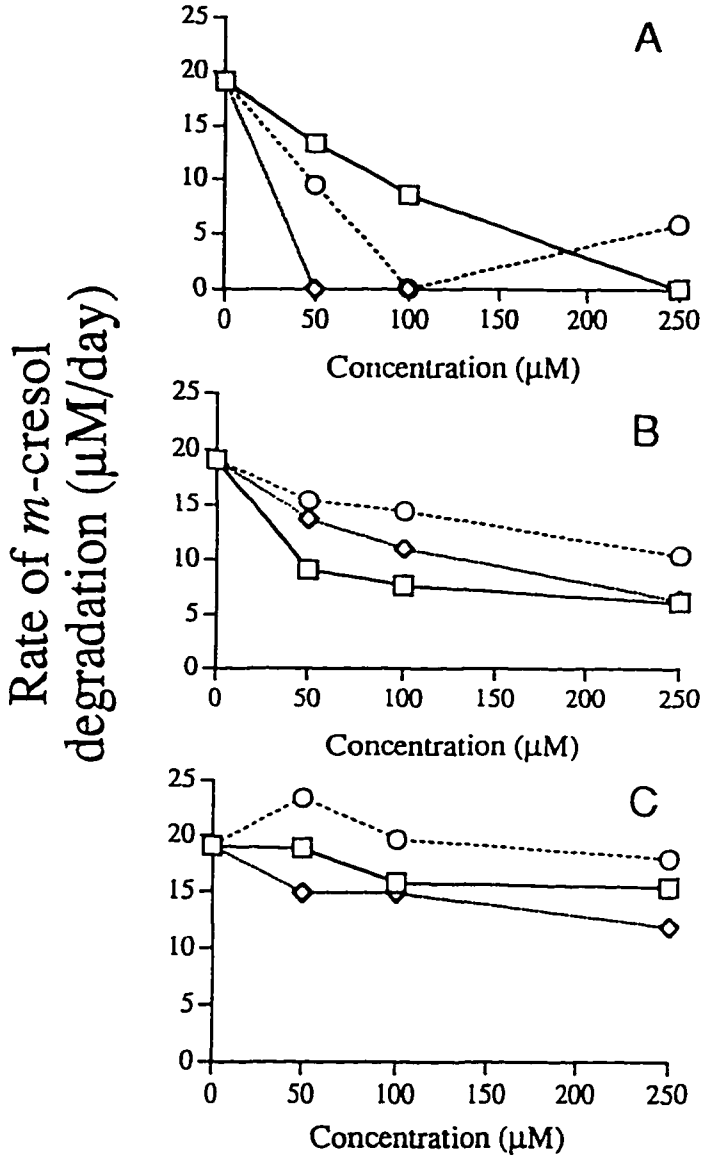

FIG. 3. Effects of fluorinated aromatic compounds on the degradation of *m*-cresol by strain Groll. Rates of *m*-cresol degradation are compared to the concentration of fluorinated analog added to the cultures. (A) 2-Fluorobenzoate (□), 3-fluorobenzoate (◊), and 4-fluorobenzoate (○) inhibited *m*-cresol degradation the most; (B) 2-fluorophenol (□), 3-fluorophenol (◊), and 4-fluorophenol (○) inhibited *m*-cresol degradation slightly; whereas (C) 4-fluoro-3-methylphenol (□), 6-fluoro-3-methylphenol (◊), and 3-hydroxybenzyl trifluoride (○) had little effect on the rate of degradation of *m*-cresol.

The fluorinated benzoates inhibited *m*-cresol degradation the most, and in particular 3-fluorobenzoate caused complete inhibition of *m*-cresol degradation at all three concentrations tested (Fig. 3A). Inhibition of *m*-cresol degradation by 2- and 4-fluorobenzoate was concentration-dependent. Of all the analogs tested, the fluorobenzoates were the only ones metabolized. Both 2-fluorobenzoate and 4-fluorobenzoate, but not 3-fluorobenzoate, were degraded by strain Groll (Table 1). Unfortunately, addition of fluorinated analogs did not result in the accumulation of any *m*-cresol metabolites that could be detected using two different HPLC techniques.

Detection of metabolites in culture fluids. Cultures of strain Groll were analyzed for the presence of intermediates that would suggest the mechanism by which *m*-cresol was metabolized. Cultures fluids were analyzed directly by two HPLC methods, or in some cases culture fluids were extracted with organic solvent, derivatized, and analyzed by GC-MS. All three analyses were performed with culture fluids from dense cell suspensions of *m*-cresol-degrading strain Groll, and were compared with cultures utilizing benzoate, as well as sterile and uninoculated controls. We were unable to detect 4-hydroxy-2-methylbenzoate, 2-methylbenzoate, or any other compounds that would indicate metabolism of *m*-cresol by any pathway other than methyl group oxidation.

Cultures amended with the putative methyl group oxidation metabolites were also analyzed by these methods. 3-Hydroxybenzoic acid was detected as a metabolite of 3-hydroxybenzyl alcohol. Fig. 4 illustrates that as 3-hydroxybenzyl alcohol was degraded, a transient accumulation of 3-hydroxybenzoic acid was observed. This metabolite was identified by its retention time in the HPLC analyses compared to an authentic standard. 3-Hydroxybenzoic acid accumulated transiently regardless of whether *m*-cresol was also added. No other metabolites were detected in these cultures, or in cultures receiving 3-hydroxybenzaldehyde, 3-hydroxybenzoic acid, or benzoic acid.

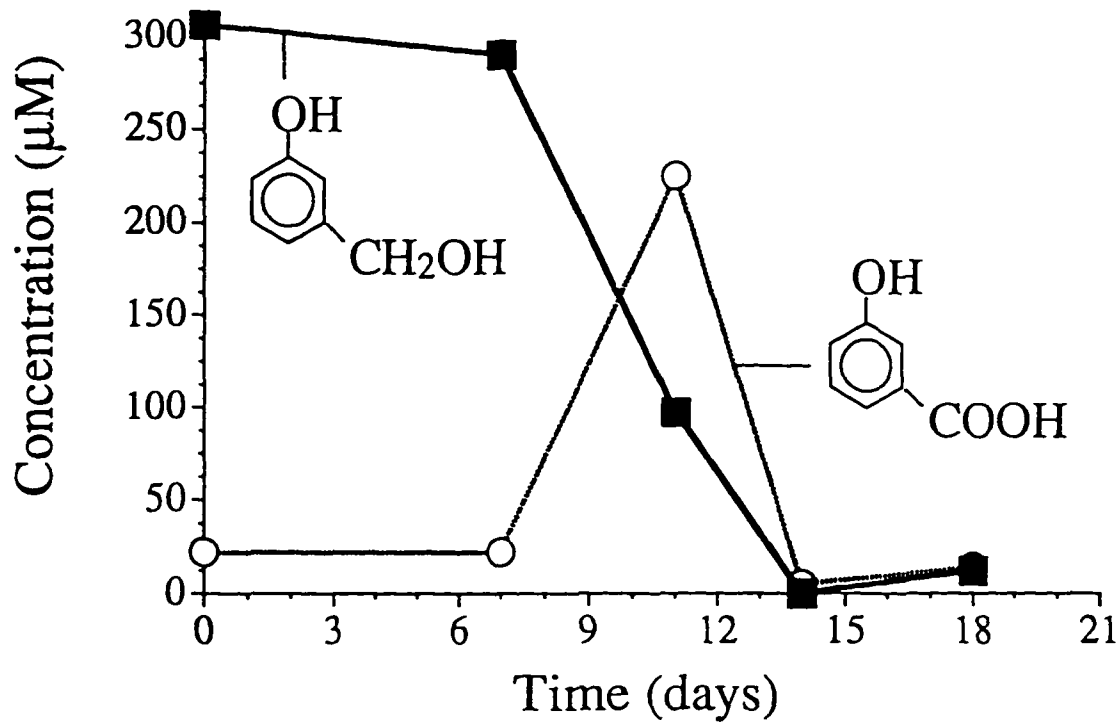

FIG. 4. Accumulation of 3-hydroxybenzoate (○) in cultures of strain Groll degrading 3-hydroxybenzyl alcohol (■). The accumulation was transient, and was observed in cultures regardless of whether *m*-cresol was also present.

HPLC analysis of dense cell suspensions revealed the presence of 3-hydroxybenzoic acid in cultures of strain Groll in which *m*-cresol degradation had ceased following the degradation of over ten amendments of *m*-cresol. HPLC analysis of culture fluids revealed a peak with the retention time corresponding to authentic 3-hydroxybenzoic acid that was not detected in cultures in which benzoate was the substrate. This compound was only detected in cultures that were stressed, presumably by the accumulation of sulfide; microscopically the cells were shrivelled and sporulating. GC-MS analyses of the extracted and derivatized culture fluid confirmed the presence of 3-hydroxybenzoic acid in cultures of strain Groll degrading *m*-cresol (Fig. 5). This compound was not detected in derivatized extracts of cultures in which butyrate or benzoate served as the starting substrate, nor in uninoculated culture fluids containing *m*-cresol. The trimethylsilyl derivative of benzoic acid was also detected in culture fluids of *m*-cresol-degrading strain Groll. Fig. 6 compares the mass spectra of a peak detected from analysis of extracted culture fluid with an authentic derivatized benzoate standard. The detection of 3-hydroxybenzoate and benzoate in culture fluids provides further evidence for their role as metabolites of *m*-cresol degradation by strain Groll.

DISCUSSION

Our evidence consistently indicated that strain Groll degrades *m*-cresol by methyl group oxidation leading to 3-hydroxybenzoic acid. The latter is subsequently converted to benzoic acid (Fig. 7). This pathway has been suggested previously for *m*-cresol degradation by a denitrifying pseudomonad (3, 16). However, previous studies of *m*-cresol degradation under sulfate-reducing conditions have suggested a different pathway involving an initial *para*-carboxylation. In studies with consortia enriched from a landfill leachate-contaminated aquifer, *m*-cresol was *para*-carboxylated to form 4-hydroxy-2-methylbenzoic acid (13). Subsequent studies extend these observations in that

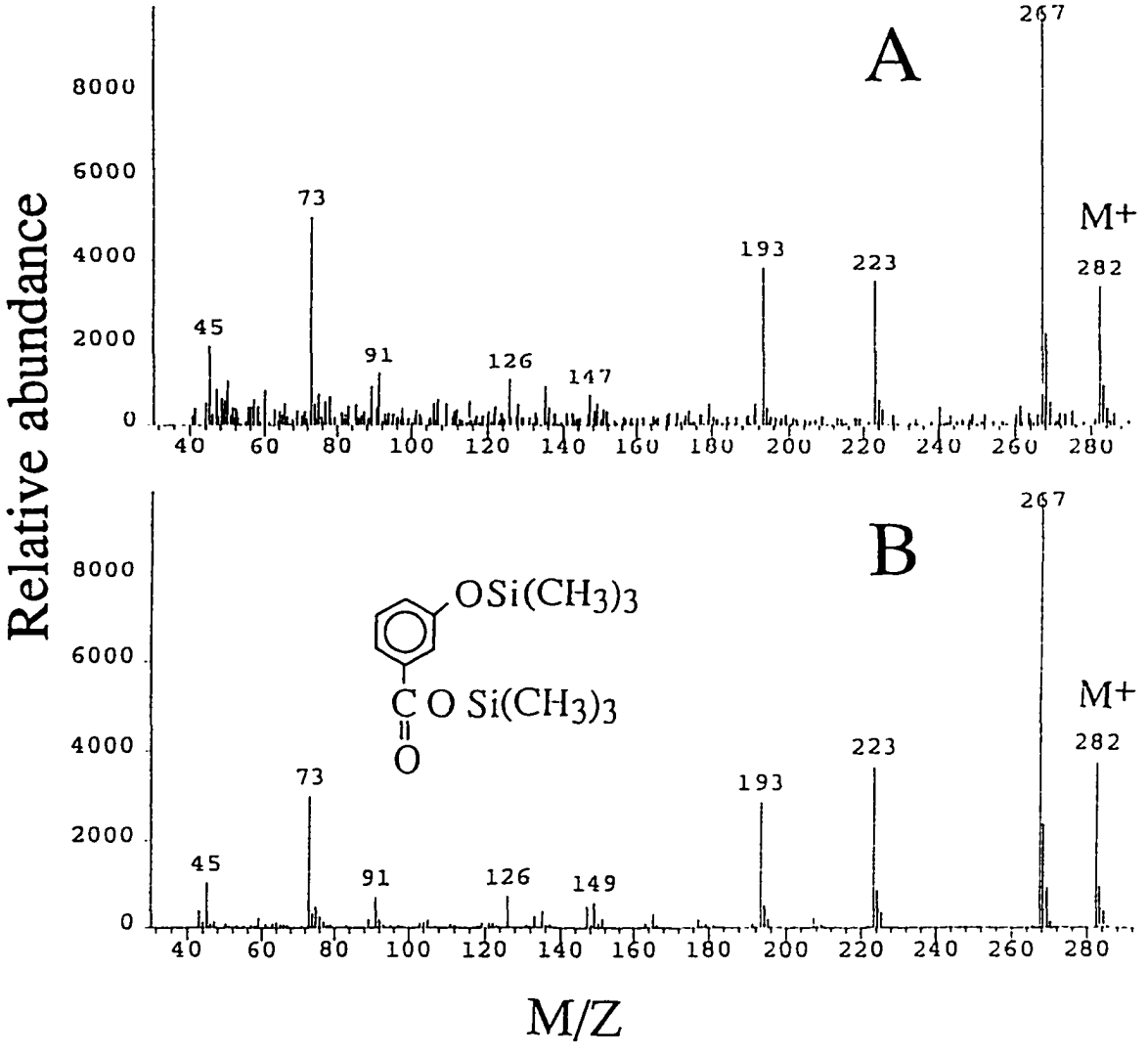

FIG. 5. From GC-MS analyses, mass spectra of a TMS-derivatized metabolite from *m*-cresol (A) and of the TMS derivative of 3-hydroxybenzoic acid (B).

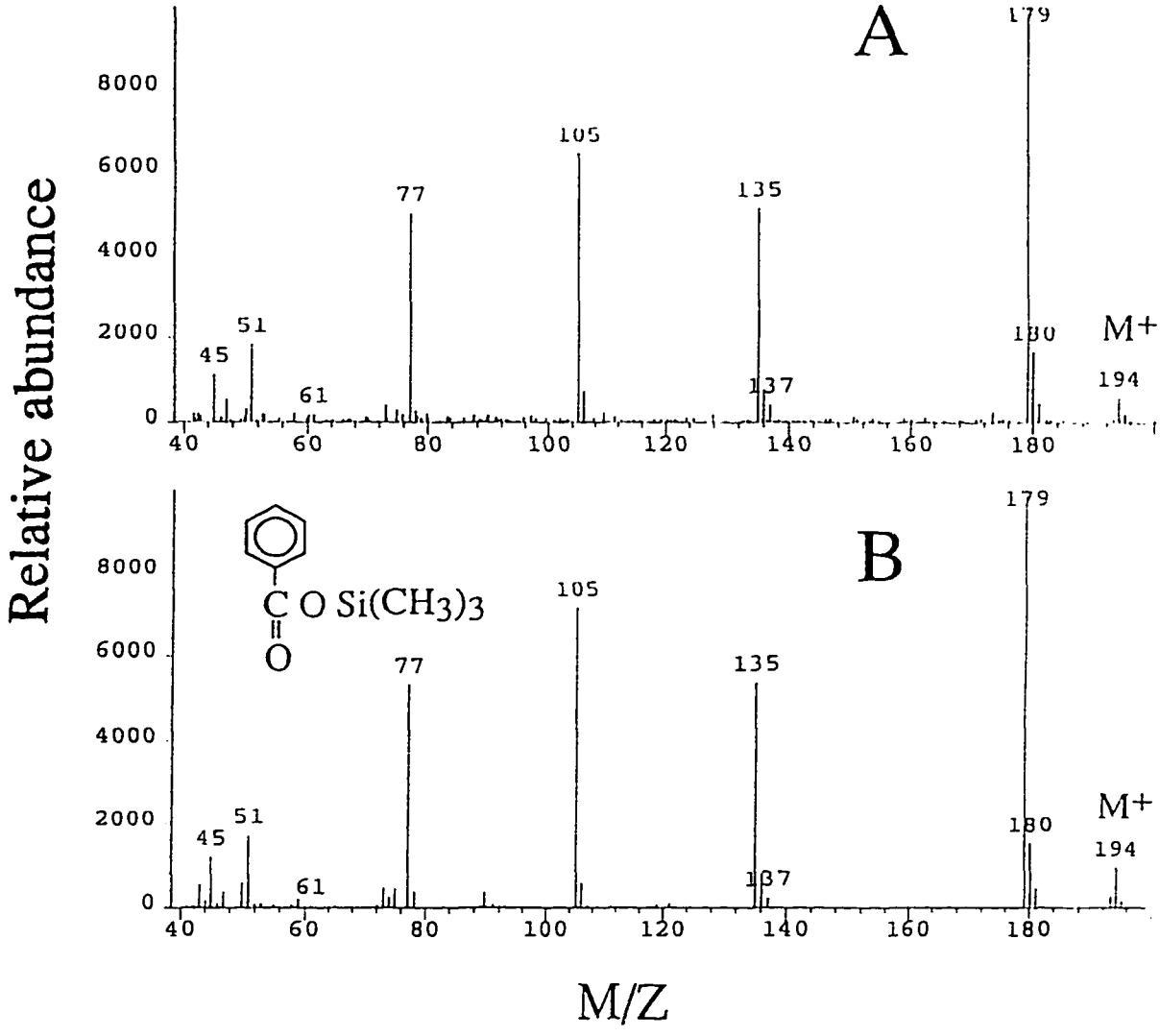

FIG. 6. From GC-MS analyses, mass spectra of a TMS-derivatized metabolite from *m*-cresol (A) and of the TMS derivative of benzoic acid (B).

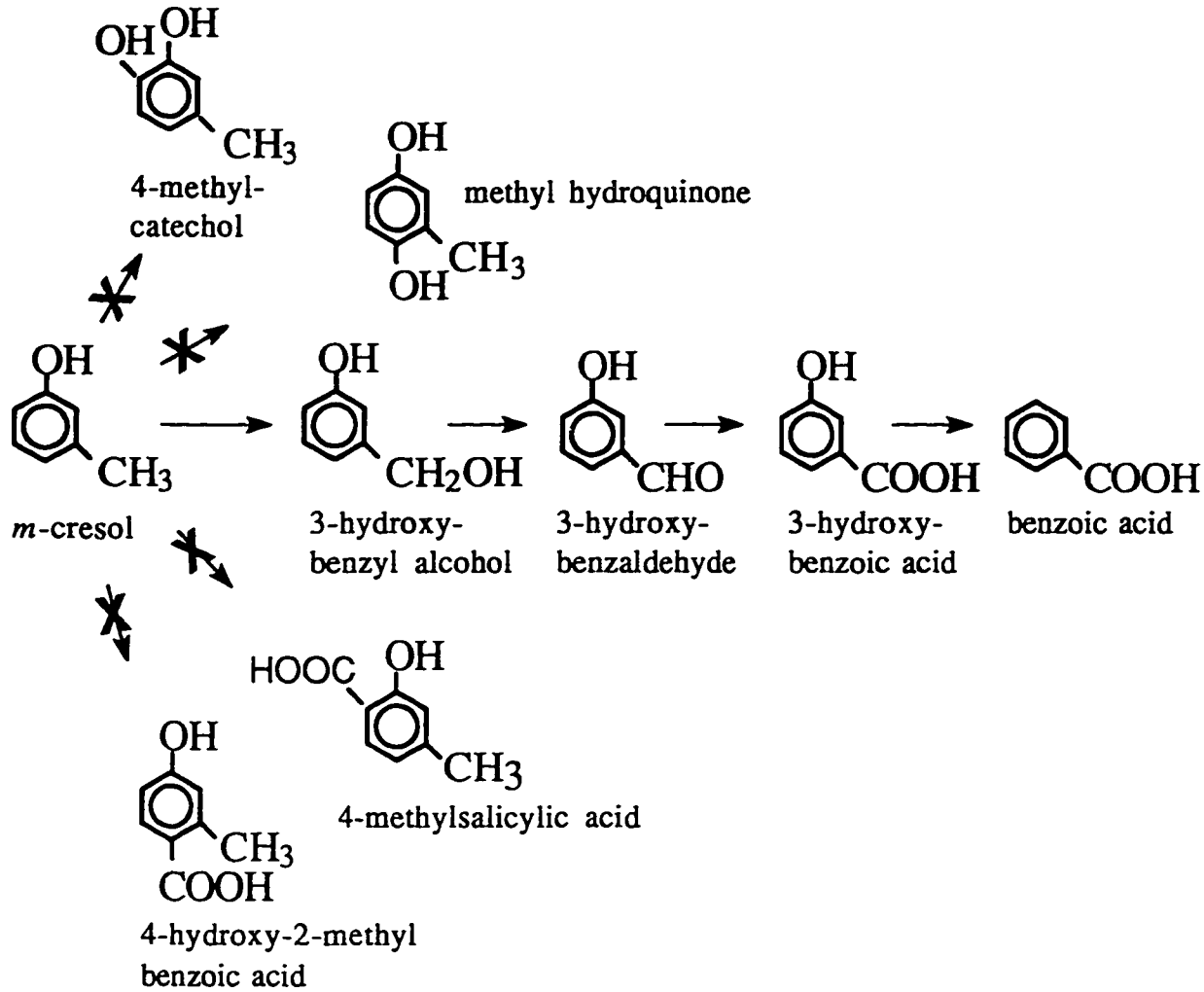

FIG. 7. Proposed reactions for the initial transformation of *m*-cresol under sulfate-reducing conditions by *Desulfotomaculum* strain Groll. *m*-Cresol is hydroxylated on the methyl group to give 3-hydroxybenzyl alcohol which is oxidized to 3-hydroxybenzaldehyde then 3-hydroxybenzoate. 3-Hydroxybenzoate is subsequently dehydroxylated to form benzoate which is further metabolized. *m*-Cresol was not metabolized by ring carboxylation or hydroxylation, as the products of these potential reactions were not degraded by strain Groll (4-hydroxy-2-methylbenzoate, 4-methylsalicylate, methylhydroquinone), or were degraded only after a longer lag period than *m*-cresol (4-methylcatechol).

both 4-hydroxybenzoic acid and benzoic acid were found as *m*-cresol metabolites (9). Thus, the pathway for *m*-cresol degradation under sulfate-reducing conditions was considered identical to that proposed for methanogenic conditions (8). However, *m*-cresol metabolism by a sulfate-reducing isolate differs substantially from the mixed consortium. For example, the specificity of substrates degraded are juxtaposed. With the consortium, 4-hydroxy-2-methylbenzoic acid and 4-hydroxybenzoic acid were readily degraded, yet 3-hydroxybenzyl alcohol and 3-hydroxybenzoic acid were not metabolized in either the presence or absence of *m*-cresol (13). The degradation of 4-hydroxybenzoic acid preceded and delayed the metabolism of *m*-cresol by the consortium (13). In contrast, strain Groll does not degrade 4-hydroxy-2-methylbenzoic acid even in the presence of *m*-cresol, yet will degrade the methyl group oxidation metabolites. Strain Groll also degraded 4-hydroxybenzoic acid, but this is a common intermediate in the degradation of aromatic compounds (6) including *p*-cresol, which is also metabolized by this organism. Furthermore, the addition of 4-hydroxybenzoic acid had no effect on *m*-cresol degradation by strain Groll.

Sulfate-reducing mixed cultures exhibited a specific requirement for carbon dioxide for the degradation of *m*-cresol but not benzoate, as bicarbonate was incorporated in the initial *para*-carboxylation reaction (13). Strain Groll had a less specific requirement for carbon dioxide, as it was required for the degradation of *m*-cresol, benzoate, as well as other carboxylated aromatic compounds. However, strain Groll did not have an absolute requirement for CO_2 as butyrate was degraded in its presence or absence. Our results are somewhat different from those of Kuever et al. (5) who reported that benzoate degradation was not CO_2 dependent, however they also found that another aromatic compound, catechol, could not be degraded in the absence of CO_2, and they similarly report that butyrate degradation was not CO_2 dependent. At present there is insufficient evidence to suggest why carbon dioxide would be required for the degradation of carboxylated aromatic compounds.

Simultaneous adaptation experiments provide indirect evidence for a *m*-cresol degradation pathway. The metabolites predicted for a methyl group oxidation pathway for *m*-cresol degradation are 3-hydroxybenzyl alcohol, 3-hydroxybenzaldehyde, and 3-hydroxybenzoic acid. 3-Hydroxybenzaldehyde was inhibitory at higher concentrations but was degraded when added at ≤ 100 μM. Since metabolites rarely accumulate even to this concentration this does not adversely affect its candidacy as a putative metabolite for this organism. Degradation of all the proposed metabolites was faster than the degradation of *m*-cresol. In cultures in which these substrates were added with *m*-cresol, the potential metabolites were preferentially degraded. Benzoic acid could likely be formed from 3-hydroxybenzoic acid by a dehydroxylation, which has been shown previously to occur under anaerobic conditions for this and other hydroxybenzoate isomers (for review see 6). In fact, 3-hydroxybenzoic acid and benzoic acid probably exist as intracellular coenzyme-A derivatives, but for convenience we refer to them as the free acids, the form added to, and detected in, our experiments.

Further evidence that this series of compounds could represent metabolites of *m*-cresol degradation came from substrate competition experiments. Each of these four metabolites caused an increase in the length of time required for *m*-cresol degradation compared to cultures in which *m*-cresol was added alone. In cultures that received ≥250 μM 3-hydroxybenzaldehyde, which was not completely degraded, *m*-cresol was not degraded at all. The inhibitory effect was observed only with these four metabolites. Other aromatic compounds, with the exception of the fluorinated analogs, whether they were degraded by strain Groll faster than *m*-cresol, slower than *m*-cresol, or not at all, did not affect *m*-cresol degradation. The preferential utilization of metabolites prior to the substrate suggests their order of reaction in the degradation sequence.

In previous studies, fluorinated analogs were very useful tools for elucidating the pathway of *m*-cresol metabolism (8). We hoped that fluorinated analogs could be used to inhibit *m*-cresol degradation and cause metabolite accumulation in cultures of strain Groll.

The effects of the various fluorinated analogs on *m*-cresol degradation depended on both the position of the fluorine, and on the other functional groups on the aromatic ring, yet none of the analogs caused an accumulation of metabolites or fluorinated transformation products in culture fluids. The fluorinated analogs of *m*-cresol had the least effect on *m*-cresol degradation, the fluorophenols had a slightly greater inhibitory effect on *m*-cresol degradation, whereas the fluorobenzoates, and particularly 3-fluorobenzoate, were the most inhibitory. The inhibitory effects were often concentration-dependent, which in previous studies was the condition for which analogs caused accumulation of metabolites (3, 8), yet it appears that the pathway for *m*-cresol degradation by strain Groll is tightly regulated and that the enzymes excluded the fluorinated analogs. 2-Fluorobenzoate and 4-fluorobenzoate were degraded by strain Groll, but no intermediates were detected so it is unknown whether the fluorine was removed prior to aromatic ring reduction and cleavage. Anaerobic degradation of these fluorobenzoates has been reported previously for nitrate-reducing organisms (19, 21).

Some of the strongest evidence for a methyl group oxidation pathway for *m*-cresol degradation by strain Groll was the detection of metabolites in culture fluids. 3-Hydroxybenzoic acid was detected as a transient metabolite in cultures degrading 3-hydroxybenzyl alcohol. 3-Hydroxybenzoic acid was also detected by HPLC and GC-MS analyses of dense cell suspensions of *m*-cresol-degrading strain Groll. Benzoic acid was detected by GC-MS analysis of extracted culture fluids. All of these metabolites were present only in trace amounts. The detection of 3-hydroxybenzoic acid from either *m*-cresol or 3-hydroxybenzyl alcohol suggests a progression of oxidation reactions as indicated in Fig. 7. The detection of benzoate indicates that 3-hydroxybenzoic acid was dehydroxylated prior to ring cleavage. 3-Hydroxybenzyl alcohol and 3-hydroxybenzaldehyde were not detected in spite of a variety of approaches taken to try to cause accumulation of these metabolites. The metabolism of *m*-cresol involves highly

efficient transformations, and the initial hydroxylation appears to be the limiting step for its degradation.

While probing the metabolic pathway for *m*-cresol degradation, we have tested a variety of aromatic compounds as possible substrates for strain Groll. Our results agreed with those of Kuever et al. (5) that strain Groll degraded *m*-cresol and *p*-cresol but not *o*-cresol. Strain Groll degraded 4-methylcatechol, a potential product of lignin hydrolysis. Kuever et al. (5) reported the degradation of 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid which would result from the oxidation of the methyl group of 4-methylcatechol, suggesting another methyl group oxidation series. Our studies, combined with the original list reported by Kuever et al. (5) indicate that at least 28 different aromatic compounds, as well as many non-aromatic compounds, can be degraded by this metabolically diverse anaerobe.

The degradation of 3-methylbenzoic acid (*m*-toluic acid) by strain Groll was an intriguing observation. There are very few reports of the anaerobic degradation of toluic acids. Londry and Fedorak (7) demonstrated that *o*-toluic acid was degraded under methanogenic conditions after a relatively long acclimation period (25 weeks compared to 8 weeks for *m*-cresol), and we are unaware of any additional reports of toluic acid biodegradation. Toluic acids are environmentally significant as they have been shown to accumulate as products of xylene metaboism under anaerobic conditions (1, 12, 18). Strain Groll does not degrade the aromatic hydrocarbons toluene or *m*-xylene (Table 1), however, probably due to the specificity of the initial methyl hydroxylating enzyme. Kuever et al. (5) reported that benzyl alcohol and benzaldehyde are metabolized by strain Groll, so biochemically only an initial hydroxylation of toluene would be required for its degradation. It may be possible to combine the organisms that transform xylene isomers with strain Groll, or to modify the methyl group hydroxylating enzyme(s) of strain Groll to achieve complete mineralization of toluene or *m*-xylene under sulfate-reducing conditions.

We conclude from these studies that there are at least two possible pathways for the degradation of *m*-cresol under sulfate-reducing conditions. Both pathways converge on benzoic acid, a common metabolite for the anaerobic degradation of aromatic compounds (6). Therefore, under sulfate-reducing conditions all three cresol isomers can be degraded by methyl group oxidation mechanisms. Furthermore, while *p*-cresol can be degraded by methyl group oxidation under many anaerobic conditions, both *m*-cresol and *o*-cresol have been shown to undergo either methyl group oxidation or an initial *para*-carboxylation, depending on the electron acceptor available. It is interesting to note that *m*-cresol metabolism has been suggested to occur by methyl group oxidation in studies with pure cultures (this study, 3), and by *para*-carboxylation in mixed consortia (8, 13). This leads us to speculate whether community dynamics can affect the mechanism by which aromatic compounds are degraded in anoxic environments. Further research will be required to conclusively establish the pathway for *m*-cresol metabolism by strain Groll by measuring enzyme activities responsible for these conversions, and to determine the environmental significance of this mechanism for degradation of aromatic compounds in anaerobic environments.

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Chapter 2.

CRESOL METABOLISM BY THE SULFATE-REDUCING BACTERIUM *DESULFOTOMACULUM* STRAIN GROLL

Abstract

The metabolism of cresols under sulfate-reducing conditions was investigated with the organism *Desulfotomaculum* strain Groll. The cell was known to degrade a variety of aromatic compounds including *para*- and *meta*-, but not *ortho*-cresol. Degradation of *p*-cresol proceeded by oxidation reactions of the methyl group to yield *p*-hydroxybenzoate, which was then dehydroxylated to benzoate. The aromatic intermediates of this pathway were readily metabolized by strain Groll, and generally preceded and inhibited the degradation of *p*-cresol. *p*-Hydroxybenzoate and benzoate were detected in culture fluids as metabolites of *p*-cresol, and *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate were detected in cultures degrading *p*-hydroxybenzyl alcohol. *m*-Cresol degradation by strain Groll also proceeded by methyl group oxidation. However, the enzymatic activities responsible for degradation of the *m*- and *p*-cresol were distinct. Enzymes responsible for the respective bioconversions were detected in cell extracts of strain Groll and were induced by growth on the various cresols. Enzyme activities were not detected for *o*-cresol derivatives. Both the *p*- and *m*-cresol pathways converge on benzoate, and this intermediate was metabolized as a coenzyme-A thioester. Thus, strain Groll could biodegrade both cresol isomers by similar reactions, but the enzymes catalyzing these transformations were biochemically discrete.

Introduction

Cresols are phenolic contaminants of fuel processing wastewaters that are of environmental concern because of their toxicity and their mobility in subsurface environments. The anaerobic biodegradation of all three cresol isomers has been demonstrated in field and laboratory studies (Londry and Fedorak 1992). A variety of mechanisms have been reported for the metabolism of cresols under anaerobic conditions. *p*-Cresol was metabolized by a series of methyl group oxidation reactions through *p*-hydroxybenzoate in methanogenic consortia (Senior and Balba 1984), sulfate-reducing consortia (Suflita et al. 1989), by nitrate-reducing isolates (Hopper et al. 1991, Rudolphi et al. 1991), and by an iron-reducing isolate (Lovley and Lonergan 1990). *m*-Cresol can be metabolized by two pathways. A denitrifying isolate degraded *m*-cresol by a methyl group oxidation mechanism (Bonting et al. 1995). Under methanogenic conditions, the methyl group was not oxidized (Roberts et al. 1987). *m*-Cresol was transformed by an initial *para*-carboxylation (Roberts et al. 1990), followed by an apparent demethylation and dehydroxylation to yield benzoic acid (Londry and Fedorak 1993). Under sulfate-reducing conditions, *m*-cresol can be degraded by methyl group oxidation or by the *para*-carboxylation pathway (Ramanand and Suflita 1991, Londry et al. 1996). *o*-Cresol is apparently degraded by either *para*-carboxylation (Bissaillon et al. 1991) or methyl group oxidation (O'Connor and Young 1996) under methanogenic conditions, methyl group oxidation under sulfate-reducing conditions (Suflita et al. 1989), and *para*-carboxylation followed by dehydroxylation under nitrate-reducing conditions (Rudolphi et al. 1991). Therefore the mechanism of cresol degradation under different conditions depends on both the isomer and the available electron acceptor.

The transformation of cresols under anaerobic conditions ultimately yields substituted benzoates or benzoate, a central metabolite for the degradation of a wide variety of aromatic compounds. Coenzyme-A (CoA) involvement in the anaerobic metabolism of benzoates has been well established, and benzoyl-CoA is an accepted precursor of ring

reduction, cleavage, and further oxidation. CoA ligases that thioesterify aromatic compounds have been characterized or detected from a variety of anaerobic bacteria (Villemur 1995) and their role in cellular transport and degradation has been investigated (Nichols and Harwood 1995). CoA ligases vary in their specificity and rates of reactions with various hydroxybenzoate isomers and benzoate, and several CoA ligases may be expressed in a single organism (Villemur 1995). The metabolism of benzoyl-CoA begins with the reduction of the aromatic ring, but the details of exactly which intermediates are formed during the conversion to pimelic acid, which is subsequently degraded by a series of ß-oxidation reactions, is still a matter of debate (Londry and Fedorak 1992).

We investigated the metabolic pathways for cresol degradation under sulfate-reducing conditions using the sulfate-reducing microorganism *Desulfotomaculum* strain Groll, a Gram-positive, spore-forming, mesophilic, strictly anaerobic rod-shaped bacterium. It is versatile, growing under freshwater or saltwater conditions, and can use a variety of electron acceptors including sulfate, but not nitrate (Kuever et al. 1993). Furthermore, it can grow at the expense of at least 28 aromatic compounds including *m*- and *p*-cresol (Kuever et al. 1993, Chapter 1 this dissertation). Previous studies showed that strain Groll degraded *m*-cresol by a series of methyl group oxidation reactions to yield *m*-hydroxybenzoate, which is dehydroxylated to benzoate and then mineralized (Chapter 1). In this report, we provide evidence that *p*-cresol is likewise metabolized by an initial series of methyl group oxidations to yield *p*-hydroxybenzoate, which is further metabolized through benzoate. Furthermore, we provide evidence for the enzyme activities required for transformation of both *m*-cresol and *p*-cresol by these pathways, and compare the initial metabolic pathways for degradation of cresol isomers by strain Groll.

Materials and Methods

Cultivation of microorganism

Desulfotomaculum strain Groll was obtained from the Deutsche Sammlung von Mikroorganismen, Braunshweig, FRG (#72313). The organism was cultivated in an anaerobic medium as previously described (Kuever et al., 1993) but with only 1 mM sulfide as reductant, in 1-5 L media bottles, with a headspace of N_2:CO_2 (80:20). Cultures prepared for use as inocula were maintained by periodic additions of substrate (250 - 500 μM) and by repeated transfers. Cultures prepared for harvesting and subsequent enzyme assays were initiated with 2 mM benzoate and a 10% inoculum of a culture previously grown on benzoate or *m*-cresol, as appropriate. Once the benzoate was depleted, benzoate, *m*-cresol or *p*-cresol (1 mM) was added, or the cultures were transferred (20% inoculum) to a medium containing 1 mM substrate, and degradation of these substrates was monitored. Butyrate-degrading cultures were grown on 2 mM butyrate using a culture that had previously degraded 5 mM butyrate as inoculum. All cultures were incubated at 32-37°C in the dark without shaking. Samples of culture fluid were withdrawn using strict anaerobic, aseptic technique and were stored frozen until analyzed. Prior to HPLC analysis, samples were thawed and centrifuged at 10 000 x g for 5 min.

Resting cell suspensions

For resting cell experiments, media prepared as above was dispensed to incubation vessels while inside an anaerobic chamber. The vessels were then sealed with butyl rubber stoppers and aluminum crimp seals, the headspace was exchanged to N_2:CO_2 (80:20), and substrates were added from neutralized, sterile, anoxic stock solutions. A 10% inoculum of a culture that degraded *m*-cresol or benzoate was then added, and sterile controls were autoclaved. Samples were withdrawn from the incubations initially and periodically thereafter to monitor depletion of substrates.

Analytical techniques

The concentrations of butyrate and aromatic compounds were determined by high performance liquid chromatography (HPLC) system as described previously (Londry et al., submitted). Sulfate concentrations were determined using a Dionex IC system as described previously (Chapter 1). Protein was determined by measuring the turbidity at 400 nm of a sample of extract in 20% trichloroacetic acid, using bovine serum albumin as a standard. Metabolites were identified by gas chromatogrphy-mass spectrometry (GC-MS) as described previously (Chapter 1).

Preparation of cell extracts and enzyme assays

Cells used for enzyme assays were harvested in the exponential phase, using strict anaerobic technique, by centrifugation (14 000 x g, 30 min). The cell pellet was resuspended in anoxic buffer (10 mM Pipes, 2 mM $MgCl_2$, pH 7.0), and stored at -20°C. Cells were thawed, broken in a cooled French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at 16 000 psi and centrifuged for 30 min at 39 000 x g (4°C). The supernatant was transferred to a serum bottle with a butyl rubber stopper and given a N_2 headspace. Cell extracts were used immediately or stored frozen (-20°C) for less than 2 weeks between enzyme assays.

Enzyme assays were performed at 35°C using anoxic solutions in 1.5 ml cuvettes, and monitored with a Beckman DU-64 spectrophotometer. Substrate-dependent reduction of 2,6-dichlorophenolindophenol (DCPIP) was followed spectrophotometrically at 600 nm ($\varepsilon_{600\ nm}$ = 22 000 M^{-1} cm^{-1}; after Rudolphi et al. 1991). The assay mixture contained: 10 mM Pipes buffer (pH 7.0), 2 mM $MgCl_2$, 5 mM NAD, 0.2 mM DCPIP, 0.1 mM pheanazinemethosulfate (PMS), 1 mM aromatic substrate added from sterile anoxic stock solutions except benzoyl-CoA which was added as a dry powder to 0.5 mM and toluene which was added from a saturated stock solution to 2 mM, and the reaction was initiated by the addition of cell extract. Rates of DCPIP reduction were corrected for background rates

determined in the absence of substrate for each cell extract. Values shown are from single assays or the average of duplicate or triplicate determinations; detection limits for activities are 110% of controls without substrate.

Aromatic acid-CoA ligase assays were performed spectrophotometrically using the molar absorption coefficients of Rudolphi et al. (1991). Absorption increases were followed using an assay mixture of 10 mM Pipes buffer (pH 7.0), 2 mM $MgCl_2$, 2 mM ATP, 1 mM CoA, cell extract, and the reaction was initiated by the addition of aromatic substrate (1 mM). Controls without aromatic substrates were determined at 290 nm as for benzoate, and all values were corrected for these rates. Rates of absorbance increase for each aromatic acid in the absence of extract were negligible. Values shown are the average of duplicates; detection limits are 110% of controls without substrate.

Chemicals

Sodium benzoate, *m*-cresol, *p*-cresol, and biochemicals (NAD, ATP, CoA, DCPIP, PMS) were obtained from Sigma Chemical Co., St. Louis, Mo.. All other aromatic compounds were obtained from Aldrich Chemical Co., Milwaukee, Wis.. The chemicals obtained were of the highest purity available (>97%) and were used without further purification.

Results

Degradation of cresols by strain Groll

m-Cresol and *p*-cresol were degraded by strain Groll, and degradation of these compounds was accompanied by growth and sulfate reduction (Kuever et al. 1993, Chapter 1). Degradation of *p*-cresol occurred after a shorter lag time than *m*-cresol, even when cells were initially grown on *m*-cresol. Previous studies established that the predicted metabolites of *m*-cresol methyl group oxidation were readily degraded by strain Groll, and that their degradation precedes and inhibits the degradation of *m*-cresol when these substrates were added together (Londry et al. 1996). *m*-Hydroxybenzoate and benzoate

were detected by GC-MS analysis of derivatized extracts of culture fluids, so the proposed pathway for *m*-cresol metabolism by strain Groll involves methyl group oxidation followed by dehydroxylation (Chapter 1). Neither *o*-cresol nor *o*-hydroxybenzoate were degraded by strain Groll in resting cell suspensions during 10 weeks incubation, which was consistent with the report of Kuever et al. (1993). However, *p*-hydroxybenzoate was attacked by strain Groll, suggesting that *p*-cresol may also be metabolized by a series of methyl group oxidation reactions.

Initial sequence of reaction in degradation of cresols

The predicted metabolites of methyl group oxidation reactions of *p*-cresol were readily metabolized by this organism. Figure 1 illustrates the degradation of *p*-cresol, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoate, as well as benzoate, the product of dehydroxylation of *p*-hydroxybenzoate. These compounds were degraded at rates similar to *p*-cresol when added individually. When *p*-hydroxybenzyl alcohol was added as a substrate to cultures of strain Groll, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate were observed to accumulate in culture fluids (Figure 2A). The transient accumulation of *p*-hydroxybenzaldehyde preceded the accumulation *p*-hydroxybenzoate that was not degraded below an apparent threshold level of 15 μM. Similarly, when *p*-hydroxybenzaldehyde was added as substrate, *p*-hydroxybenzoate accumulated to 30 μM then decreased to the threshold value of 15 μM (Figure 2B). No metabolites were observed during the degradation of *p*-cresol or *p*-hydroxybenzoate using HPLC techniques.

GC-MS analysis of derivatized extracts of *p*-cresol-degrading cultures of strain Groll revealed trace amounts *p*-hydroxybenzoate and benzoate. As shown in Figure 3, a peak was detected in culture fluids which matched the retention time (18.58 min compared to 18.60 min) and spectrum (90%) of the TMS derivative of authentic *p*-hydroxybenzoate.

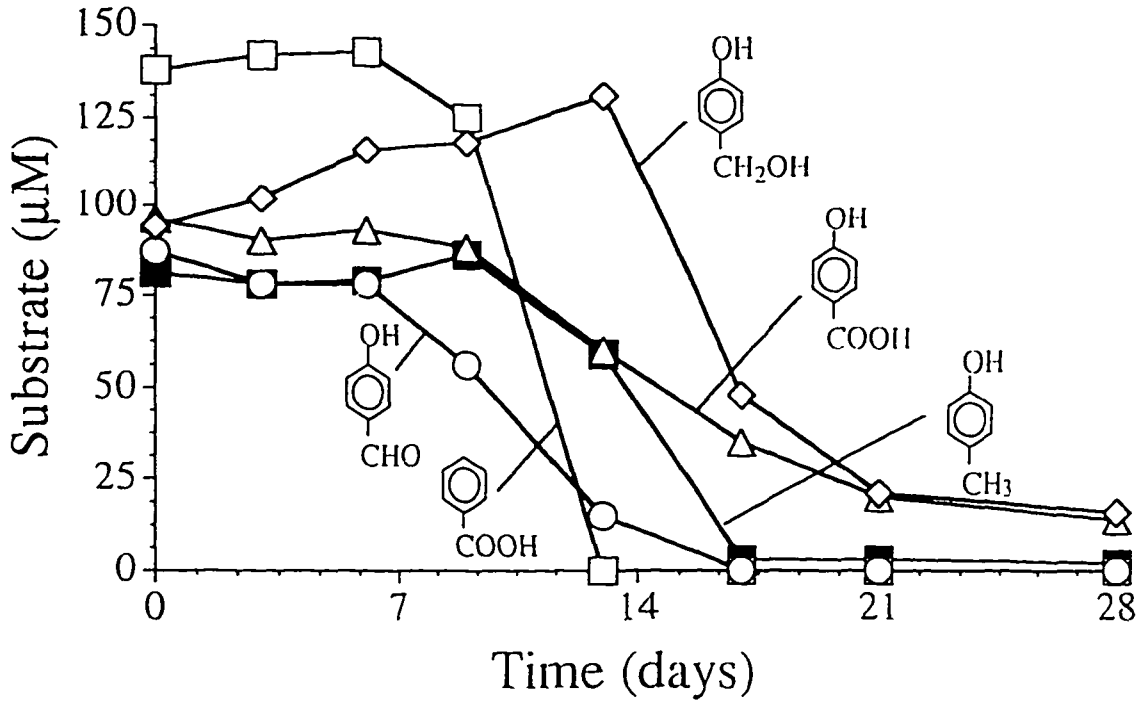

Fig. 1. Degradation of individual proposed *p*-cresol metabolites by strain Groll. *p*-Cresol (■), *p*-hydroxybenzyl alcohol (◊), *p*-hydroxybenzaldehyde (○), *p*-hydroxybenzoate (Δ), and benzoate (□).

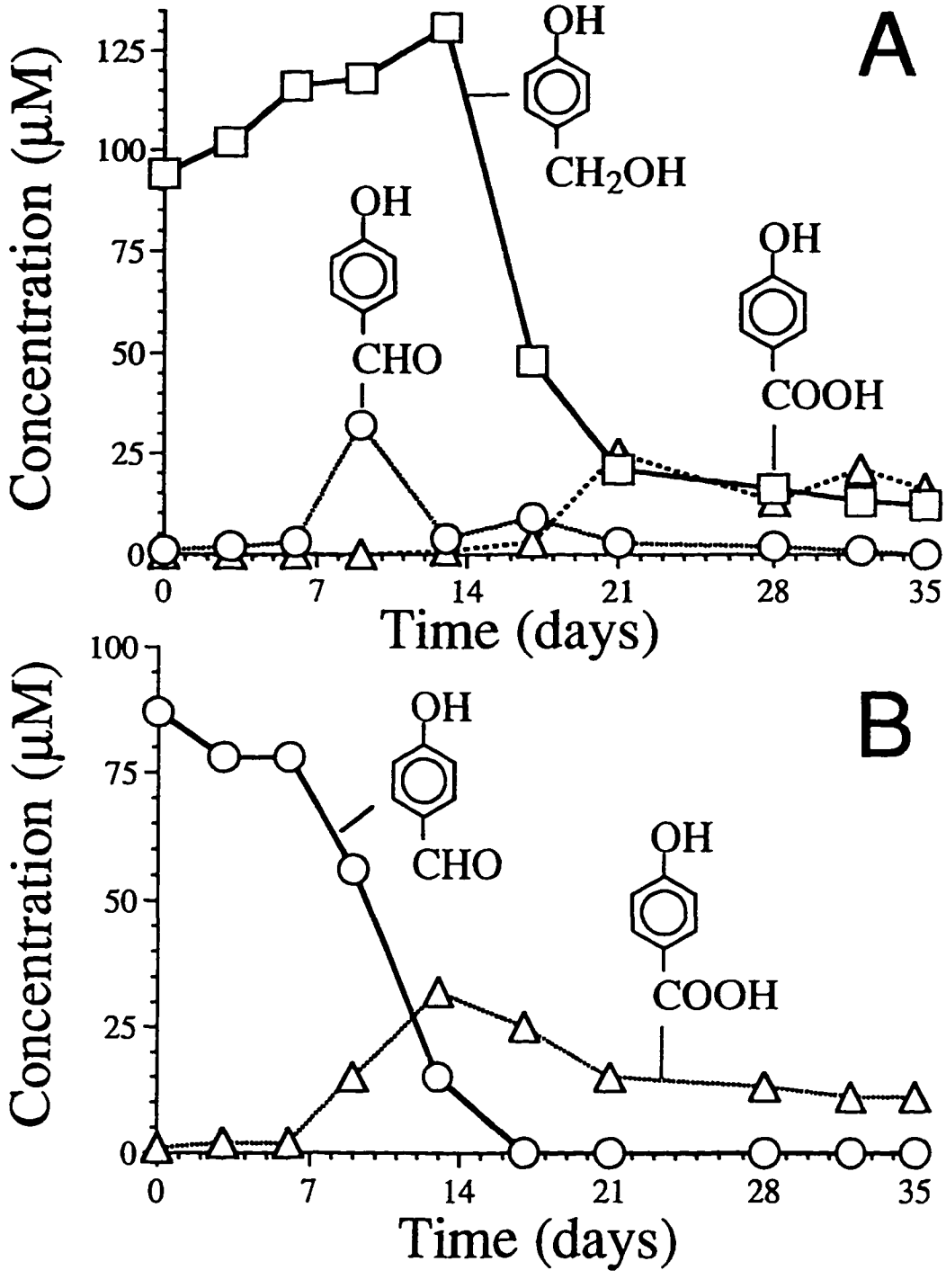

Fig. 2. Accumulation of metabolites in cultures degrading *p*-hydroxybenzyl alcohol (A) and *p*-hydroxybenzaldehyde (B); *p*-hydroxybenzyl alcohol (□), *p*-hydroxybenzaldehyde (○), *p*-hydroxybenzoate (Δ).

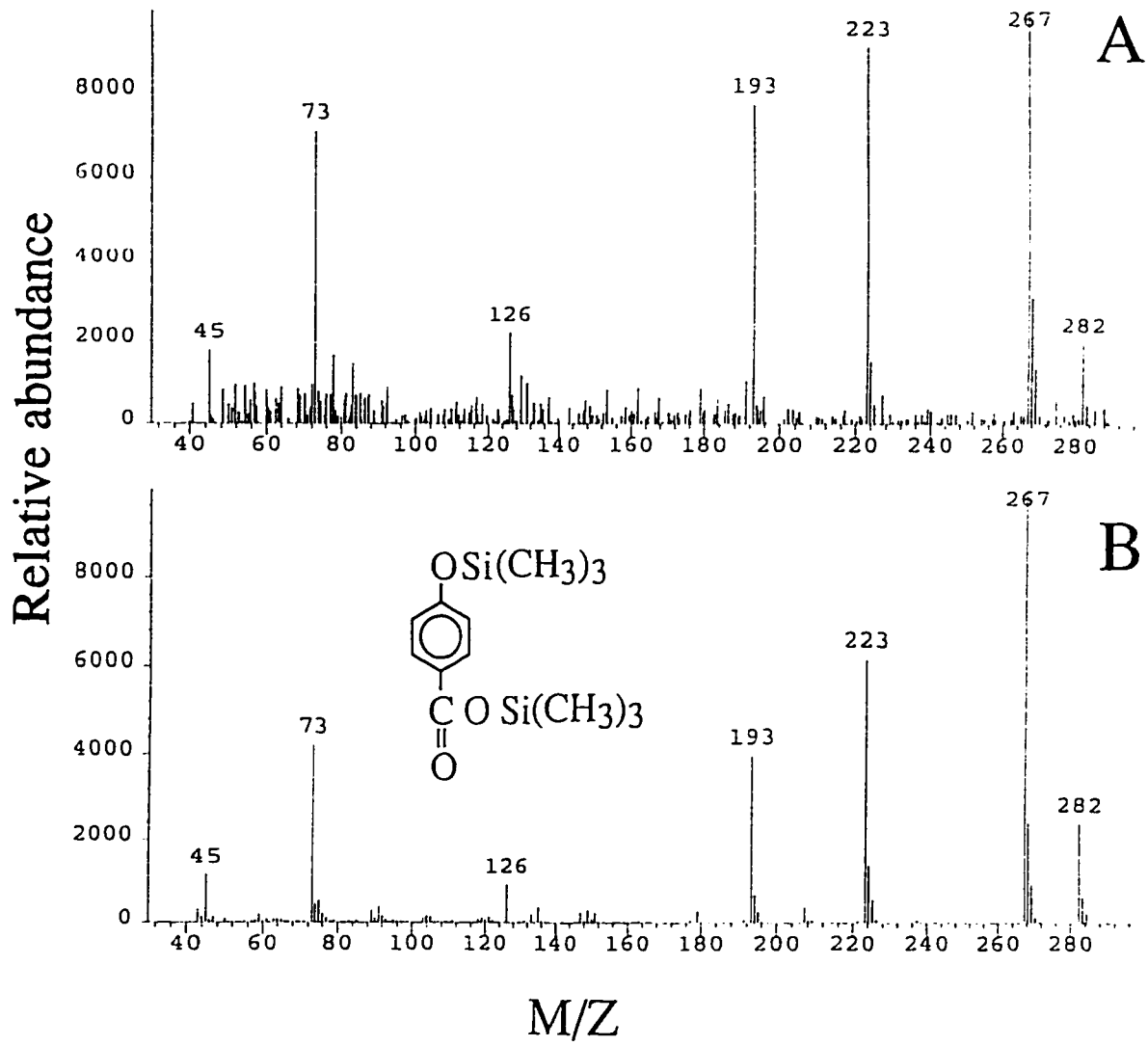

Fig. 3. From GC-MS analyses, mass spectra of a TMS-derivatized metabolite from *p*-cresol (A) and of the TMS derivative of *p*-hydroxybenzoic acid (B).

Another peak detected in extracts of culture fluids matched the retention time (10.45 min compared to 10.36 min) and spectrum (93%) of the TMS derivative of benzoate (Figure 4). Neither of these compounds were detected in extracts of butyrate-grown strain Groll, and *p*-hydroxybenzoate was not detected in cultures degrading benzoate or *m*-cresol.

When the proposed *p*-cresol metabolites were added as substrates with equimolar concentrations of *p*-cresol, the proposed metabolites were degraded prior to *p*-cresol and in most cases caused a temporary inhibition of *p*-cresol degradation (Figure 5). The addition of *p*-hydroxybenzoate had no effect on *p*-cresol degradation, benzoate and *p*-hydroxybenzaldehyde retarded *p*-cresol degradation, and *p*-hydroxybenzyl alcohol prevented *p*-cresol degradation entirely. When added alone, *p*-hydroxybenzyl alcohol was metabolized, but in the presence of *p*-cresol or *m*-cresol it was only partly transformed and all degradation activity ceased. In contrast, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate did not affect *m*-cresol degradation when added in equimolar concentrations (not shown). Addition of *p*-cresol or *m*-cresol had no effect on the degradation of *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate, or benzoate (not shown).

Presence of enzyme activities responsible for cresol metabolism

Cell extracts prepared from cultures of strain Groll grown on *m*-cresol, *p*-cresol, benzoate, and butyrate were assayed for the presence of enzymatic activities required for the proposed initial transformation reactions of cresol metabolism. The ability of proposed substrates and metabolites to serve as electron donors for the reduction of DCPIP was assayed as a measure of the transformation activities present in the crude cell extracts. The results are summarized in Table 1.

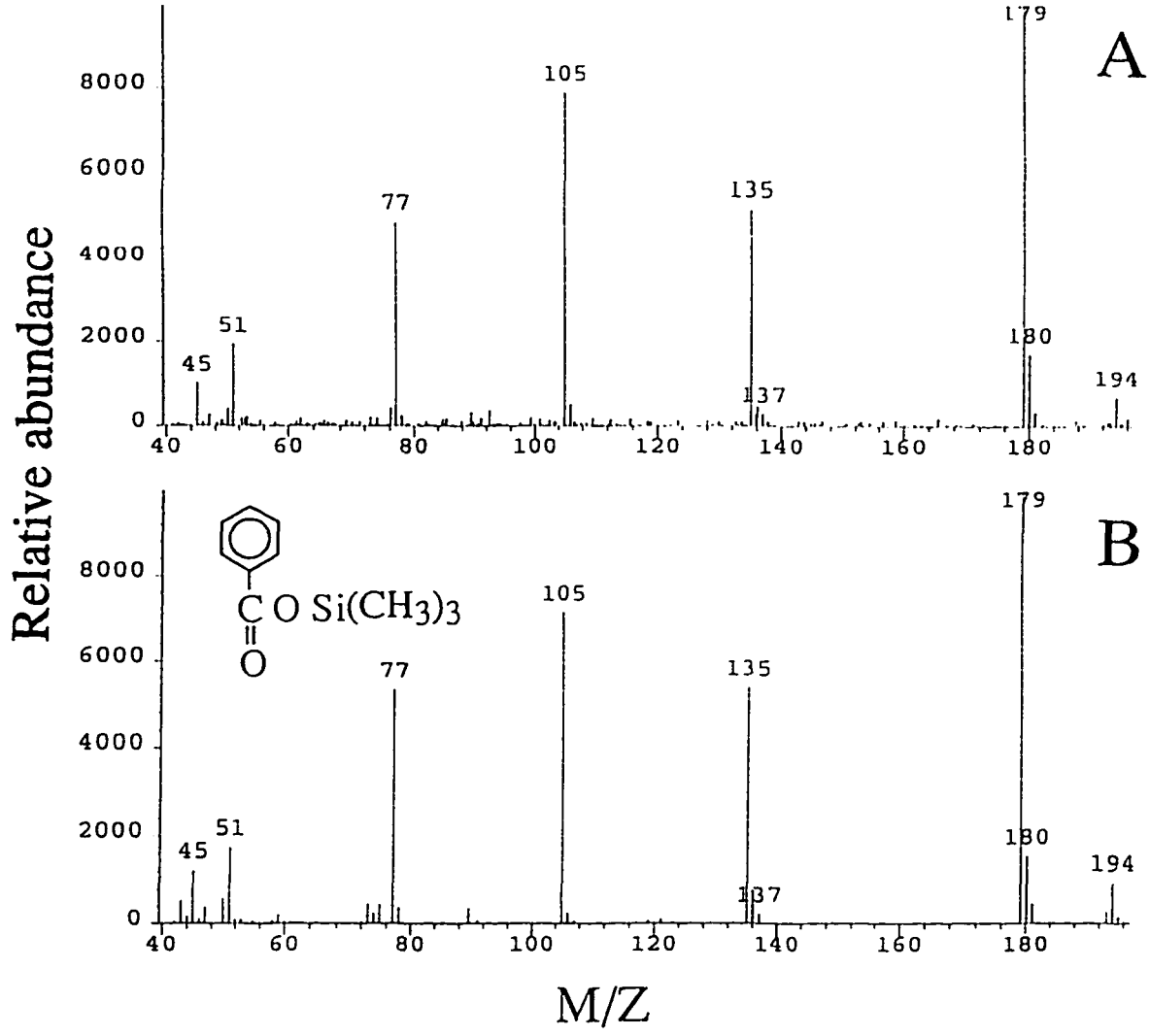

Fig. 4. From GC-MS analyses, mass spectra of a TMS-derivatized metabolite from *p*-cresol (A) and of the TMS derivative of benzoic acid (B).

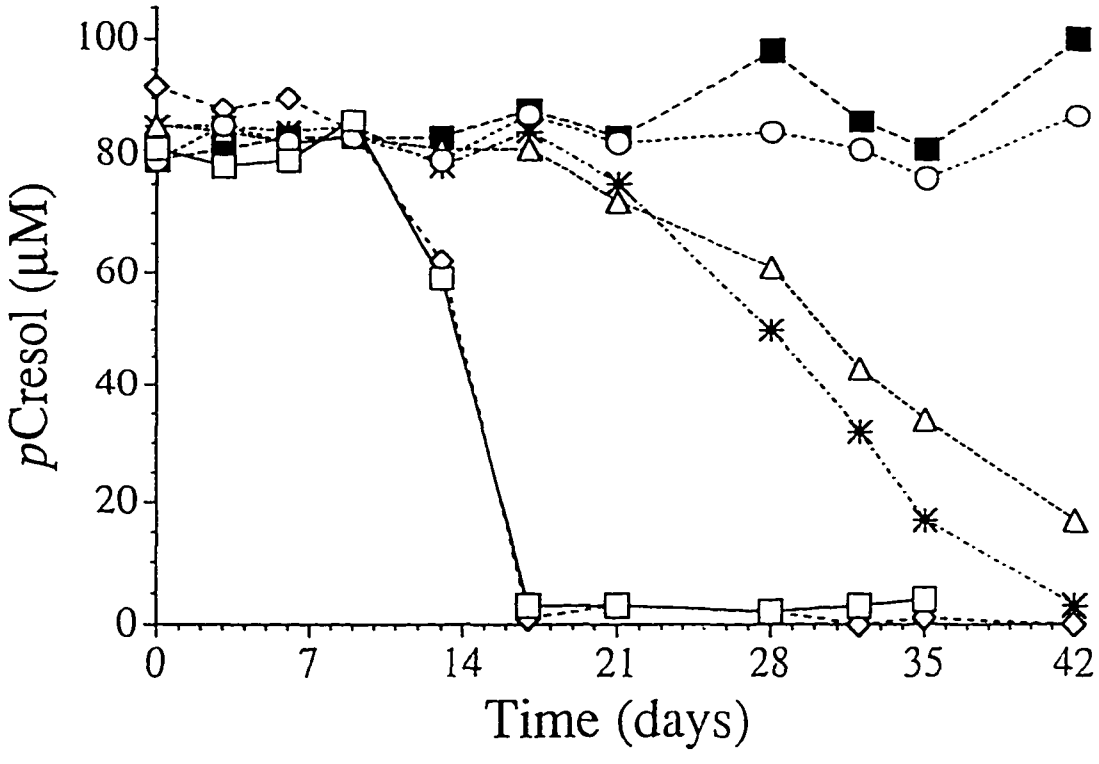

Fig. 5. Inhibition of *p*-cresol degradation by the addition of equimolar amounts of proposed *p*-cresol metabolites. *p*-Cresol only (□), with *p*-hydroxybenzyl alcohol (○), with *p*-hydroxybenzaldehyde (Δ), with *p*-hydroxybenzoate (◊), with benzoate (*), autoclaved control (■).

Table 1 Oxidation of aromatic compounds coupled with DCPIP reduction (μmol min^{-1} (mg protein)$^{-1}$) by cell extracts of strain Groll grown on different substrates.

Assay substrate	Growth substrate			
	butyrate	benzoate	*m*-cresol	*p*-cresol
o-cresol	0.05	0.29	BDL	BDL
o-hydroxybenzoate	BDL*	BDL	BDL	BDL
m-cresol	BDL	BDL	0.26	0.26
m-hydroxybenzyl alcohol	BDL	BDL	0.24	0.20
m-hydroxybenzaldehyde	0.09	0.26	0.62	BDL
m-hydroxybenzoate	0.16	BDL	0.63	0.33
p-cresol	BDL	BDL	0.31	1.05
p-hydroxybenzyl alcohol	0.09	BDL	BDL	0.71
p-hydroxybenzaldehyde	0.07	0.24	0.13	0.57
p-hydroxybenzoate	0.18	0.63	BDL	0.79
benzoate	0.27	1.46	BDL	0.21
benzoyl-CoA	0.05	0.48	0.45	0.99
toluene	0.07	BDL	BDL	BDL
detection limit	0.01	0.11	0.04	0.09

*BDL= below detection limit

Toluene, included as a negative control, was not used as a substrate for the reaction and with all but butyrate-grown cell extracts inhibited DCPIP reduction compared to substrate-unamended controls. Neither *o*-cresol nor *o*-hydroxybenzoate served as substrates since rates of DCPIP reduction were at or below detection limits for all of the cell extracts, consistent with the lack of degradation of these compounds in whole cell studies.

Enzymatic activities measured were quite low with extracts of cells grown on butyrate. DCPIP reduction with *m*-cresol, *p*-cresol, or *m*-hydoxybenzyl alcohol was below the detection limit, and rates with *p*-hydroxybenzyl alcohol, and the *m*- and *p*-isomers of hydroxybenzaldehyde and hydroxybenzoate were all less than 0.2 μmol min^{-1} mg^{-1}. There was somewhat greater reduction of DCPIP with benzoate as a substrate, but very little activity with benzoyl-CoA. Similar results were obtained with benzoate-grown cell extracts. Enzyme activities with *m*- and *p*-cresol, *m*- and *p*-hydroxybenzyl alcohol, as well as *m*-hydroxybenzoate were below the detection limit, and rates with *m*- and *p*-hydroxybenzaldehyde were not much greater. There was substantially more reduction of DCPIP with *p*-hydroxybenzoate and especially benzoate as reaction substrates, although once again activity with benzoyl-CoA was less than with benzoate. With the exception of benzoate, and perhaps *p*-hydroxybenzoate, these substrates were not readily used in this assay by cell extracts if strain Groll was grown on butyrate or benzoate.

Cell extracts prepared from*m*-cresol grown cells catalyzed the highest rates of DCPIP reduction with the *m*-cresol series of metabolites as substrates, and all four activities were detected at rates which could account for the rate of *m*-cresol degradation by whole cells. Rates of DCPIP reduction ranged from 0.63 to 0.24 μmol min^{-1} mg^{-1} for *m*-hydroxybenzoate and *m*-hydroxybenzyl alcohol respectively. The activity with *p*-cresol was very similar to *m*-cresol whereas the activities with the other *para*-substituted compounds was near or below the detection limit. In contrast to the previous cell extracts, those from *m*-cresol-grown cells had very little activity with benzoate yet were quite active with benzoyl-CoA. By comparison, cell extracts prepared from*p*-cresol-grown cells

catalyzed similar rates of DCPIP reduction with *m*-cresol series of compounds as reaction substrates, as for *m*-cresol-grown cell extracts. Nevertheless, in these *p*-cresol grown cell extracts, rates of DCPIP reduction with the *m*-cresol series of metabolites were less than with the *p*-cresol series of metabolites. All four *para*-substituted compounds tested served as substrates for DCPIP reduction, and the rates measured could account for the rate of *p*-cresol degradation in whole cell cultures. In addition, significant DCPIP reduction was observed with benzoyl-CoA as substrate although much less so with benzoate, just as for *m*-cresol-grown cells. The favorable reduction of benzoyl-CoA in these extracts suggests the involvement of benzoyl-CoA in the metabolism of cresols, so CoA ligase activities were also determined for cell extracts.

Role of CoA in degradation of cresols

Benzoate and the three isomers of hydroxybenzoate were tested as substrates for CoA ligase reactions using cell extracts of strain Groll grown under different conditions (Table 2). With butyrate-grown cells, slight benzoyl-CoA ligase activity was detected, but all benzoyl- and hydroxybenzoyl-CoA ligase activities were below the relatively high detection limit of 4.1 nmol min^{-1} mg^{-1}. In contrast, benzoate-grown cells had detectable activity with benzoate and even some activity with *m*-hydroxybenzoate as reaction substrate, although the other hydroxybenzoate isomers gave activities below the detection limit of 1.3 nmol min^{-1} mg^{-1}. *m*-Cresol-grown cells had the most CoA ligase activity with benzoate and some activity with *p*-hydroxybenzoate, but with *o*- and *m*-hydroxybenzoate, CoA ligase activities were below the detection limit (2.6 nmol min^{-1} mg^{-1}). Significant activities with benzoate, *m*- *p*-hydroxybenzoate were observed with *p*-cresol-grown cells, although activity with *o*-hydroxybenzoate was once again below the detection limit, 2.2 nmol min^{-1} mg^{-1}.

Table 2 CoA ligase activities (nmol min^{-1} (mg protein)$^{-1}$) for cell extracts of strain Groll grown on different substrates.

Assay substrate	Growth substrate			
	butyrate	benzoate	*m*-cresol	*p*-cresol
benzoate	BDL*	7.2	45.8	27.1
o-hydroxybenzoate	BDL	BDL	BDL	BDL
m-hydroxybenzoate	BDL	3.8	BDL	10.5
p-hydroxybenzoate	BDL	BDL	6.2	6.1

*BDL= below detection limit

Comparing extracts, the highest activities of benzoyl-CoA ligase were in *m*-cresol and *p*-cresol grown cells. CoA ligase activity was always greater for benzoate than any of the hydroxybenzoate isomers. The least activity was observed with *o*-hydroxybenzoate regardless of growth substrate, and in all cases activity was below detection limits. This was consistent with the lack of degradation of this compound by whole cells. *m*-Hydroxybenzoate and *p*-hydroxybenzoate were used as substrates for the CoA ligase reactions but the rates did not directly correlate with growth substrate. Benzoate-grown cells had significant activity with *m*-hydroxybenzoate but almost no activity with *p*-hydroxybenzoate, which is opposite to the dehydrogenase activities in which this extract had activity for *p*-hydroxybenzoate but not *m*-hydroxybenzoate. *m*-Cresol- and *p*-cresol-grown cells both had activity for both *m*-hydroxybenzoate and *p*-hydroxybenzoate, and although the differences between the rates were modest, the activity was higher for the opposite isomer of assay substrate from growth substrate.

Discussion

The sulfate-reducing bacterium *Desulfotomaculum* strain Groll degrades two of the three cresol isomers. *p*-Cresol was degraded faster than *m*-cresol, whereas *o*-cresol was not metabolized at all. Even *o*-hydroxybenzoate was not attacked by whole cells, and neither *o*-cresol nor *o*-hydroxybenzoate were substrates for DCPIP reduction or CoA ligase activities, even in cells that actively degraded *m*-cresol or *p*-cresol. The recalcitrance of *o*-cresol is consistent with previous reports that indicate that *o*-cresol is depleted slower than the other cresols, or not at all, in microcosms and enrichment cultures under a variety of anaerobic conditions (Londry and Fedorak 1992). Strain Groll appears to lack the ability to transform any aromatic compound with substituents in *ortho*-positions other than catechols (Kuever et al. 1993, Chapter 1).

The pathway proposed for *m*-cresol degradation is illustrated in Figure 6, which summarizes all the reactions investigated. Previous studies had indicated evidence for a

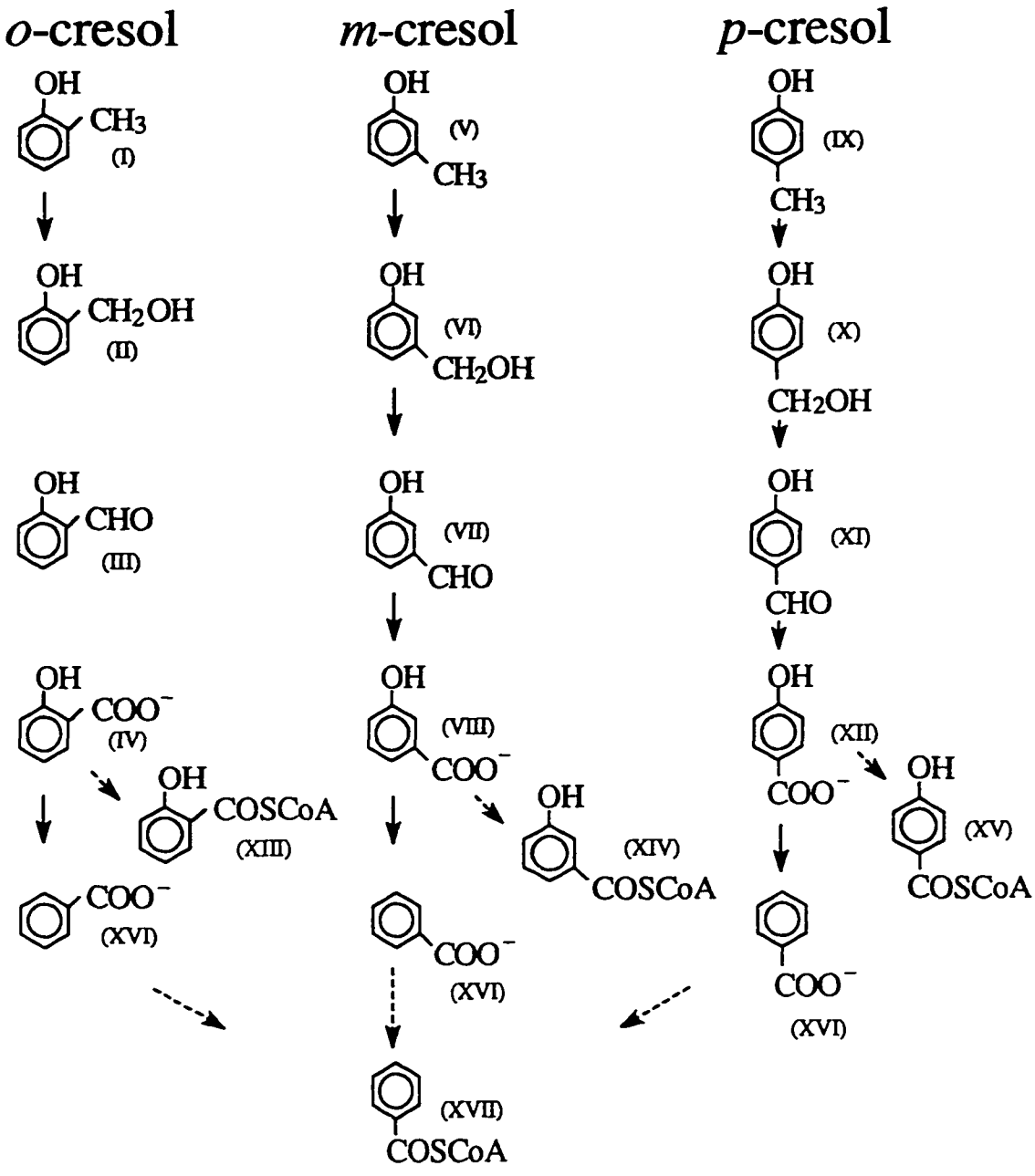

Fig. 6. Proposed reactions for the initial transformation of cresol isomers by strain Groll. Compounds: (I) *o*-cresol, (II) *o*-hydroxybenzyl alcohol, (III) *o*-hydroxybenzaldehyde, (IV) *o*-hydroxybenzoate, (V) *m*-cresol, (VI) *m*-hydroxybenzyl alcohol, (VIII) *m*-hydroxybenzaldehyde, (VIII) *m*-hydroxybenzoate, (IX) *p*-cresol, (X) *p*-hydroxybenzyl alcohol, (XI) *p*-hydroxybenzaldehyde, (XII) *p*-hydroxybenzoate, (XIII) *o*-hydroxybenzoyl-CoA, (XIV) *m*-hydroxybenzoyl-CoA, (XV) *p*-hydroxybenzoyl-CoA, (XVI) benzoate, (XVII) benzoyl-CoA. Hydroxylation, dehydroxylation, and dehydrogenase reactions are indicated by solid arrows, CoA ligase reactions are indicated by dashed arrows.

methyl group oxidation pathway in which *m*-cresol is hydroxylated to *m*-hydroxybenzyl alcohol by *m*-cresol methyl hydroxylase, *m*-hydroxybenzyl alcohol is oxidized by *m*-hydroxybenzyl alcohol dehydrogenase to *m*-hydroxybenzaldehyde, which is further oxidized to *m*-hydroxybenzoate by *m*-hydroxybenzaldehyde dehydrogenase, then *m*-hydroxybenzoate is dehydroxylated to benzoate by *m*-hydroxybenzoate dehydroxylase. Each of the *meta*-substituted metabolites served as substrates for the reduction of DCPIP in assays with cell extracts of strain Groll grown on *m*-cresol (Table 1) at rates that account for the overall conversion of *m*-cresol by whole cells. Furthermore, CoA ligase activities have been detected in cell extracts of this organism that indicate that benzoate is converted to benzoyl-CoA, and that benzoyl-CoA is the preferred substrate for further reduction. *m*-Cresol, *p*-cresol, and benzoate are apparently mineralized, as evidenced by sulfate reduction stoichiometry, but the reactions involved in the further metabolism of benzoyl-CoA have not been investigated.

m-Cresol metabolism in this sulfate-reducing bacterium is similar to the nitrate-reducing isolate, strain S100 (Bonting et al. 1995). Initial evidence for strain S100 had indicated this pathway was not used, as the predicted metabolites were not degraded by this organism and the specific dehydrogenase activities could not be detected (Tschech and Fuchs 1987, Rudolphi et al. 1993). However, Bonting et al. (1995) subsequently reported the degradation of all the proposed metabolites except 3-hydroxybenzyl alcohol. Bonting et al. (1995) also reported detection of 3-hydroxybenzyl alcohol dehydrogenase, 3-hydroxybenzaldehyde dehydrogenase, 3-hydroxybenzoate CoA ligase, benzoate-CoA ligase, and benzoyl-CoA reductase activities, although *m*-cresol methyl hydroxylase activity was not detected. We have discovered that cell extracts of strain Groll catalyze DCPIP reduction with all the predicted metabolites in the conversion of *m*-cresol to benzoyl-CoA, and at higher rates than reported for strain S100. Like strain S100, the dehydrogenase activities in strain Groll were induced by growth on *m*-cresol and not by

growth on benzoate or non-aromatic substrates. However, unlike strain S100, strain Groll did not appear to have a CoA ligase specific for 3-hydroxybenzoate.

The metabolic pathway proposed for *p*-cresol degradation is also illustrated in Figure 6. Each of the proposed methyl-group oxidation metabolites is readily degraded by strain Groll (Figure 1) and all except *p*-hydroxybenzoate retard *p*-cresol degradation. *p*-Hydroxybenzoate and benzoate were detected as TMS derivatives in extracted *p*-cresol-degrading cultures by GC-MS (Figure 4, 5); *p*-hydroxybenzoate was detected in *p*-hydroxybenzaldehyde-degrading cultures, and both *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate were detected as metabolites in *p*-hydroxybenzyl alcohol-degrading cultures using HPLC techniques. In addition to the whole cell studies, the proposed pathway is also supported by detection of the enzymatic activities in cell extracts of *p*-cresol-grown cells (Table 1). Similarly, CoA ligase activities have been detected that convert benzoate to benzoyl-CoA which is a substrate for further transformation and eventually mineralization. The metabolism of *p*-cresol by methyl group oxidation through benzoyl-CoA by sulfate-reducing strain Groll is consistent with the metabolism of *p*-cresol by other anaerobic microorganisms and in microcosms.

Benzoyl-CoA ligase activity was detected in cell extracts of strain Groll regardless of growth substrate. *m*-Hydroxybenzoate and *p*-hydroxybenzoate were used as substrates for the CoA ligase reactions but the rates did not directly correlate with growth substrate, and the rates were less than the rates for benzoate. This suggests that the addition of CoA probably occurs predominantly after the substrates have been converted to benzoate by a dehydroxylation reaction. CoA ligase activities appear to be induced, for the most part, by growth on aromatic substrates. The evidence does not preclude alternative mechanisms such as hydroxybenzoate thioesterification and hydroxybenzoyl-CoA dehydroxylation to form benzoyl-CoA. However, the relative rates of dehydrogenase and CoA ligase activities supports the proposed mechanism. The involvement of CoA transferases can not be

excluded, particularly for the metabolism of benzoate when it is added as an abundant substrate rather than formed as a trace intermediate.

As Figure 6 indicates, *m*-cresol and *p*-cresol are metabolized by this organism by a comparable series of reactions that lead to the formation of benzoyl-CoA. However, several lines of evidence indicate that the metabolism of these two isomers is separate and distinct. First, degradation of *p*-cresol is faster, and occurs after a shorter lag period than *m*-cresol, indicating that the first reaction, catalyzed by methylhydroxylase(s) was not induced to the same extent by both substrates, or was catalyzed by enzymes with different affinities for the two isomers. Second, the pattern of inhibition caused by the addition of proposed metabolites to cresol-degrading cultures differed. In general, *m*-cresol intermediates inhibited *m*-cresol metabolism, *p*-cresol metabolites inhibited *p*-cresol degradation, but *p*-cresol metabolites did not affect *m*-cresol utilization. Third, the accumulation of metabolites in cultures degrading proposed metabolites of *m*-cresol and *p*-cresol was different, which would not be expected if the same enzymes were responsible for transformation of both series of metabolites. *m*-Hydroxybenzyl alcohol-degrading cultures transiently accumulated *m*-hydroxybenzoate (Londry et al. submitted), whereas *p*-hydroxybenzyl alcohol-degrading cultures transiently accumulated both *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate (Figure 3A). No metabolites were observed from *m*-hydroxybenzaldehyde whereas *p*-hydroxybenzoate was observed in cultures degrading *p*-hydroxybenzaldehyde (Figure 3B). The sensitivity of strain Groll to the proposed intermediates was also dependent on the position of various function groups, as *p*-hydroxybenzyl alcohol and *m*-hydroxybenzaldehyde were inhibitory. Inhibition by aromatic alcohols has been reported for a number of organisms for which such compounds are proposed metabolites (Rudolphi et al. 1991). Fourth, enzymatic reduction of DCPIP was greatest with proposed *m*-cresol metabolites with cell extracts of strain Groll grown on *m*-cresol, whereas the proposed*p*-cresol metabolites led to the greatest rates of DCPIP reduction with *p*-cresol-grown cell extracts. Therefore, it is clear that although *m*-cresol

and *p*-cresol are degraded by chemically analagous pathways, the metabolism of these isomers is enzymatically separate and distinct. Purification and characterization of each enzyme involved in the two pathways would further clarify the biochemical relationship between the various isomers.

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Chapter 3.

ANAEROBIC BIODEGRADATION OF *n*-ALKANES, *n*-ALKANOLS, AND *n*-ALKANOIC ACIDS

Abstract

The fate of *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids under nitrate-reducing, sulfate-reducing, and methanogenic conditions was investigated. A multi-phase incubation system was utilized in which substrates were introduced as a mixture of compounds ten to twenty carbons in length in an organic overlay of heptamethylnonane. Oily sludge served as an inoculum and autoclaved incubations served as sterile controls. *n*-Alkanes were not degraded under any condition. *n*-Alkanol degradation was observed under all three conditions after an initial adaptation period of appoximately six weeks. Consumption of *n*-alkanols was associated with reduction of electron acceptors or methanogenesis. Susceptibility to degradation depended on chain length, as shorter *n*-alkanols were degraded faster and to a greater extent than longer *n*-alkanols. In separate incubations, decanol, undecanol, and dodecanol were oxidized to the corresponding alkanoic acids, which accumulated in the medium under sulfate-reducing conditions. Undecanol and *n*-alkanoic acids could further react to form higher molecular weight esters. *n*-Alkanoic acids were degraded under nitrate- and sulfate-reducing conditions, but not under methanogenic conditions at the concentrations tested. These results suggest that the initial aerobic oxidation products of *n*-alkane metabolism are more amenable to anaerobic decay than the parent hydrocarbons.

Introduction

Oily wastes associated with petroleum extraction, refining, transportation, utilization, and disposal are often released accidentally or intentionally into the environment. If released on land, these wastes can enter the subsurface where the readily degradable components will be metabolized by microorganisms at the expense of the available oxygen reserves. If released in aqueous environments, oily wastes will eventually deposit in sediments, which also quickly become anoxic. The type of anaerobic conditions that develop will depend on the availability of electron acceptors such as nitrate, iron, sulfate, or carbon dioxide, which in turn affects the susceptibility of components in the oily wastes to biodegradation. An understanding of the fate of petroleum compounds in anaerobic environments is necessary in order to predict the impact of anaerobes on all aspects of the oil industry including prospects for intrinsic bioremediation of hydrocarbon components and the feasibility of anaerobic treatment processes for oily wastes.

Intermittent periods of oxygenation of oils could result in partial oxidation of *n*-alkanes by aerobic oil-degrading bacteria, releasing *n*-alkanols and *n*-alkanoic acids into anaerobic environments. Jobson et al. (1979) have shown that products released from aerobic degradation of oil supported growth of sulfate-reducing bacteria, although the nature of these products was not determined. In most cases, the aerobic degradation of *n*-alkanes is initiated by the introduction of molecular oxygen at the terminal carbon to yield *n*-alkanols, which are further oxidized to the corresponding *n*-alkanoic acids and degraded by a series of ß-oxidation reactions (Watkinson and Morgan 1990). Studies of long-chain fatty acids have indicated that these compounds are degraded under anaerobic conditions (Roy et al. 1986, Lorowitz et al. 1989, Angelidaki and Ahring 1995), but little work in this regard has been done with *n*-alkanols. Similarly, the mechanism for anaerobic degradation of *n*-alkanes has not been determined, as alkane-degrading sulfate-reducing bacteria have only recently been isolated (Aekersberg et al. 1991, Rueter et al. 1994). One of these organisms, Hxd3, was reported to degrade hexadecane, *n*-hexadecanol and *n*-hexadecanoic

acid, which suggests that the latter two compounds may be metabolites of *n*-alkanes under sulfate-reducing conditions. In addition, a wide range of free or bound *n*-alkanoic acids, *n*-alkanols, and *n*-alkanes are released from decomposing matter, including microorganisms (Lichtfouse et al. 1995).

We have investigated the biodegradation and biotransformation of a range of *n*-alkanes and the corresponding terminal *n*-alkanols and *n*-alkanoic acids under methanogenic, sulfate-reducing, and nitrate-reducing conditions. In our studies, *n*-alkanols and *n*-alkanoic acids were susceptible to anaerobic degradation whereas the *n*-alkanes were not transformed. Our results indicated that environmental factors such as availability of electron acceptors, as well as structural features of the substrates, affect both the rate and extent of *n*-alkanol degradation under anaerobic conditions. Furthermore, we report transformation of *n*-alkanols to alkanoic acids, and present evidence for esterification reactions as an alternative fate process for partially oxidized hydrocarbons.

Materials and methods

Inoculum and incubation conditions

Oily sludge used as inoculum was collected from a settling tank (Tank #63) at the U.S. Navy Craney Island Fuel Depot in Portsmouth, VA, and stored in sealed glass bottles at room temperature. The sludge (0.5 ml) was added to serum bottles (25 ml) containing a brackish medium designed for the cultivation of sulfate-reducing bacteria (Widdel and Bak 1992). The medium was amended as needed with sterile anoxic solutions of sodium sulfate, sodium nitrate, or no electron acceptors other than CO_2 (methanogenic conditions). Resazurin (0.0002%) and sodium sulfide (1 mM) were added as redox indicator and reductant, respectively. The medium was dispensed into serum bottles, each culture was inoculated, and bottles were sealed with a composite stopper (Mormile and Suflita 1996) while inside an anaerobic chamber. The stoppers were secured with aluminum crimp seals and the headspace of the serum bottles was adjusted to N_2/CO_2 (80:20). Sterile controls

were autoclaved and positive controls were amended with sodium lactate (10 mM unless otherwise indicated) from a neutralized sterile anoxic stock solution. Strict anaerobic technique was used at all times. Cultures were incubated in the dark at room temperature without shaking. Samples of the aqueous phase were periodically withdrawn by syringe and stored frozen until analyzed. Prior to analysis, the samples were thawed and centrifuged (10 000 x g) for 5 min to remove particulate debris.

Initial toxicity study

A mixture of *n*-alkanes 10 to 20 carbons long was prepared by adding each compound to a serum bottle, flushing the bottle with N_2, and autoclaving the mixture. The solution was heated to fluidity and added to cultures by syringe to give either 2 µmol or 20 µmol of each compound per 10 ml culture. Mixtures of *n*-alkanols or *n*-alkanoic acids were prepared and added to cultures the same way. Dodecane, undecanol, and hexadecanoic acid were added separately to 2,2,4,4,6,8,8 heptamethylnonane (HMN) which was thoroughly sparged with N_2. The HMN mixtures were added to cultures by syringe to give 500 µmol dodecane or undecanol, or 20 µmol hexadecanoic acid per 10 ml culture. All cultures were prepared in duplicate and contained 14 mM lactate and 13 mM sulfate, positive controls received lactate but no HMN or compound mixtures.

Alkanol transformation experiments

Undecanol or hexadecanol were added as sterile, anoxic HMN solutions at 250 µmol per 15 ml of medium containing 20 mM sulfate without lactate. Incubation mixtures were inoculated with spent culture fluid (0.5 ml) from one replicate of the initial toxicity screen. Triplicate cultures were compared with an autoclaved control for each substrate. In a subsequent experiment, decanol, undecanol, and dodecanol were added separately (100 µmol per culture in HMN) to triplicate cultures and sterile controls prepared as above except with 10 mM sulfate. Undecanol (100 µmol) was also added to duplicate cultures

and sterile controls which contained 100 µmol decanoic acid, undecanoic acid, or dodecanoic acid. All transfers (10 ml) were inoculated with spent culture fluid (0.5 ml) from a replicate which exhibited undecanol transformation.

Biodegradation survey

For biodegradation experiments, each of the eleven *n*-alkanes was dispensed into a serum bottle, HMN was added, the mixture was flushed with N_2, autoclaved, and 2.5 ml was added to each culture to give 100 µmol of each *n*-alkane per culture. *n*-Alkanol and *n*-alkanoic acid HMN mixtures were prepared the same way except that decanol was not included, and hexadecanol, octadecanol, nonadecanol, and eicosanol were added to give 50 µmol per culture for the *n*-alkanol mixture. Cultures (10 ml) were prepared as above and amended with nitrate (20 mM), sulfate (20 mM), or no additional electron acceptors (methanogenic). For each incubation condition, triplicate cultures and an autoclaved control were prepared with each of the *n*-alkane, *n*-alkanol, and *n*-alkanoic acid mixtures. In addition, a positive control amended with lactate and the *n*-alkanes mixture, another that received lactate only, and a negative control that contained HMN but neither lactate nor substrates, were prepared for each incubation condition. Substrate amounts in sterile controls were averaged from the single incubations established under each condition. Subsequent transfers (0.5 - 1 ml inoculum) received sterile, anoxic HMN solutions with *n*-alkanes or *n*-alkanols ten to sixteen carbons long and were incubated under nitrate-reducing, sulfate-reducing, or methanogenic conditions.

Analytical techniques

Substrates were analyzed by gas chromatography (GC) using a Hewlett Packard (Palo Alto, CA) 5890 series II GC with a flame ionization detector, equipped with a carbograph VOC capillary column (30 m, Alltech Associates, Inc., Deerfield, IL). The oven temperature programs were as follows: *n*-alkanes - an initial temperature of 40°C raised at

8°C/min to 200°C and held for 5 min; *n*-alkanols - initially 100°C raised at 8°C/min to 240°C for 12.5 min; and *n*-alkanoic acids - initially 200°C raised at 5°C/min to 240°C for 12 min. Tridecane could not be quantified due to chromatographic interference with HMN. Samples of the HMN organic overlay (1 μL) were removed from the cultures and injected directly without extraction or derivatization. For the initial toxicity experiments, the response of undecanol and products were expressed relative to an internal standard of hexadecane. For subsequent undecanol transformation experiments, the amount of alkanols and alkanoic acids were calculated from the ratio of the peak area of each alkanol to the peak area of external standards analyzed the same day. For GC-MS analysis, the Hewlett Packard GC was used with a 5970 mass selective detector and a DB-5 fused silica capillary column (30 m, J&W Scientific, Folsom, CA). For the biodegradation survey experiments, the *n*-alkanes, *n*-alkanols, or *n*-alkanoic acids eluted sequentially and the amounts were calculated by ratio of peak areas to standard solutions analyzed the same day.

Sulfate and nitrate were analyzed by ion chromatography using a Dionex DX 500 IC system (Dionex, Sunnyvale, CA) with an AS4A-SC 4 mm column, a CD20 conductivity detector, and a mobile phase of 1.8 mM Na_2CO_3 + 1.7 mM $NaHCO_3$ at 2 ml/min. Lactate was analyzed using a high performance liquid chromatography (HPLC) system (Beckman Instruments, Inc., Berkeley, CA) with an Aminex HPX-57H organic acid column (Bio-Rad Laboratories, Richmond, CA) with a mobile phase of 0.016N H_2SO_4 at 0.9 ml/min and UV detection (214 nm). Methane was analyzed with a Varian (Palo Alto, CA) 3300 GC with an FID detector, a 1.8 m x 0.32 cm 80/100 Porapak Q column, and N_2 as a carrier gas at a flow of 30 ml/min. The temperature of the injector, column, and detector were 100°C, 105°C, and 120°C, respectively.

Chemicals

n-Alkanes, *n*-alkanols, and *n*-alkanoic acids were obtained from Aldrich Chemical Co. (Milwaukee, Wis.), at least 97% pure, and were used without further purification. All fatty

alcohols and fatty acids used in this study had terminal hydroxyl or carboxyl groups so for simplicity they are collectively referred to as *n*-alkanols and *n*-alkanoic acids, respectively. HMN was obtained from Sigma Chemical Co. (St. Louis, MO). Undecanol esters were synthesized by adding undecanol (1 mmol), and decanoic, undecanoic, or dodecanoic acid (100 μmol), concentrated sulfuric acid (0.1 ml), and anhydrous sodium sulfate (0.1 g, to adsorb water) to HMN (2 ml) and heated to 80°C for 30 min.

Results

Effects of *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids on microbial activity

An initial toxicity evaluation was performed to determine whether mixtures of *n*-alkanes, *n*-alkanols, *n*-alkanoic acids, or individual compounds in the HMN carrier would inhibit microbial activity in the sludge. Lactate degradation, sulfate reduction, sulfide production, and methanogenesis did not occur in sterile controls (Table 1). Neither the HMN solutions nor substrate mixtures affected the rate or extent of lactate degradation or sulfate reduction compared to positive controls with lactate only. In most cases, sulfide accumulation and methanogenesis were likewise unaffected (Table 1). However, the high concentration alkanoic acid mixture inhibited sulfide formation and the small amount of methanogenesis that occurred even in the presence of sulfate (Table 1). While hexadecanoic acid degradation was suggested by the >50% decrease of this substrate from the HMN layer during 9 weeks incubation, this result could not be confirmed by other comparison of other parameters such as sulfate reduction or by the accumulation of metabolites.

Undecanol biotransformation

In the initial toxicity study, the amount of undecanol in the cultures declined during prolonged incubation, following lactate depletion. As the amount of undecanol decreased

Table 1. Effects of *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids on microbial activity in lactate-amended[a] oily sludge incubations.

Conditions	CH_4 (%)[b]	SO_4^{2-} (mM)[c]	S^{2-} (mM)[d]
Sterile control	0.01 ±0.00	0.00 ±0.18	1.72 ±0.01
Positive control	0.04 ±0.02	5.55 ±0.57	5.56 ±3.46
n-Alkanes			
mix (2 µmol each)	0.05 ±0.02	6.42 ±0.54	7.81 ±0.13
mix (20 µmol each)	0.05 ±0.01	7.28 ±0.66	11.84 ±6.43
dodecane (440 µmol)	0.03 ±0.01	6.75 ±0.59	6.48 ±0.38
n-Alkanols			
mix (2 µmol each)	0.04 ±0.01	9.01 ±2.86	11.18 ±5.10
mix (20 µmol each)	0.05 ±0.01	7.33 ±0.80	6.80 ±3.81
undecanol (480 µmol)	0.04 ±0.01	7.94 ±1.10	11.80 ±6.55
n-Alkanoic acids			
mix (2 µmol each)	0.05 ±0.02	5.87 ±0.31	7.55 ±1.54
mix (20 µmol each)	0.01 ±0.00	6.29 ±0.62	0.80 ±1.08
hexadecanoic acid (20 µmol)	0.02 ±0.00	6.80 ±0.37	5.67 ±0.21

[a] 14 mM lactate degraded in all cultures except sterile controls

[b] Amount of methane in cultures after 2 weeks incubation, expressed as per cent of headspace, ± one standard deviation for duplicates.

[c] Decrease in sulfate concentration over 2 weeks incubation

[d] Sulfide concentration after 2 weeks incubation

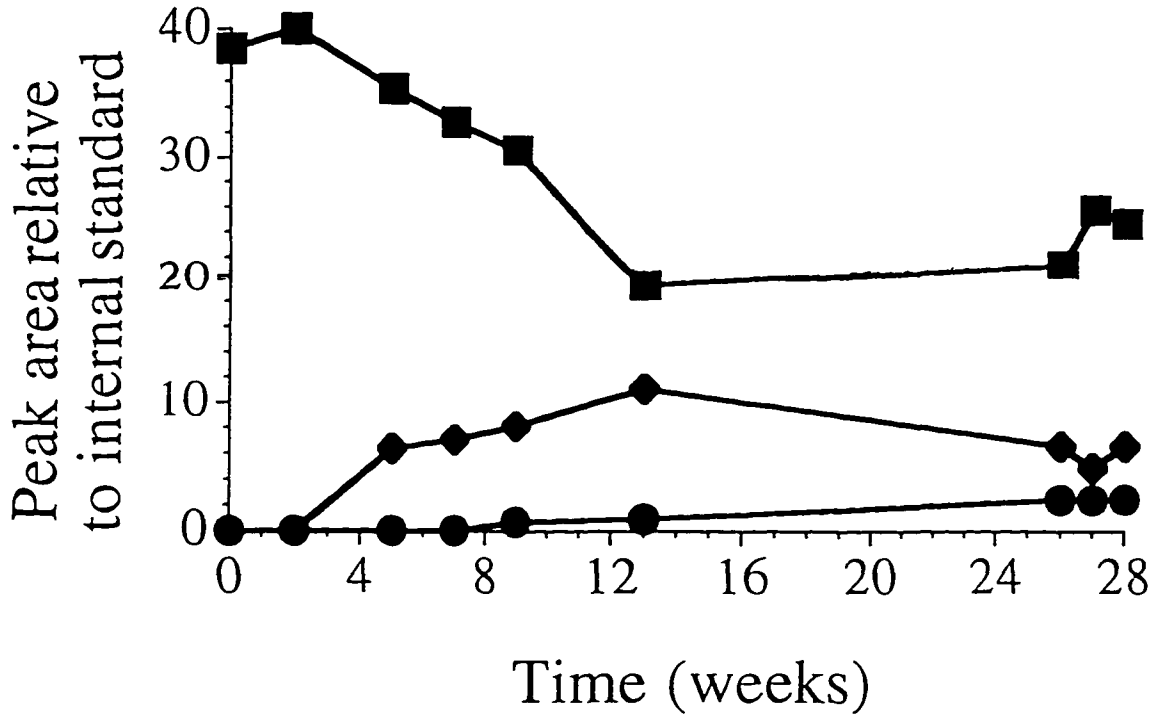

Fig. 1. Transformation of undecanol and accumulation of two products under sulfate-reducing conditions. Undecanol (■) depletion was associated with accumulation of two products identified as undecanoic acid (◆) and the ester of undecanol and undecanoic acid (●). Amounts are expressed as peak areas relative to an internal standard in the organic overlay.

in the cultures, two products accumulated (Fig. 1). The estimated amount of products in the cultures, based on a comparison of peak areas to standards of known concentration, were 212 μmol of one product, and 40 μmol of the other, compared with 290 μmol of undecanol remaining from the calculated amount of 480 μmol added initially. Undecanol transformation stopped after twelve weeks, presumably due to lack of sulfate. Of the 13.5 mM sulfate present at the start of the experiment, only 5.6 mM remained after 2 weeks and no sulfate was present when the cultures were analyzed after 26 weeks.

The first product to accumulate was identified by GC-mass spectrometry (GC-MS) as undecanoic acid. The retention time (6.13 min) and mass spectral profile of the compound (Fig. 2) matched authentic undecanoic acid in HMN (not shown). The second product was tentatively identified by the molecular ion and the fragmentation pattern as the undecanol ester of undecanoic acid. The retention time (17.1 min) of the ester and the mass spectral characteristics (Fig. 2) matched those of a synthesized authentic standard (not shown).

An undecanol-degrading culture was used as inoculum in transfers to confirm undecanol metabolism, to determine whether sulfate reduction was associated with the biotransformation, and to ascertain if hexadecanol could be similarly transformed. Undecanol (280 μmol) was depleted in the transfers within 24 weeks and undecanoic acid accumulated (32 μmol), whereas hexadecanol was not transformed within 24 weeks incubation. Compared to sterile controls and transfers containing hexadecanol, undecanol-degrading cultures consumed 0.35 mol sulfate per mol parent substrate depleted (6.1 mM sulfate). Transformation of undecanol was associated with the proliferation of at least four different cellular morphologies as observed by phase-contrast microscopy, as well as increased culture turbidity. Only a trace amount of the undecanol ester of undecanoic acid was detected in the transfers, but less of the parent substrate was potentially available for esterification. The corresponding aldehyde intermediate was not detected in any of our

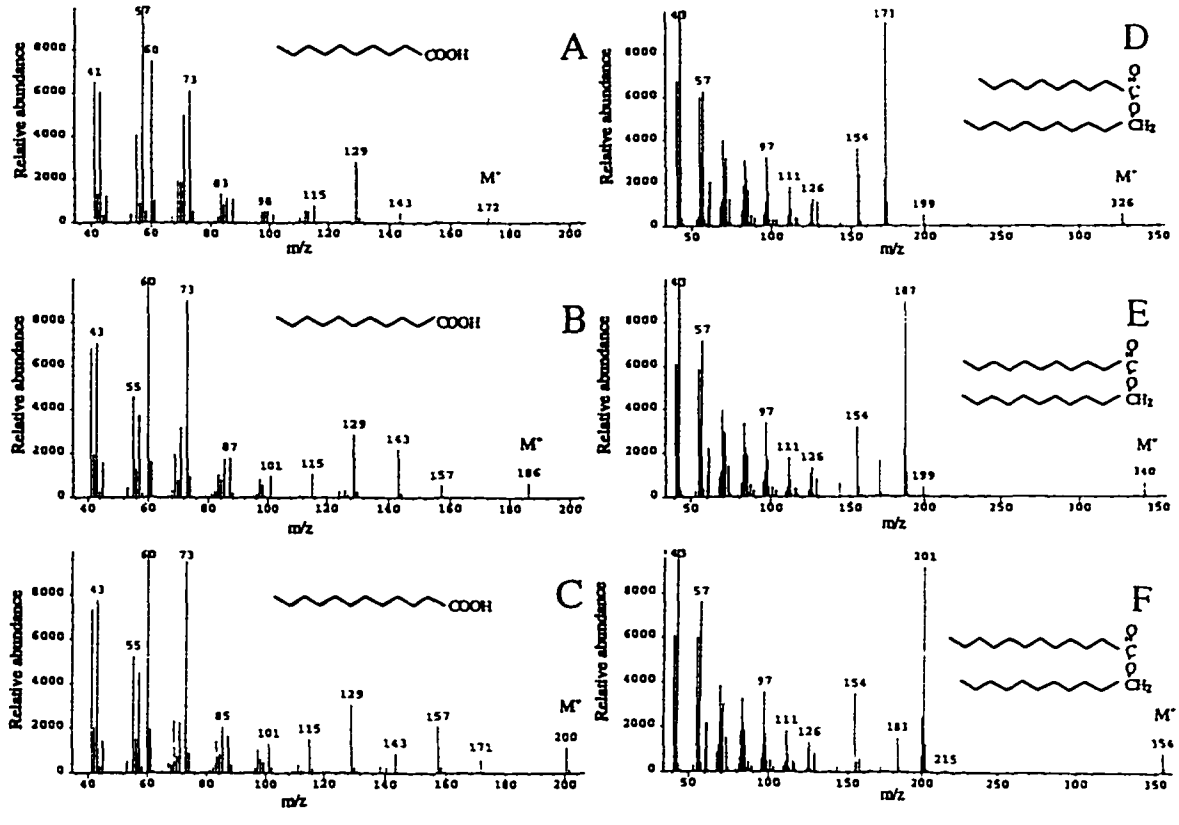

Fig. 2. Mass spectra of products formed by microbial metabolism of alkanols. Decane was oxidized to decanoic acid (A), undecane was oxidized to undecanoic acid (B), and dodecane was oxidized to dodecanoic acid (C). Undecanol esters of decanoic acid (D), undecanoic acid (E), and dodecanoic acid (F) were detected in cultures containing undecanol and the corresponding alkanoic acids. The molecular ions are indicated by M^+; relative abundances have been normalized.

experiments. Undecanol was not transformed in sterile controls, indicating that the oxidation was biologically catalyzed. Since undecanoic acid was not formed in sterile controls, conclusion as to whether the esterification reaction was biologically catalyzed was not possible.

A second series of transfers were established with decanol, undecanol, or dodecanol as parent substrates. All three alkanols were transformed at similar rates (5 μmol/week), with concomitant increase in culture turbidity, and no substrate depletion or sulfate reduction occurred in sterile controls. Transformation of the alkanols to alkanoic acids was associated with sulfate reduction. From equations [1] and [2] one would predict 0.50 mol sulfate reduced per mol alkanol oxidized.

$$CH_3(CH_2)_nCH_2OH + H_2O \rightarrow CH_3(CH_2)_nCOOH + 4\ H^+ + 4\ e^- \quad [1]$$

$$SO_4^{2-} + 8\ e^- + 8\ H^+ \rightarrow S^{2-} + 4\ H_2O \quad [2]$$

Consistent with previous results, about 0.37 mol of sulfate were reduced per mol alkanol oxidized, or about 75% of that theoretically expected. However, the theoretical value does not account for sulfur incorporation by cells, so the values obtained are in reasonably good agreement with the predicted stoichiometry. Oxidation of the alkanols was accompanied by formation of the corresponding alkanoic acid products. That is, decanoic acid, undecanoic acid, and dodecanoic acid accumulated in cultures with decanol, undecanol, and dodecanol respectively (Fig. 2). Products were identified by GC-MS by comparison to authentic standards and a spectral library. The esters of the corresponding alkanols and alkanoic acids were not detected in these transfers, presumably because the alkanols were completely transformed and not available for esterification.

To investigate the esterification process, decanoic, undecanoic, and dodecanoic acid were added to cultures along with undecanol. In each case, undecanol was oxidized to undecanoic acid which accumulated in the medium, although the reaction was retarded in the presence of decanoic acid (data not shown). However, in the presence of the abundant

(100 μmol) alkanoic acids, undecanol was also esterified to form the undecanol esters of decanoic acid, undecanoic acid, and dodecanoic acid (Fig. 2). These products were identified by their ion fragmentation patterns and by comparison of retention times and mass spectra to synthesized authentic standards, and to the spectrum in a library for the dodecanoic ester. Undecanol was not oxidized in sterile controls. However, trace amounts of the esters were detected in sterile controls after extended incubation (30 weeks), indicating that the esterification does occur abiotically at slow rates. There was no sulfate reduction associated with the esterification process, as the amount of sulfate consumed in these cultures was no greater than cultures that received only undecanol. Our results do not exclude an active role for microorganisms in catalyzing the formation of the esters, but abiotic transformation certainly occurred. The pH of all cultures after 30 weeks incubation was unchanged at 7.18 ± 0.10.

Biodegradation of *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids

For the biodegradation survey, substrates were added as mixtures to determine of the range of compounds potentially metabolized, and to better simulate the complex mixtures of components typically encountered in natural environments. Lactate-amended positive controls were included in the experiment to confirm microbial activity under each condition, and to test if the mixtures were inhibitory. Lactate was readily degraded under all incubation conditions (data not shown) and was associated with either methanogenesis, sulfate reduction, or nitrate reduction. The addition of the *n*-alkanes mixture to positive controls did not affect these microbial activities (methanogenesis depicted in Fig. 3). Negative controls with the HMN overlay but no substrate amendments did exhibit methanogenesis (1% in 30 weeks), sulfate reduction (30 mM between 18 and 50 weeks) and nitrate reduction (12 mM from 18 to 43 weeks), indicating the availability of electron donors in the oily sludge inoculum. However, the lag times associated with this

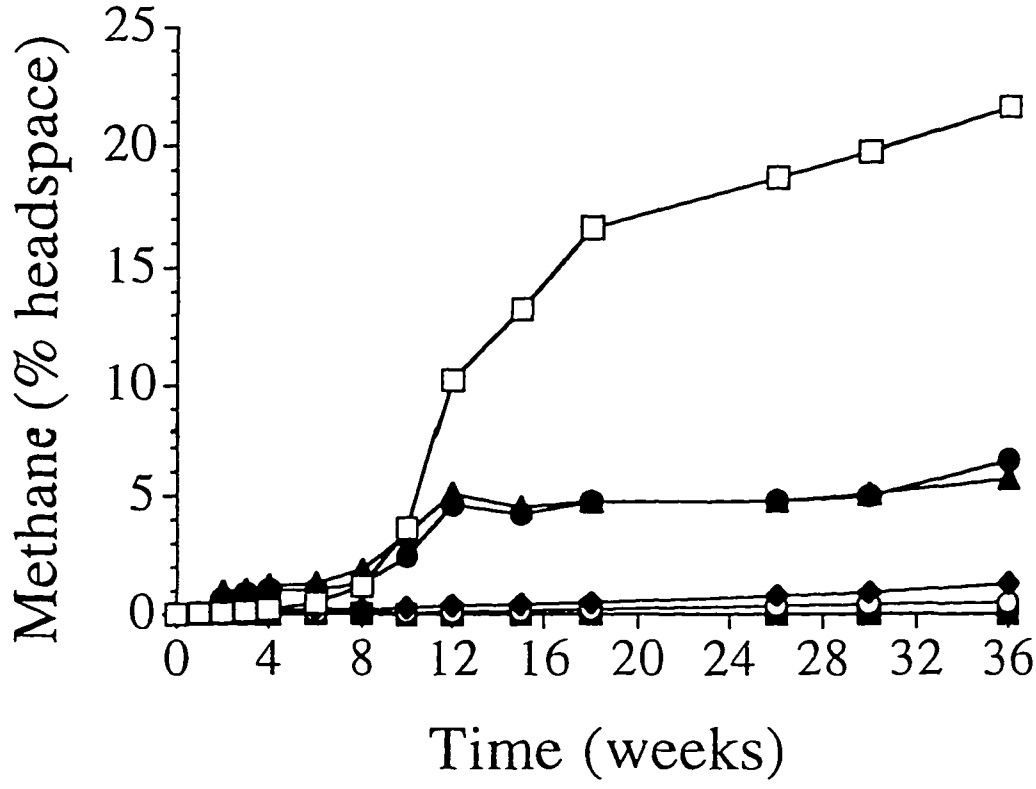

Fig 3. Methanogenesis in cultures from the biodegradation survey. Methane production in *n*-alkanol-degrading cultures (□) was greater than in lactate-degrading positive controls with (▲) and without (●) *n*-alkanes, a negative control with HMN without substrate (◆), cultures with *n*-alkanes (○), or sterile controls (■).

metabolism and rates of electron acceptor reduction did not interfere with the detection of degradation of the amended substrates. Under methanogenic conditions, cultures amended with *n*-alkanes accumulated slightly less methane than negative controls which did not receive substrate (Fig. 3). In addition, sulfate and nitrate concentrations remained unchanged in cultures with *n*-alkanes over 50 weeks incubation (data not shown). In all but the sterile controls, cultures that received nitrate turned pink after 2-4 weeks incubation, presumably due to oxidation of resazurin by nitrous oxide (Jenneman et al. 1986). Sterile controls with the various substrates exhibited no methanogenesis, sulfate reduction, nitrate reduction, or substrate decay.

n-Alkanes (C_{10}-C_{20}) were not degraded under any of the conditions tested. There was no evidence for *n*-alkane loss, no enhanced reduction of electron acceptors, no methane production over controls, and no increase in culture turbidity over a 50 week incubation period (data not shown). In contrast, *n*-alkanoic acids (C_{10}-C_{19}) were readily degraded under nitrate-reducing conditions, and to a lesser extent under sulfate-reducing conditions, as evidenced by a decrease in the amount of parent substrate, electron acceptor reduction, emulsification of the HMN overlay, increased culture turbidity, and a proliferation of microorganisms as observed microscopically. In alkanoic acid-degrading cultures, nitrate was reduced rapidly during the first two weeks, as were five subsequent additions of nitrate (10 mM) added over two week intervals. In sulfate-amended incubations, the sulfate was depleted in 16 weeks, and subsequent amendements were reduced at similar rates. In contrast, methane production in cultures containing *n*-alkanoic acids (data not shown) was identical to cultures containing *n*-alkanes, and comparable to negative controls.

The average decrease in the amount of the *n*-alkanoic acids is depicted in Fig. 4. After 10 weeks incubation, quantifying the substrates became unreliable for the nitrate-reducing cultures because of emulsification of the HMN layer due to microbial activity, yet

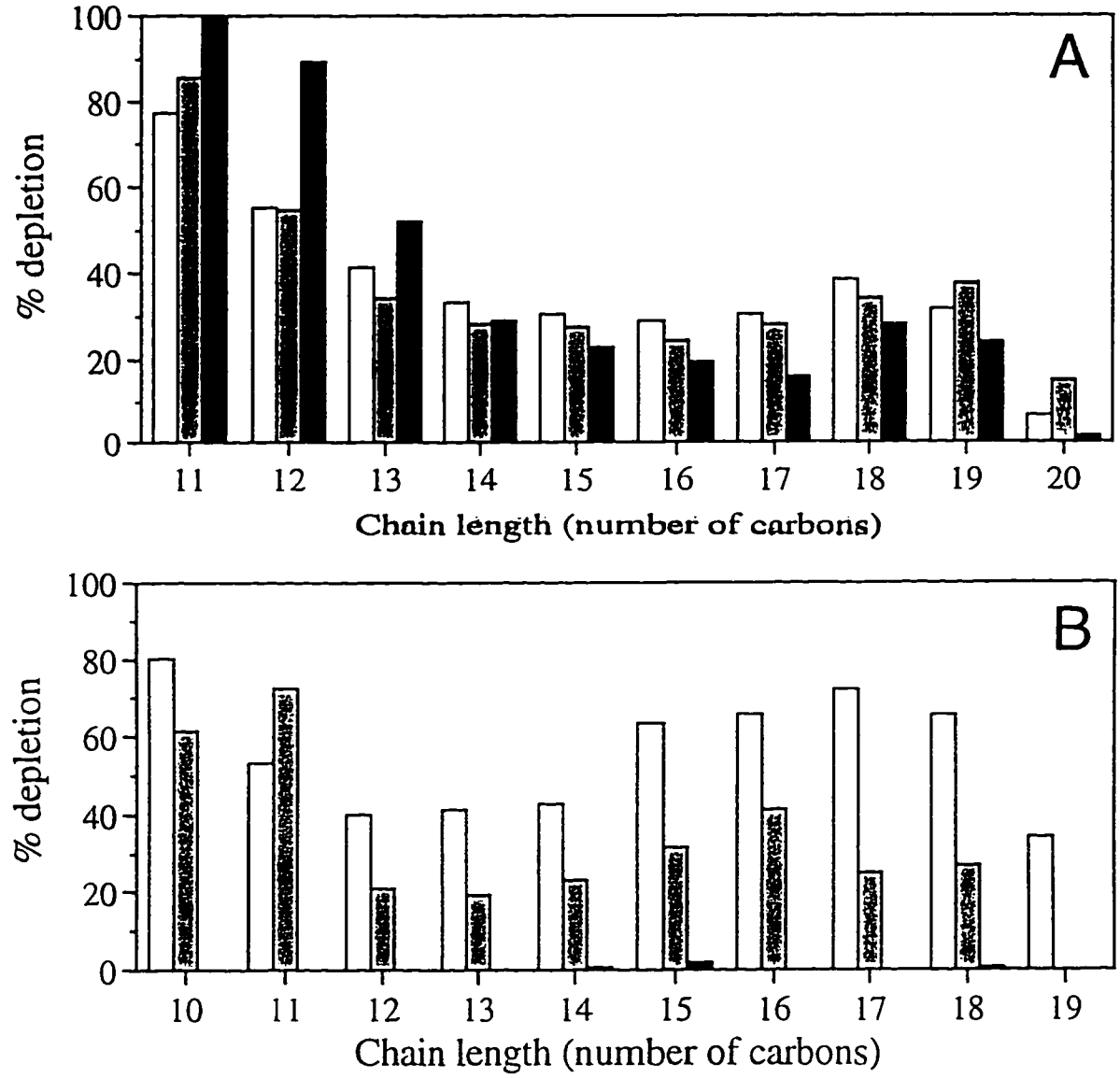

Fig 4. Profiles of *n*-alkanol (A) and *n*-alkanoic acid (B) biodegradation. Depletion of substrates under nitrate-reducing (white), sulfate-reducing (gray), and methanogenic conditions (black) was corrected for changes in sterile controls. Values for *n*-alkanols were calculated as percent depletion based on average values after 43 and 50 weeks incubation, *n*-alkanoic acid values were calculated after 10 and 30 weeks incubation under nitrate- and sulfate-reducing conditions, respectively. Eicosanoic acid (C_{20}) could not be accurately quantified.

the decrease in substrate concentration was greater under nitrate-reducing conditions after 10 weeks than under sulfate-reducing conditions after 30 weeks (Fig. 4). The lag period prior to the onset of *n*-alkanoic acid degradation was 2 weeks under nitrate-reducing conditions, and from 4 to 24 weeks under sulfate-reducing conditions. Under both sulfate- and nitrate-reducing conditions, the decrease in parent substrate concentration was affected by chain length, but there was no pronounced difference between even- and odd-numbered substrates. The smallest (decanoic and undecanoic acid) and mid-sized *n*-alkanoic acids (C_{15}-C_{18}) were degraded to a greater extent than the other available substrates. No substrate loss was observed under methanogenic conditions during 30 weeks incubation.

Select *n*-alkanols were degraded under all incubation conditions, as manifested by depletion of substrates, increased culture turbidity in the aqueous phase, emulsification of the organic overlay, increased pressure in the headspace of the cultures, methanogenesis, and electron acceptor consumption. Degradation of *n*-alkanols resulted in abundant methane production after an initial 6 week lag period (Fig. 3). By comparison, degradation of 100 µmol of lactate in positive controls resulted in methane production to 5% of the headspace and less than 1% methane was produced in substrate-unamended controls (Fig. 3). Nitrate and sulfate (18 mM) were consumed within 2 and 16 weeks, respectively, and subsequent additions of either electron acceptor (10 mM) were removed at even faster rates. Decanol was not included in the *n*-alkanol mixture so only ten compounds were tested. Degradation of the *n*-alkanols began after an initial lag period of about 7 weeks but stopped after 24 weeks under all conditions, when less than half of the total amount of substrate was consumed. Similarly, rates of methanogenesis, sulfate- and nitrate-reduction decreased substantially after *n*-alkanol degradation abated.

Biodegradation of the *n*-alkanols was not uniform in that the smaller molecular weight compounds were degraded preferentially under all test conditions. After 50 weeks of incubation, the degradation of the smallest *n*-alkanols (C_{11} - C_{13}) was greatest under methanogenic conditions, removal of mid-sized *n*-alkanols (C_{14} - C_{16}) was comparable

under each test condition, while the larger *n*-alkanols (C_{17} - C_{19}) decayed the least under methanogenic conditions (Fig. 4). Degradation of octadecanol was somewhat greater than similar *n*-alkanols, but undecanol and dodecanol were depleted the most under all three conditions. The rate and extent of *n*-alkanol degradation under methanogenic conditions is illustrated in Fig. 5. The shorter chain-length *n*-alkanols (C_{11} - C_{12}) were degraded almost completely after the shortest lag periods and at the fastest rates, mid-sized *n*-alkanols (C_{13} - C_{14}) were only partially transformed, whereas the longer chain length *n*-alkanols (C_{15} - C_{20}) apparently were not transformed (Fig. 5).

The stoichiometry of nitrate and sulfate reduction associated with the loss of *n*-alkanols and *n*-alkanoic acids in these cultures was compared to predicted values calculated based on the average total amounts of substrates depleted in the cultures during the time course of incubation. Although sulfate and nitrate were rapidly consumed in these cultures, the total amounts of these anions added to the cultures could not account for complete mineralization of the substrates. This suggests that partially oxidized products may have accumulated in these cultures. Sulfate respiration by sulfate-reducing bacteria results in the production of sulfide, and the amount of sulfide predicted to accumulate from the observed depletion of sulfate would have exceeded 50 mM in these cultures, which could have a substantial toxicity effect. Therefore, while the incubation systems employed were favorable for measuring the relative loss of substrates, they were not appropriate for demonstrating complete mineralization of all the substrates added to the cultures.

n-Alkanol-degrading enrichments

The *n*-alkanol-degrading cultures were transferred under each of the three anoxic electron-accepting conditions, to confirm substrate removal and degradation patterns. Since microbial activity was observed primarily with shorter chain-length *n*-alkanols, only C_{10}-C_{16} *n*-alkanols were tested as substrates. After a 3 week lag period, methanogenesis was evident in these cultures with 25% of the headspace consisting of methane after 12 weeks.

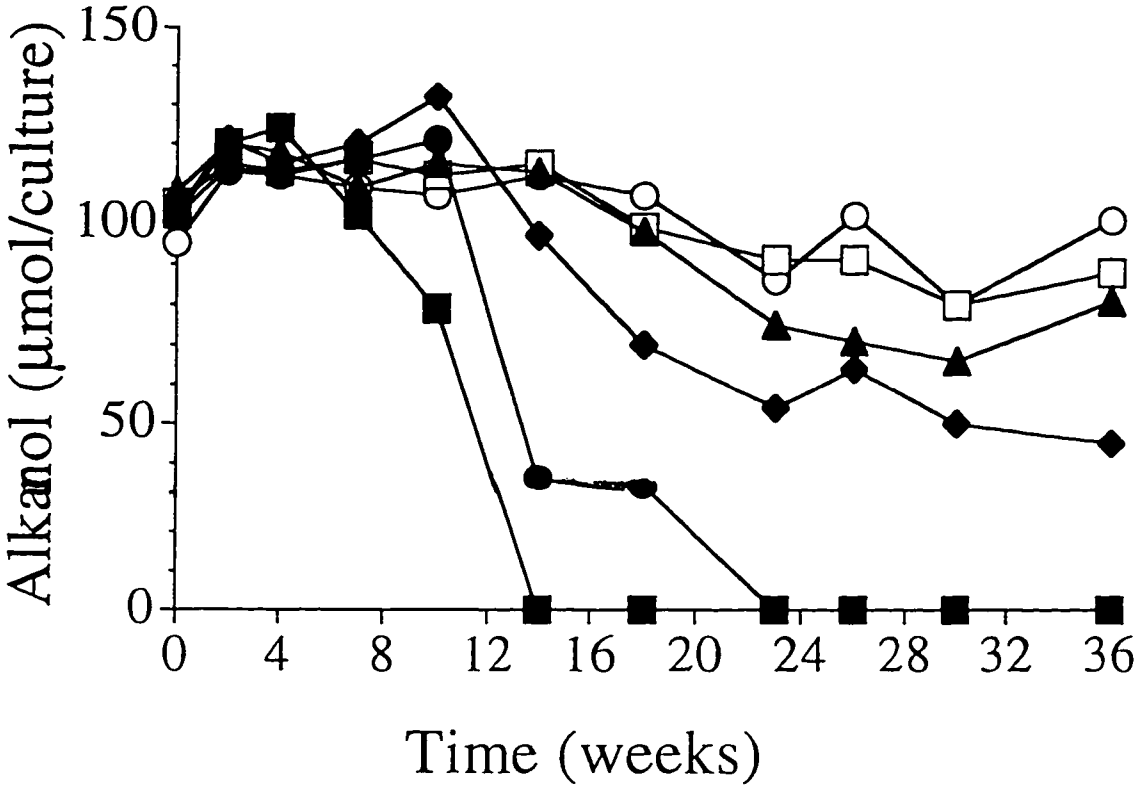

Fig. 5. Chain-length dependence of *n*-alkanol degradation under methanogenic conditions. Undecanol (■) and dodecanol (●) were completely degraded, tridecanol (◆) and tetradecanol (▲) were partially transformed, whereas pentadecanol (□) and hexadecanol (○) were not substantially depleted over the 50 weeks of incubation. Larger *n*-alkanols (C_{17}-C_{20}) remained unchanged but are not shown for the sake of clarity.

Similarly, sulfate- and nitrate- reduction proceeded rapidly after lag periods of 2 and 6 weeks, respectively (data not shown). Degradation of the smallest *n*-alkanols (C_{10}-C_{12}) was evident after a 3 week lag period, while the larger substrates (C_{13}-C_{16}) were not transformed over a 34 week incubation period. The rate and extent of *n*-alkanol degradation was chain-length dependent, with decanol degraded at a faster rate than undecanol and dodecanol respectively (data not shown).

n-Alkanols are potential intermediates of the metabolism of *n*-alkanes under anaerobic conditions, and enrichment on *n*-alkanols could favor the development of a greater biomass of organisms capable of *n*-alkane degradation. Indeed, the *n*-alkane-degrading sulfate-reducing isolate Hxd3 was originally enriched on hexadecanoic acid (Aeckersberg et al. 1991). Enrichment cultures were prepared with a mixture of *n*-alkanes (C_{10}-C_{16}) with culture fluid from *n*-alkanol-degrading cultures. No *n*-alkane loss was evident under any incubation condition (data not shown). Moreover, no significant methane production, sulfate consumption, or nitrate depletion greater than control incubations was evident for over 24 weeks. Thus, the *n*-alkanol-degrading cultures were incapable of transforming the corresponding *n*-alkanes.

Discussion

While investigating the potential for anaerobic biodegradation of *n*-alkanes and the corresponding partially oxidized alcohols and fatty acids, we observed sulfate-dependent oxidation of undecanol to undecanoic acid. Decanol, undecanol, and dodecanol, but not hexadecanol, were all transformed to the corresponding fatty acids. In addition, undecanol combined with fatty acids to form higher molecular weight ester products. The accumulation of alkanoic acids in these cultures was unexpected, since hexadecanoic acid was degraded in parallel cultures in the initial toxicity survey. Nevertheless, this attribute allowed us to demonstrated that the catalytic activity existed to convert *n*-alkanols to *n*-alkanoic acids, which supports part of the proposed pathway for *n*-alkane degradation

under sulfate-reducing conditions (Aeckersberg et al. 1991). The separation of the oxidative reactions, presumably alcohol dehydrogenase and aldehyde dehydrogenase, from the complete mineralization indicates that different members of microbial consortia may be responsible for sequential transformation reactions. The esterification reaction may have been promoted by the use of the bi-phasic system. The hydrophobic environment provided by the organic overlay has been shown to reduce toxicity of hydrophobic substrates and products (Inoue and Horikoshi 1991) and to stabilize enzymatic activities including lipases (Ogino et al. 1994). It is likely that the esterified products detected in these cultures would be readily hydrolyzed in natural environments as a variety of esterases are common in microorganisms.

Both the oxidation of the alkanols to alkanoic acids and the esterification of the compounds by these microorganisms have potential biotechnological applications. Carboxylic acids such as octanoic and decanoic acids are important for the preparation of perfumes and cosmetics, and are currently synthesized from alkanols using toxic metals such as chromium or manganese (Oda et al. 1994). The aerobic conversion of octanol and decanol to the corresponding acids by a variety of fungi and bacteria has been demonstrated (Oda et al. 1994). Furthermore, enzymatic conversion of alcohols and acids by lipase in non-aqueous environments to esters of biotechnological importance is currently under development (Geigert et al. 1984, Williams et al. 1988). Combined microbial oxidation of alkanols and accumulation of alkanol esters of the corresponding fatty acids in organic solvents has also been reported (Takazawa et al. 1984, Ueda et al. 1986). It was previously shown that esterification of tetradecanol and tetradecanoic acid takes place in the absence of water with acetone-dried cells, so living cells are not strictly required for these reactions (Ueda et al. 1986). Our observations are consistent with this because esterification was greatest when oxidative activity ceased, and in our studies we have also shown that esterification occurs in sterile controls.

The initial toxicity evaluation prior to the biodegradation experiments indicated that *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids could be added to multi-phase oily sludge-derived cultures without measurable inhibitory effects. The mixtures of the three compound classes for the most part did not affect microbial activity. The mixture of alkanoic acids inhibited methane and sulfide production when 0.50 g/L total fatty acids were added to cultures, whereas inhibition by *n*-alkanes was not observed even when they were added to a total of 0.42 g/L. This is consistent with a previous report that octane and other gasoline hydrocarbons did not affect biogas production in anaerobic aquifer slurries at concentrations of 52 mg/L (Mormile and Suflita 1996). Our results expand those of Mormile and Suflita (1996) in that mixtures of chemically related compounds were tested rather than individual compounds, yet support the conclusion that hydrocarbons are not particularly inhibitory to anaerobic microorganisms.

n-Alkanes were not degraded in enrichment cultures under any of the anaerobic conditions tested, nor were *n*-alkanes degraded in transfers inoculated from cultures enriched on *n*-alkanols. The addition of *n*-alkanes did not affect lactate degradation or electron-accepting processes in positive controls, which was consistent with the toxicity experiment. However, in the absence of readily degraded lactate, the rates of methanogenesis, sulfate reduction, or nitrate reduction in cultures that received *n*-alkanes were less than in negative controls that received neither form of substrate. Therefore, the full effects of *n*-alkanes on these communities is uncertain, as these compounds may affect the organisms that are responsible for initial transformation reactions but not the terminal members of anaerobic consortia. Deleterious effects of hydrocarbons on microorganisms are well-recognized, and mechanisms of toxicity have been reviewed recently (Sikkema et al. 1995). These inhibitory effects were limited to the *n*-alkanes, and neither the *n*-alkanols nor *n*-alkanoic acids had similar effects even though they were also added as organic HMN mixtures. Nevertheless, although there are reports of degradation of *n*-alkanes by sulfate-reducing bacteria (Aeckersberg et al. 1992; Reuter et al. 1994), our results are consistent

with many studies indicating that *n*-alkanes are not readily degraded under anaerobic conditions (Jobson et al. 1979, Schink 1985).

n-Alkanols were biodegraded under anaerobic conditions in our oily sludge-derived cultures. However, parent substrate removal was limited to incomplete removal of some *n*-alkanols, while others were not attacked at all. The lower limit of *n*-alkanol chain length for degradation was not established, but we would expect shorter alkanols to be degraded, as ethanol and other short chain alcohols are readily metabolized under anaerobic conditions. The upper size limit for alkanols appeared to be tetradecanol, although higher chain length *n*-alkanols might be susceptible to degradation if shorter *n*-alkanols are not present, and previous studies have indicated that hexadecanol is susceptible to anaerobic degradation (Schink 1985, Aeckersberg et al. 1991). The limited activity could be due to accumulation of metabolites or products, or nutrients such as nitrogen and phosphorous may have become limiting after substantial amounts of *n*-alkanols were degraded. No metabolites were detected in any of these cultures, so the mechanism of *n*-alkanol degradation was unknown but probably involved oxidation of the *n*-alkanols to the corresponding *n*-alkanoic acids followed by ß-oxidation reactions leading to complete mineralization. A similar mechanism has been suggested for the degradation of *n*-alkanes and *n*-alkenes (Aeckersberg et al. 1991, Schink 1985), and we have shown that *n*-alkanols can be transformed to *n*-alkanoic acids, and that the latter can be degraded under anaerobic conditions.

The chain-length specificity of substrate degradation could be related to the physical properties of these compounds, as the smaller molecular weight *n*-alkanols have greater water solubility than the longer chain counterparts. Surface tension has been shown as a limiting factor for degradation of *n*-alkanes, and while the rate of *n*-alkane degradation may be affected by microbiological parameters, the pattern of *n*-alkane degradation is linked to substrate physico-chemical characteristics such as molecular structure, molecular weight, and density (Setti et al. 1995). The anaerobic degradation of *n*-alkanes by the sulfate-

reducing bacterium strain TD3 also proceeded in a chain-length dependent manner with decreasing degradation as chain length increased from 8 to 16 carbons long (Rueter et al. 1994). Our results indicate that structural characteristics also affect the degradation of *n*-alkanols, as the same pattern of degradation was observed under all conditions tested, whereas the rate of degradation was dependent on electron acceptor availability and hence the microbial ecology. Future studies will explore the limits of *n*-alkanol chain length that can be degraded, the effects of branching on alkanol degradation, and the effect of hydroxyl group position on the susceptibility to degradation. The microorganisms responsible for biodegradation and oxidation of *n*-alkanols will be isolated in order to determine the mechanism of *n*-alkanol degradation, and the environmental, physiological, and metabolic factors that affect the anaerobic degradation of alkanols.

The third series of substrates tested for susceptibility to anaerobic attack were the *n*-alkanoic acids. These substrates were readily degraded under nitrate-reducing conditions, but were metabolized slowly under sulfate-reducing conditions, and no evidence for degradation of the *n*-alkanoic acids under methanogenic conditions was apparent. This was surprising since the anaerobic degradation of various *n*-alkanoic acids has been reported previously (Roy et al. 1985, Lorowitz et al. 1989, Angelidaki and Ahring 1995). The lack of degradation could be a toxicity effect, as methanogens are sensitive to high concentrations of alkanoic acids (Hanaki et al. 1981, Koster and Cramer 1986). Additional evidence for toxicity included the observation that the background rate of methanogenesis in cultures with *n*-alkanoic acids was less than in negative controls without substrates. This apparently prevented us from determining the relative susceptibility of the various *n*-alkanoic acids to biodegradation under methanogenic conditions. Under nitrate-reducing and sulfate-reducing conditions, the pattern of substrate degradation was such that the intermediate chain length fatty acids typically associated with cell membranes and especially the lower molecular weight fatty acids were most amenable to degradation.

The main conclusion from the biodegradation experiment was that partially oxidized hydrocarbons were more susceptible to anaerobic degradation than the corresponding straight-chain hydrocarbons. Therefore, the initial oxidation of the hydrocarbons is a limiting step for their degradation under anaerobic conditions. However, if oxidation of an *n*-alkane to the corresponding *n*-alkanol occurs, for example by the activity of aerobic microorganisms, then the subsequent degradation can occur under anaerobic conditions. Additional environmental factors may also limit degradation of substrates under anaerobic conditions, such as toxicity as observed with the alkanoic acids under methanogenic conditions, or unknown limiting factors such as for the larger *n*-alkanols. Further research will be required before we can reliably predict the fate of components in oily wastes released into anaerobic environments and the potential for intrinsic bioremediation of aliphatic hydrocarbons.

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Chapter 4.

USE OF NITRATE TO CONTROL SULFIDE GENERATION BY SULFATE-REDUCING BACTERIA ASSOCIATED WITH OILY WASTE

Abstract

Sulfide is a toxic and corrosive product of sulfate-reducing bacteria that can accumulate in oily waste streams to nuisance levels. Sludge associated with an oily waste stream was collected from a settling tank and used to assess sulfide generation activities. Methanogenesis was a predominant process in sludge in the absence of electron acceptors other than CO_2. Sulfate reduction and sulfide formation were evident when sulfate was available. Nitrate inhibited sulfate reduction and prevented sulfide accumulation under freshwater, brackish, and saltwater conditions. Sodium, potassium, and calcium nitrate were equally effective in preventing sulfide formation. Inhibition of sulfate reduction varied as a function of the nitrate concentration, with 50 mM nitrate inhibiting sulfate reduction, and as little as 16 mM nitrate prevented sulfide accumulation. Sulfide was oxidized in nitrate-reducing incubations, and sulfate accumulation was observed in select cultures. Nitrate reduction was accompanied by production of nitrite and nitrous oxide, which presumably helped prevent sulfate reduction in extended incubations. Our results suggest that nitrate amendments control the formation of sulfide in oily waste streams both by preventing sulfate reduction and by stimulating anaerobic sulfide oxidation.

Introduction

Oily wastes associated with petroleum extraction, refining, utilization, transportation, and disposal are subject to microbiological alteration under anaerobic conditions. In operations such as the collection and treatment of oily wastes, or the subsurface injection of seawater for oil recovery, alteration of the physico-chemical properties of oils can occur [16, 17, 30]. Furthermore, substantial amounts of sulfide can be generated by the action of sulfide-producing microorganisms, particularly sulfate-reducing bacteria which are virtually ubiquitous in most anaerobic environments. These nutritionally diverse organisms can use a variety of xenobiotic compounds, including petroleum components as electron donors and couple this metabolism to the reduction of sulfate and the production of sulfide [12, 29, 30]. Whether present in planktonic form or as biofilms, a wide variety of sulfate-reducing bacteria is clearly associated with oil production systems, although the relative prevalence of various species is still under investigation [5, 26, 33]. Sulfide production by sulfate-reducing bacteria is a serious concern because of its odor, toxicity, corrosiveness, and ability to form insoluble iron sulfide precipitates that plug oil-bearing strata and stabilize undesirable oil-water emulsions [9]. In addition, sulfide contamination increases the sulfur content of fossil fuels and results in the devaluation of energy reserves. Processes for control of sulfide production as well as other activities of sulfate-reducing bacteria are needed from both an economic and environmental perspective [16].

Thermodynamically, the microbial reduction of nitrate to nitrite, nitrogen, or ammonia provides more Gibbs free energy than the reduction of sulfate [21], and therefore nitrate might be used as a preferred electron acceptor when both anions are potentially available. Nitrate may provide a competitive advantage for nitrate-reducing bacteria over sulfate-reducing bacteria when competing for available electron donors. Nitrate can also serve as an alternative electron acceptor for sulfate-reducing bacteria [10]. In practical applications, nitrate has long been used to control odors associated with sewage systems

[4, 19], resulting in both transient and long-term inhibition of sulfide production [1, 3, 7 13, 15, 25]. Nitrate has also been used to control sulfide production in sandstone cores with subsurface formation water from a gas storage facility [22]. The addition of high nitrate concentrations also leads to the buildup of nitrous oxide, which raises the redox potential, contributing to long-term inhibition of sulfide production [1, 18, 25, 28].

We investigated whether nitrate could be useful for preventing sulfide formation associated with the collection and treatment of oily wastes produced on board marine vessels. Our results indicate that substantial sulfide production occurs in sludge incubations due to the degradation of exogenous substrates under a variety of salinity conditions. The addition of nitrate inhibits sulfate reduction and prevents sulfide formation, and also reduces the amount of pre-existing sulfide. Therefore nitrate amendment is a promising treatment for prevention of sulfide production associated with oily waste streams.

Materials and methods

Media and experimental conditions

A brackish medium designed for the cultivation of sulfate-reducing bacteria [34] was prepared using strict anaerobic technique. Resazurin (0.0002%) was included as a redox indicator, the sulfate concentration was 20 mM unless otherwise indicated, and the medium was reduced with sodium sulfide (1 mM). The medium was dispensed into 25 ml serum bottles inside an anaerobic chamber and each bottle was inoculated with 0.5 ml of oily sludge. The sludge was collected from a settling tank (Tank #63) at the U.S. Navy Craney Island Fuel Depot in Portsmouth, Virginia, and stored in sealed glass bottles at room temperature. Serum bottles were sealed with 1-cm-thick butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ), then removed from the anoxic chamber and secured with aluminum crimp seals. The headspace of the serum bottles was adjusted to N_2/CO_2 (80%:20%). Sterile controls were autoclaved. Sodium lactate was added from a

neutralized sterile anoxic stock solution to an initial concentration of 10 mM unless otherwise indicated. Cultures were incubated at room temperature, in the dark, without shaking. Samples were withdrawn periodically using strict anaerobic technique and stored frozen until analyzed. Prior to analysis, the samples were thawed and centrifuged (10 000 x g) for 5 min to remove particulate debris.

For the initial survey of anaerobic activity, the medium did not receive an electron acceptor (methanogenic conditions), or was amended with either sodium sulfate (20 mM) or sodium nitrate (20 mM) from sterile, anoxic stock solutions. Acetate, when used, was added from a stock solution to an initial concentration of 10 mM. Results from this experiment are averages for cultures established with 0.5, 1, or 2 mM initial sulfide concentration. The effect of nitrate on sulfate-reducing and methanogenic cultures was determined in triplicates containing either lactate (10 mM), benzoate (1 mM), or no exogenous substrate, with or without 100 mM nitrate.

To test the effect of sodium nitrate under different salinity conditions, a freshwater medium was prepared (1 g/L NaCl), and a salt concentrate was added to give brackish (7 g/L) and saltwater (20 g/L) conditions [34]. To compare the three forms of nitrate, sterile anoxic solutions of $NaNO_3$, KNO_3, and $Ca(NO_3)_2$ were added to cultures to give 50 mM nitrate. Likewise, NaCl, KCl, and $CaCl_2$ were prepared as stock solutions and added to give 50 mM additional chloride. All values are averages of triplicate cultures except for sterile controls.

The effect of nitrate on sulfate depletion and sulfide accumulation was determined by amending cultures with 0-80 mM sodium nitrate. A negative control without lactate, as well as an autoclaved sterile control, were included at each nitrate concentration. Samples were taken initially then after weekly intervals for 7 weeks to follow the rate of transformation of the analytes, and again after 25 weeks to determine long-term effects. Sulfide was analyzed after 6 and 25 weeks incubation.

Sulfate accumulation was measured in sludge incubations amended with nitrate but not sulfate. These cultures were amended with a variety of substrates including lactate (positive controls), *n*-alkanes (which were not degraded), *n*-alkanols (biodegraded), or *n*-alkanoic acids (biodegraded) [20]. Values shown are averages for triplicate cultures after 18 to 30 weeks incubation, long after lactate and acetate were depleted in positive controls.

Analytical techniques

Sulfate, nitrate, nitrite, lactate, and acetate were analyzed by ion chromatography using a Dionex DX500 system (Dionex, Sunnyvale, Cal) with an AS11 4 mm column, a CD20 conductivity detector, and an aqueous mobile phase at 2 ml/min. The mobile phase was initially a rinse for five minutes with 0.4 mM NaOH. Two min after injection, the mobile phase was changed to 5 mM NaOH over four minutes, and to 18.5 mM NaOH over the next four minutes to elute sulfate. Concentrations were determined by comparison to external standards analyzed the same day. Methane and hydrogen were analyzed by gas chromatography as previously described [24]. The amount of methane or hydrogen in the headspace of cultures was calculated as percent by volume by comparison to external standards. Sulfide was analyzed by a methylene blue assay as previously described [8]. Sulfate-reducing bacteria were enumerated according to the method of Tanner [32]. Chemicals were obtained from Aldrich Chemical Co. (Milwaukee, Wis.), were of at least 97% purity, and were used without further purification.

Results

Microbial activity in anaerobic sludge

The oily sludge was a thick, shiny, black, heterogeneous composite with small particulates, and a strong petroleum odor. Analyses of the sludge indicated the presence of sulfide, a suite of alkanes typical of refined petroleum, and methane which accumulated in storage containers. Microscopic examination of sludge diluted in anaerobic media revealed

a diverse assemblage of motile and non-motile rods, cocci, and spirilla. An estimate of sulfate-reducing bacteria in the sludge indicated $>10^6$ cells ml^{-1}.

Anaerobic cultures were established using the oily sludge as inoculum with various substrates and electron acceptors. Lactate and acetate were degraded under nitrate-reducing conditions within two weeks although there was a 4-5 day lag prior to substrate utilization (Figure 1). The active nitrate-reducing cultures turned pink after 1 week, indicating resazurin oxidation as a result of nitrous oxide production (18), whereas sterile controls and cultures without nitrate remained colorless. Under sulfate-reducing conditions, acetate was degraded more slowly than lactate, with no appreciable decrease until after a two week lag period (Figure 1). In cultures with either sulfate or nitrate as electron acceptor, hydrogen in the headspace of cultures was consumed, although a transient increase in hydrogen concentration was associated with the degradation of lactate or acetate under sulfate-reducing conditions (data not shown). In the absence of sulfate or nitrate, endogenous components in the sludge as well as added lactate were transformed eventually to methane. However methanogenesis was not observed in cultures containing either sulfate or nitrate (data not shown). Reducing the medium with 0.5 mM to 2 mM sodium sulfide did not affect microbial activity, and no degradation of lactate or acetate was observed in sterile controls. These observations indicated the presence of lactate-degrading, hydrogen-consuming, and to a lesser extent acetate-degrading microorganisms, as well as nitrate-reducing, sulfate-reducing, and methanogenic bacteria in the oily sludge.

Effects of nitrate on sulfate reduction

Anaerobic cultures were established with oily sludge, sulfate, with and without benzoate or lactate. With only endogenous substrates, 11.4 mM sulfate was consumed in 18 weeks, following an 8 week lag period, indicating that some degradable organic matter

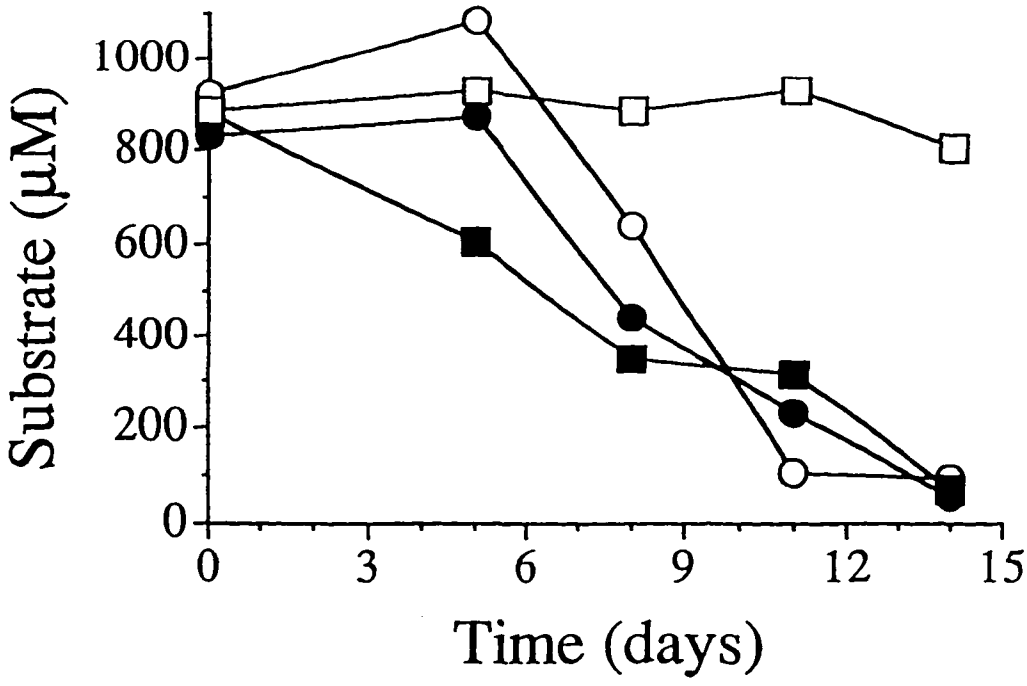

Figure 1 Degradation of fatty acids by oily sludge incubations. Lactate [squares] and acetate [circles] were degraded under nitrate-reducing conditions (filled symbols), and lactate was transformed under sulfate-reducing conditions (open symbols), whereas acetate was not immediately degraded under sulfate-reducing conditions.

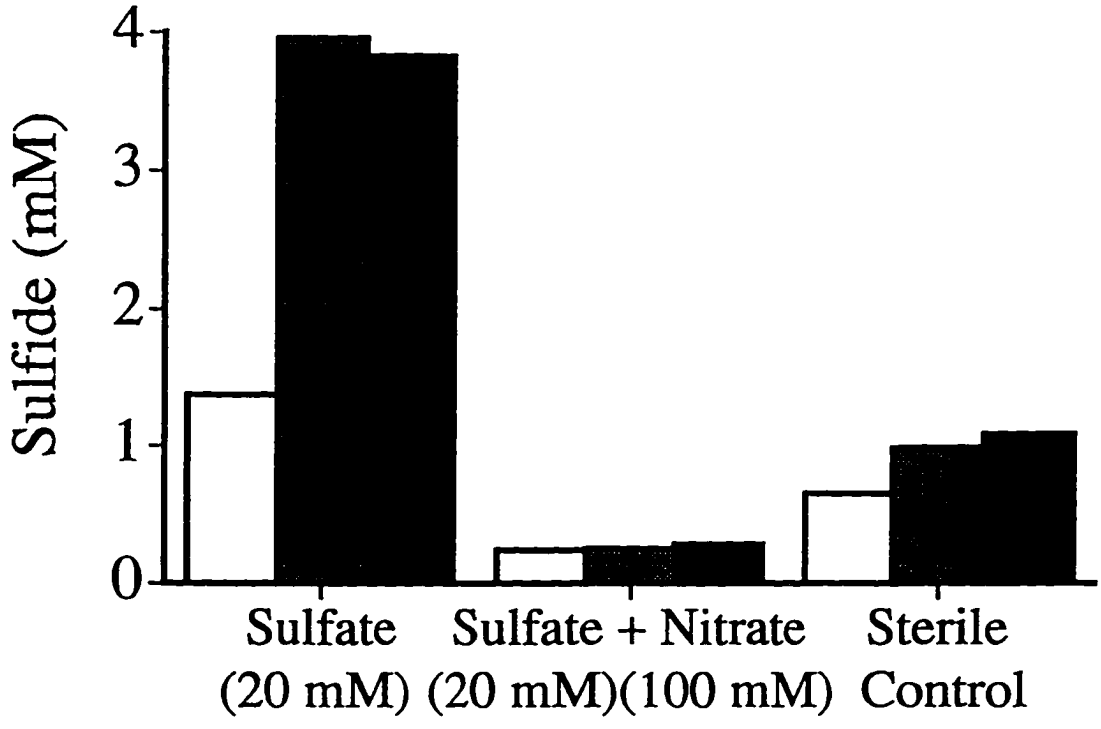

Figure 2 Sulfide concentrations 10 weeks incubation in cultures amended with sulfate, or sulfate and nitrate, compared to sterile controls. Cultures were inoculated with oily sludge that contained endogenous substrate [white], or were also amended with the exogenous electron donors lactate [gray] or benzoate [black].

was present in the sludge. In substrate-amended cultures without nitrate, sulfate was reduced concomitant with the degradation of lactate (10.3 mM sulfate in 6 weeks) and benzoate (16.3 mM sulfate in 18 weeks). In nitrate-amended cultures, lactate or benzoate addition resulted in nitrate reduction whereas nitrate loss was minimal with endogenous substrate only. With nitrate also present in the incubations, less than 1 mM sulfate was reduced. To confirm the inhibition of sulfate reduction by nitrate addition, the sulfide concentration in cultures was measured after 10 weeks incubation. Cultures that received lactate or benzoate had almost 4 mM sulfide, and even cultures with endogenous substrate had more sulfide than sterile controls (Figure 2). In contrast, cultures that received nitrate as well had less sulfide than the corresponding sterile controls (Figure 2). In sulfate-free cultures, methane was produced from endogenous substrates (0.5% of headspace), or with benzoate (1%), or lactate (5%) as substrates. If sulfate or nitrate were added, little or no methane was produced (<0.1%). Thus, nitrate also inhibited methanogenesis in these cultures. No substrate degradation, sulfate or nitrate reduction, or methanogenesis occurred in sterile controls.

Form of nitrate

The effects of sodium-, potassium- and calcium nitrate on lactate degradation, sulfate reduction, sulfide production, nitrate reduction, and nitrite accumulation in cultures established with oily sludge were compared with the effects of the corresponding chloride salts. Lactate was depleted in all incubations except sterile controls. However, in the chloride-amended cultures, lactate degradation was incomplete since acetate accumulated up to the stoichiometrically expected amount (data not shown). Thus, there was variation between cultures in the amount of electrons released from the oxidation of lactate, and the amount of electron acceptor reduced. Using equations 1-2, the theoretical amount of electrons released by the oxidation of lactate to acetate and the degradation of acetate were calculated for each culture. Similarly, the recovery of electrons accepted was calculated

based on the actual amount of sulfate and nitrate reduced, correcting for nitrite accumulation (equations 3-5). All values were corrected for slight changes in sterile controls due to analytical variations. The average amount of electron equivalents accepted was 122% of the average number released, indicating that the assumed complete reduction of sulfate and nitrate did not occur which would lead to an overestimation of actual electron equivalents accepted, although contribution of electrons from components in the sludge were not accounted for.

Lactate oxidation: $CH_3CH_2OHCOOH + H_2O \rightarrow CH_3COOH + CO_2 + 4\ H^+ + 4\ e^-$ [1]

Acetate oxidation: $CH_3COOH + 2\ H_2O \rightarrow 2\ CO_2 + 8\ H^+ + 8\ e^-$ [2]

Sulfate reduction: $SO_4^{2-} + 8\ e^- + 8\ H^+ \rightarrow S^{2-} + 4\ H_2O$ [3]

Nitrate reduction: $NO_3^- + 2\ e^- + 2\ H^+ \rightarrow NO_2^- + H_2O$ [4]

Nitrite reduction: $2\ NO_2^- + 6\ e^- + 8\ H^+ \rightarrow N_2 + 4\ H_2O$ [5]

In cultures that received the chloride salts, the sulfate-reducing bacteria were active, as sulfate was reduced and sulfide was produced (Table 1). The decrease in sulfate consumption was greater than the increase in sulfide production by 3 mM in all cultures except sterile controls, suggesting that an intermediate oxidation state of sulfur might accumulate independent of nitrate addition. To determine the amount of activity of sulfate-reducing bacteria in these cultures, the recovery of electrons calculated based on the average of the measured sulfate depletion and sulfide production was determined. The recoveries were 112%, 100%, and 89% for cultures that received sodium, potassium, and calcium chloride, respectively, for an overall average of 100% in the absence of nitrate. Therefore, sulfate-reducing bacteria are responsible for most of the electron-accepting activity observed in these cultures. The results were independent of whether sodium, potassium, or calcium chloride were added, confirming that the cations were not responsible for the inhibitory effects on sulfate-reducing bacteria observed with the various nitrate salts.

Table 1 Comparison of the effects of sodium, potassium, or calcium salts of chloride and nitrate on lactate degradation, sulfate reduction, and nitrate reduction in cultures established with oily sludge.

Conditions	Change in concentration (mM)[a]				
	lactate	acetate	nitrate	sulfate	sulfide
Chloride salts (50 mM Cl^-)					
sodium	-11.73	+4.17	-	-15.87	+11.96
potassium	-11.65	+9.78	-	-7.47	+3.67
calcium	-11.54	+5.49	-	-10.41	+6.48
Nitrate salts (50 mM NO_3^-)					
sodium	-11.01	+0.03	-19.88	-4.34	-0.57
potassium	-11.01	+0.04	-17.02	-5.53	-0.58
calcium	-11.24	+0.09	-29.77	-2.80	-0.43

[a] averages of triplicates; (-) decrease, (+) increase; corrected for sterile controls

The addition of nitrate consistently inhibited both sulfate reduction and sulfide production. In cultures with nitrate, lactate was degraded but acetate did not accumulate (Table 1). Substantial nitrate reduction occurred in all cultures regardless of which nitrate salt was added. However, there were slight differences between the effects of the three forms of nitrate. Nitrite accumulated in cultures to which sodium nitrate (2.43 mM NO_2^-) and potassium nitrate (0.78 mM NO_2^-) were added but not in cultures with calcium nitrate. Nitrous oxide accumulated in all cultures containing nitrate as evidenced by the conversion of resazurin to its pink oxidized state. Sulfate reduction occurred even in the presence of nitrate, but sulfide did not accumulate, and in fact the sulfide levels decreased below initial amounts and values in sterile controls (Table 1). In contrast to the cultures without nitrate, the average recovery of electrons based on sulfate depletion and sulfide production was only 12%, 16% and 7% for sodium, potassium, and calcium nitrate, respectively. Therefore, all three forms of nitrate inhibited the activity of sulfate-reducing bacteria in these incubations.

Salinity conditions

The effects of varying the medium salinity conditions was tested in comparable experiments, using the sodium salt of nitrate. As with the previous experiment, cultures without nitrate sometimes accumulated acetate so that not all the reduction potential was realized during the incubation period. Nevertheless, transformation of lactate was associated with both sulfate reduction and sulfide production under all three salinity regimes (Table 2). Accounting for the expected electron transfers from the conversion of lactate to acetate and acetate degradation, recovery of released electrons as sulfate reduction to sulfide was 92%, 112%, and 100% under freshwater, brackish, and saltwater conditions, respectively.

Table 2 Effects of sodium nitrate addition on lactate degradation, sulfate reduction, and sulfide accumulation in cultures established with oily sludge under different salinity conditions.

Conditions	Change in concentration (mM)[a]				
	lactate	acetate	nitrate	sulfate	sulfide
Without nitrate					
freshwater	-11.21	+6.05	-	-11.97	+6.09
brackish	-10.17	+6.96	-	-8.67	+7.20
saltwater	-10.48	+6.29	-	-9.31	+6.00
With nitrate (50 mM)					
freshwater	-11.38	+0.07	-25.93	-9.32	-0.58
brackish	-10.54	+0.03	-20.11	-5.75	-0.63
saltwater	-10.22	0.00	-18.44	-1.48	-0.49

[a] averages of triplicates; (-) decrease, (+) increase; corrected for sterile controls

The addition of nitrate once again inhibited sulfate reduction and sulfide accumulation relative to controls without nitrate. Lactate was degraded and no acetate accumulated in cultures containing nitrate (Table 2). Nitrate was reduced (Table 2) and 0.47 mM, 1.92 mM, and 1.19 mM nitrite accumulated under freshwater, brackish, and saltwater conditions, respectively. Nitrous oxide production in all nitrate-reducing cultures was evidenced by the oxidation of resazurin. Sulfate reduction also occurred even though sulfide concentrations decreased below initial levels and values for sterile controls (Table 2). The decrease in sulfate concentration was greater than any increase in sulfide production in cultures with nitrate, particularly under freshwater conditions (8.76 mM) compared to brackish (5.19 mM) and saltwater conditions (0.92 mM), which also suggested incomplete reduction of sulfate. The average recovery of electrons associated with sulfate reduction to sulfide was 28%, 17%, and 2% under freshwater, brackish, and saltwater conditions, respectively. As in the previous experiment, the net recovery of electron equivalents assuming complete reduction of electron acceptors was 124%, suggesting that endogenous substrate contributed electron donors during the incubation or that incomplete reduction of electron acceptors occurred. No degradation of lactate or reduction of sulfate or nitrate occurred in sterile controls under the three salinity conditions.

Nitrate concentration

In order to determine how much nitrate must be added to control sulfate reduction, cultures were established with lactate as a substrate and varying amounts of nitrate. In cultures without nitrate, lactate was depleted within 4 weeks, coupled with accumulation of acetate. After 25 weeks incubation, acetate values decreased to <2 mM. In cultures with nitrate, lactate degradation occurred primarily between two and four weeks incubation, but lactate was not completely removed in 7 weeks, although no fatty acids could be detected after 25 weeks. In cultures with nitrate, acetate accumulated transiently during the first four weeks, up to 6 mM, with greater concentrations accumulating with decreased nitrate

concentration (data not shown). There was no evidence for lactate degradation, sulfate or nitrate reduction, or sulfide production in sterile controls.

Nitrate was reduced in these cultures, and the amount of nitrate reduced relative to the potential amount predicted if all of the reducing potential from the degradation of lactate were transferred to nitrate (based on equations 1, 2, 4, and 5) was 75%. This indicates that nitrate reduction accounted for most of the electron accepting processes in these cultures. The total reduction of electron acceptors was 75% to 108% of expected amounts based on substrate decay.

The degradation of lactate was associated with both sulfate reduction and sulfide production in cultures lacking nitrate. Over a six week incubation period, 4.0 mM sulfate was reduced, and 3.5 mM sulfide accumulated above sterile controls (Table 3). Based on equations 1-3, this represents 75% of potential sulfate reduction based on the substrate decay observed. The depletion of sulfate was dependent on the concentration of nitrate added, with greater nitrate concentrations decreasing the amount of sulfate reduced (Figure 3). Sulfide accumulated to amounts greater than in sterile controls only in cultures lacking nitrate, and sulfide continued to accumulate over the extended incubation period. Cultures with nitrate had sulfide concentrations of less than 0.5 mM even after 25 weeks incubation (Table 3).

Sulfide oxidation to sulfate

In all of the previous experiments, cultures to which nitrate was added also contained sulfate, so it was difficult to determine whether sulfide oxidation resulted in sulfate accumulation. In separate oily sludge incubations without added sulfate, sulfate accumulation was indeed associated with nitrate reduction. Sulfate accumulation was found only in cultures that were not actively degrading a substrate such as lactate or fatty acids, and was always associated with accumulation of nitrite. In sterile controls and

Table 3 Effects of sodium nitrate on lactate degradation and sulfate reduction in cultures with oily sludge inoculum and 20 mM sulfate and varying concentrations of nitrate.

Nitrate addition	Change in concentration (mM)[a]						
	lactate	nitrate		nitrite		sulfate	
	6 wks	6 wks	25 wks	6 wks	25 wks	6 wks	25 wks
0 mM	-7.4	-	-	-	-	-4.0	-4.8
17 mM	-6.3	-10.7	-18.1	+1.6	+4.3	-3.9	-0.0
35 mM	-4.7	-10.5	-30.1	+3.0	+8.8	-1.5	-0.0
50 mM	-3.7	-13.0	-34.2	+2.7	+4.8	-1.0	-0.8
70 mM	-5.3	-11.4	-40.0	+0.4	+2.6	-0.8	-0.3

[a] averages of triplicates; (-) decrease, (+) increase; corrected for sterile controls

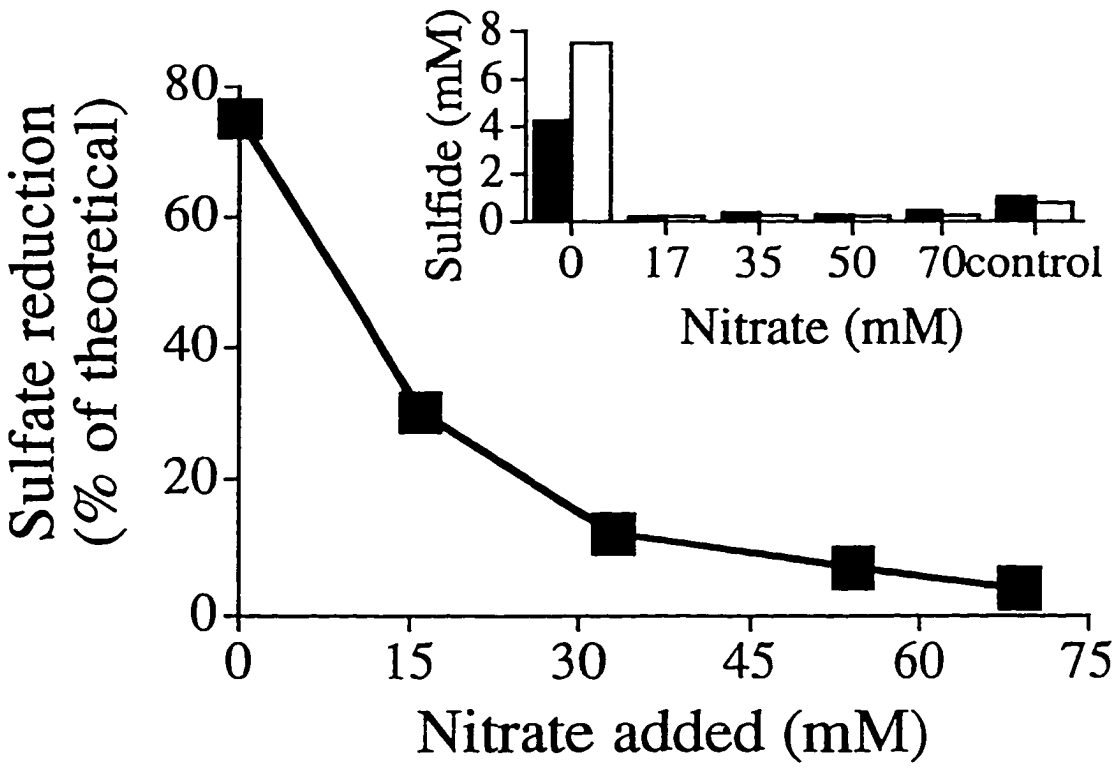

Figure 3 Effect of nitrate concentration on sulfate depletion and sulfide accumulation. Increased nitrate concentration resulted in decreased sulfate reduction as indicated by the percent of the theoretical amount expected. The concentration of sulfide in cultures (insert) after 6 weeks (black) and 25 weeks (white) was also affected by the addition of nitrate.

cultures degrading exogenous substrates, the sulfate concentration between 18 and 30 weeks incubation was <0.1 mM, whereas in substrate-unamended controls sulfate was 2.23 mM during this time. Cultures established with *n*-alkanes, which were not degraded, had accumulated 2.04 mM sulfate. In these same cultures there was < 0.1 mM nitrite, 1.3 mM, and 1.8 mM nitrite, respectively. There was very little nitrate reduction in sterile controls and cultures containing *n*-alkanes (< 2 mM), whereas approximately 30 mM and 60 mM total nitrate were reduced in lactate-degrading positive controls and cultures with degradable substrate such as fatty acids, respectively. Sodium sulfide (1 mM) added to the medium is presumably the source for the sulfate production, and the production of nitrous oxide as indicated by the oxidation of resazurin also occurred under these conditions.

Discussion

Generation of sulfide in the petroleum industry is associated with a myriad of detrimental consequences, so industries have invested in strategies to manage this nuisance gas and the organisms that produce it, including the use of biocides, special coatings, and mechanical cleaning [16]. However, rarely is an ecological approach to sulfide control employed. The addition of nitrate as a preferred electron acceptor is one such approach that has been successful for preventing sulfide formation in contaminated sediments (22, 6). Nitrate is a thermodynamically preferred electron acceptor relative to sulfate, but unlike other electron acceptors, nitrate is readily soluble in water and does not form precipitates. We attempted to determine the feasibility of using nitrate for the treatment of oily waste streams that produce copious amounts of sulfide.

Our studies confirmed that sulfate-reducing bacteria were indigenous to oily wastes streams that are typical of those encountered on board Navy ships and at treatment facilities. In the oily sludge incubations, these organisms oxidized exogenous substrates, reduced sulfate, and produce sulfide. Lactate, a preferred substrate for incomplete oxidizing sulfate-reducing bacteria, was readily degraded in these cultures, whereas acetate

when metabolized at all was removed after a longer lag period. This may indicate that completely oxidizing sulfate-reducing bacteria were less numerous or active in our sludge incubations. Hydrogen, a source of electrons for many anaerobes including sulfate-reducing bacteria, was readily depleted in our cultures regardless of available electron acceptor. Benzoate was also metabolized in these cultures, although a substantial lag period was typically encountered. These results are consistent with the nutritional diversity of sulfate-reducing bacteria [12] and indicate a metabolic capacity to couple the degradation of a wide variety of compounds in oily sludge to sulfate reduction. In addition, physiological diversity was indicated by sulfate-reducing activity present under freshwater as well as saltwater conditions.

In the absence of sulfate or nitrate, the sludge inoculum converted lactate initially to acetate, and the latter was eventually converted to methane. Methanogenesis was also detected in the containers used to store the oily waste, suggesting that methanogens are abundant in the highly reduced oily sludge. Methanogens would not be expected to compete well with other organisms for common electron donors in oily waste streams in the presence of sulfate or other electron acceptors, but could predominate if other electron acceptors were depleted [27]. Our results indicate that sulfate and especially nitrate curtail methanogenic activity, so that the nitrate additions proposed here for prevention of sulfide formation would effectively preclude methane production as well.

Nitrate inhibited sulfate reduction and sulfide formation in cultures, regardless of substrate, salinity, or nitrate form. Differences were noted for the amount of nitrate reduced and the amount of nitrite accumulating depending on the nitrate salt added. Nevertheless, all three forms of nitrate would be effective for preventing sulfidogenesis. Our research indicates that nitrate-reducing organisms in oily sludge were sufficiently active that additional inoculation would not be required, and that nitrate amendments as part of a controlled remediation strategy could effectively reduce sulfide emissions in separation and storage facilities.

Due to the heterogeneous nature of oily wastes, any remediation strategy would have to be applicable to a variety of environmental conditions. We tested the effects of nitrate under different salinity regimes, as freshwater, marine, and mixed wastes are all encountered on board ships and at treatment facilities. The addition of 50 mM nitrate prevented sulfide accumulation in lactate-degrading cultures under all the above salinity conditions. Less sulfate reduction was noted under saltwater conditions in the presence of 50 mM nitrate relative to freshwater conditions. The sulfate-reducing bacteria may have been more active under freshwater conditions, or conversely the nitrate-reducing or sulfide-oxidizing bacteria may have been relatively more active under saltwater conditions. Nevertheless, salinity was not a limiting environmental factor for the prevention of sulfide production by nitrate.

Nitrate did not affect sulfate removal and sulfide accumulation equally. We found that the addition of even the lowest concentration of nitrate tested (16 mM) prevented sulfidogenesis, and reduced the sulfide concentration to below values in sterile controls. The lower limit of nitrate required to prevent sulfide formation is therefore unknown. Previous reports from studies with cultures established with dilute sewage sludge, pond sediment, or oil field brines, and amended with acetate, glucose, or hydrogen indicated that 59 mM nitrate inhibited biogenic sulfide production [17]. Inhibition was incomplete with lower amounts of nitrate (6 or 20 mM) or if the sulfate concentration was increased from 20 mM to 159 mM [17]. Recent studies have indicated nitrate and nitrite can prevent sulfide production at concentrations as low as 0.71 mM in sandstone columns containing a biofilm of sulfate-reducing bacteria [28], so the potential to use low concentrations of nitrate is promising from an economic point of view. Further studies would be required to determine the lower limit required for prevention of sulfidogenesis in actual oil-water separators and storage tanks, but our results suggest that the amount of nitrate required could be lower than the amount of sulfate present, depending on the organic substrate load.

In contrast to sulfide production, prevention of sulfate reduction was concentration dependent over the range of nitrate concentrations tested. 50 mM Nitrate inhibited sulfate reduction by 90% whereas greater than 80 mM would be required for complete inhibition of sulfate reduction. At <50 mM nitrate, the flow of electrons was divided between sulfate- and nitrate-reduction which indicates that both acceptors are competing for available electrons. Our results demonstrate that it is important to distinguish whether sulfate depletion or sulfide accumulation is used as the indicator of the activity of sulfate-reducing bacteria. Further investigation into the competition for electron donors by sulfate- and nitrate-reducing bacteria, as well as the relative contribution of sulfide-oxidizing bacteria to sulfide removal in cultures is needed.

Sulfate reduction did not result in stoichiometric sulfide accumulation, so intermediate oxidation state products were probably formed, the most likely of which are elemental sulfur and thiosulfate. Sulfate-reducing bacteria have been shown to produce elemental sulfur as an intermediate product in the oxidation of sulfide [14]. In addition, thiosulfate has been shown as an intermediate in the sulfur cycle that can be oxidized, reduced, or disproportionated [2]. The addition of nitrate could either prevent complete reduction of sulfate to the fully reduced sulfide, or sulfide may be produced but then re-oxidized to an intermediate product. We have not detected thiosulfate in our incubations, and further research will be required to establish whether elemental sulfur production is important in our cultures.

In all the cultures established with nitrate, sulfide levels decreased during the incubation relative to initial values and sterile controls. In addition, sulfate was detected in cultures containing nitrate in which biological activity was substrate-limited. Therefore, nitrate can also be used for the oxidation of pre-existing sulfide in these systems. Some species of bacteria including *Thiobacillus denitrificans* [23] can use nitrate as an electron acceptor to oxidize sulfide to sulfur or sulfate. Even some species of sulfate-reducing bacteria are able to oxidize sulfide with nitrate as the electron acceptor [11, 14]. High

concentrations of sulfide have been shown to inhibit growth of *T. denitrificans* [31] and cause incomplete denitrification to gaseous nitrogen oxides, as well as dissimilatory nitrate reduction to ammonia, in nitrate-amended sediment slurries that oxidize reduced sulfur compounds [6]. However, concentrations of sulfide up to 2 mM (65 ppm) did not affect nitrate utilization in our cultures. Bacteria able to utilize nitrate for the oxidation of sulfide are apparently naturally present in the oily wastes we have used, so that inoculation with sulfide-tolerant sulfide-oxidizing bacteria would not be required as part of a treatment strategy for these oily waste streams.

Several intermediates of nitrate reduction were detected in these cultures. Nitrite was detected in concentrations up to 3 mM in cultures with sodium nitrate, although it did not uniformly accumulate in all cultures. Nitrite has the effect of raising the redox potential of the medium and preventing the activity of sulfate-reducing bacteria [18]. Nitrous oxide was also detected as indicated by the oxidation of the redox indicator to its pink oxidized state. Only nitrous oxide and not nitrite or nitrate will oxidize resazurin [18]. Furthermore, there was no evidence for contamination of these cultures with oxygen because sterile controls and cultures established without nitrate remained reduced. Resazurin might be useful in oil-water separator and storage containers as an indicator of nitrate reduction and the existence of an elevated redox potential in the system that would preclude sulfide production.

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Chapter 5.

TOXICITY EFFECTS OF ORGANOSULFUR COMPOUNDS ON ANAEROBIC MICROBIAL METABOLISM

Abstract

Four classes of organosulfur compounds were tested for their effects on anaerobic microbial activity as indicated by lactate degradation, nitrate or sulfate reduction, and methanogenesis. Each class consisted of six to ten chemically-related compounds added concurrently to anaerobic cultures established with an oily sludge inoculum. Effects on anaerobic activities were evaluated at different amounts of the chemicals, expressed as moles per volume of aqueous culture due to their limited water solubility, and were concentration-dependent in all cases. Thiophenes and thiols inhibited microorganisms under methanogenic and nitrate-reducing conditions at ≥ 5 mmol/L, but had little influence under sulfate-reducing conditions even at 10-50 mmol/L. Thiophenic acids did not affect most microbial processes, but they were used in much lower concentrations (≤0.50 mmol/L) relative to the other compound classes because of their greater water solubility. The aromatic sulfides were particularly inhibitory to microbial activity under sulfate-reducing conditions (≥5 mmol/L), and had more effect on electron acceptor reduction than lactate oxidation. Overall, the latter group was more inhibitory to anaerobic metabolism relative to the four classes of test materials. The biodegradation of organosulfur compounds in laboratory cultures or in anoxic environments could be predisposed to failure by the toxicity associated with these compounds to anaerobic microbial metabolism.

INTRODUCTION

The environmental significance of organosulfur compounds has received increased attention lately, in part because of their prevalence in petroleum and fossil fuels. A wide variety of naturally occurring organosulfur compounds have been identified, and the sources, biogeochemistry, and microbiology of organosulfur compounds in the environment was reviewed by Kelly and Smith (1). In addition, over 200 organosulfur compounds were identified as part of the small-scale sample program of API research project 48 (2). The major classes of organosulfur compounds in marine sediments and petroleum include thiols, organic sulfides, disulfides, and thiophene derivatives, which can make up a significant proportion of the sulfur content of oils. Crude oils may contain as much as 14% sulfur, with some fractions comprising 70% sulfur-containing compounds (3). In addition, elemental analysis indicates that sulfur can comprise as much as 14.6 weight percent of kerogens (4). Although there is considerable variation, the sulfide and thiophene contents were as much as 16% and 7.6% of Alberta petroleums, respectively (5). The total sulfur in coal ranges from 0.2 to 11 percent, although in most cases it is between 1 and 3 percent, and the amount of organic sulfur in coal is usually one-half to one-third of the total sulfur (6). In some high-sulfur coals 70% of organic sulfur comprises simple mononuclear thiophene derivatives (7). The major part of the organically-bound sulfur is produced by reactions of organic matter with reduced inorganic sulfur species during the early stages of diagenesis (8). The number and structural complexity of the organosulfur compounds identified has continued to increase significantly, as has the characterization and biogeochemistry of organically-bound sulfur in kerogen, coal asphaltenes, resins, and petroleum (8).

Although a wide variety of organosulfur compounds has been detected from environmental sources, very little is known about their potential toxicity. Seymour et al. (9) evaluated the acute toxicity of condensed thiophenes and their microbial metabolites

using the Microtox and *Daphnia magna* bioassays. Benzothiophene, 3-, and 5-methylbenzothiophenes, and dibenzothiophene had IC50 values in the Microtox assay of 1.7, 0.67, 0.70, and 0.16 mg/L respectively, making them more toxic than phenol. The partially oxidized benzothiophenes tested were both more water soluble and less toxic than the parent compounds from which they were derived. In addition, the toxicity of culture supernatants removed from cultures of a *Pseudomonas* strain capable of oxidizing the thiophenes decreased as the parent substrates were transformed (9). However, thiophene sulfoxides are more mutagenic than their parent thiophenes (10). Benzothiophene was previously reported to cause fatty distrophy of the liver and decreased leukocyte and erythrocyte counts when administered orally to rats or mice (LD50 1.26 and 0.96 g/kg respectively), whereas benzothiophene sulfone had higher LD50 values (3.75 and 1.57 g/kg, respectively) but significant cumulative toxicity (11). Furthermore, an LC50 value of 0.466 mg/L was reported for dibenzothiophene in a toxicity study of a series of aromatic sulfur heterocycles and structurally similar polycyclic aromatic hydrocarbons using the *D. magna* assay (12). The human health effects of organosulfur compounds depend on chemical structure, as different isomers exhibit varying degrees of carcinogenic activity (6). Benzonaphtho(1,2-d)thiophene and benzonaphtho(2,1-d)thiophene are noncarcinogenic, benzophenanthro(2,3-d)thiophene and benzophenanthro(3,2-d)thiophene are moderately carcinogenic, and benzophenanthro(3,4-d)thiophene is a very potent carcinogen (6, 13).

Oily wastes associated with petroleum extraction, refining, transportation, utilization, and disposal are often released accidentally or intentionally into the environment. If released on land, these wastes can enter the subsurface where the readily degradable components will be metabolized by microorganisms at the expense of the available oxygen reserves. Once oxygen reserves are depleted, the type of anaerobic conditions that develop will depend on the availability of electron acceptors such as nitrate, iron, sulfate, or carbon dioxide, which in turn affects the susceptibility of components in

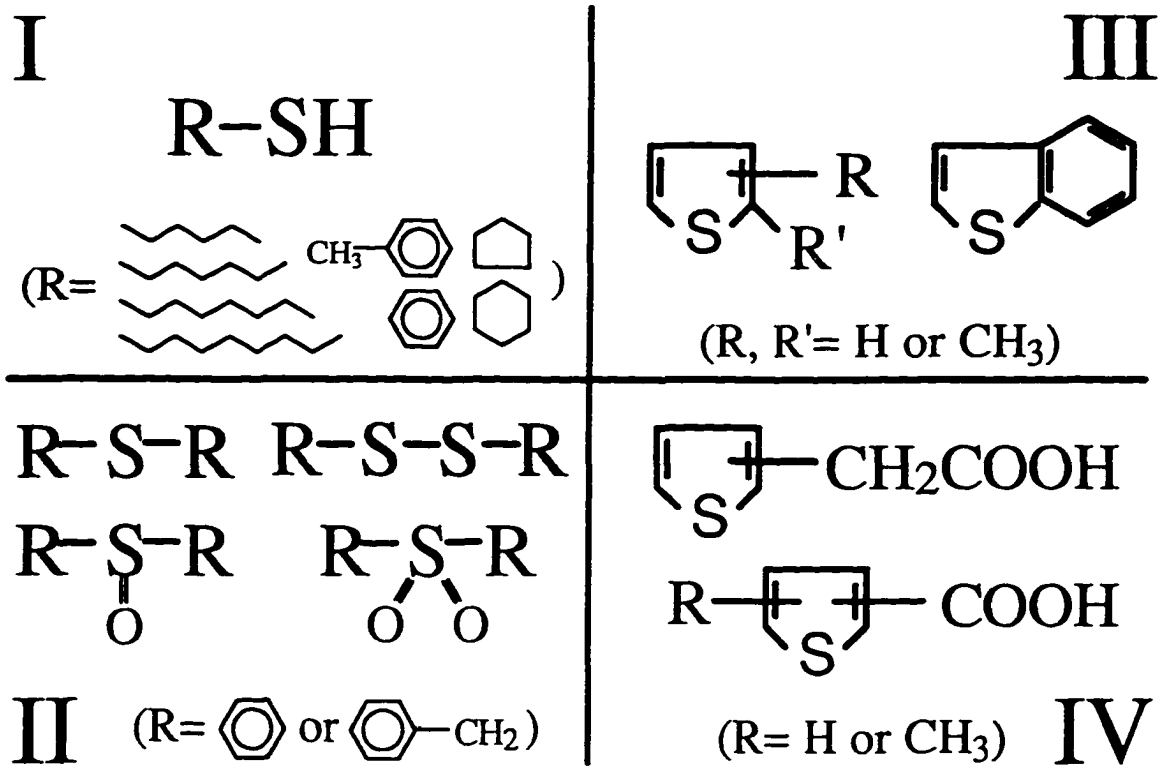

Fig. 1: General structures of organosulfur compounds under investigation. (I) Alkyl-, cyclic-, and aromatic thiols; (II) aromatic sulfides, sulfoxides, sulfones, and disulfides; (III) alkyl- and benzo-thiophene; and (IV) thiophene carboxylic and acetic acids.

the oily wastes to biodegradation. The inhibition of the indigenous microorganisms by organosulfur compounds has the potential to limit the microbial attack on these compounds and to influence the degree to which components of petroleum are transported in the environment.

We investigated the toxicity of model compounds from four representative classes of organosulfur compounds to anaerobic microbial activity. These included (i) alkyl, cyclic, and aromatic thiols, (ii) thiophene, alkyl thiophenes, and benzothiophene, (iii) thiophene carboxylic acids, thiophene acetic acids, and methylthiophene carboxylic acids, and (iv) aromatic sulfides, sulfoxides, sulfones, and disulfides (Fig. 1). All of these compounds have been detected in petroleum (2, 4, 14). In addition, some have also been detected in polluted ground waters (15), or were accumulated by algae, mosquitoes, and snails (16). We compared the relative toxicity of the four classes of compounds to microbial activity under methanogenic, sulfate- and nitrate-reducing conditions. Our results indicate that organosulfur compounds are inhibitory to anaerobic microorganisms and that the level of toxicity depends on both the structure of the organosulfur compounds and the terminal electron-accepting process in the incubations. This information is an important step in the evaluation of the transport and fate characteristics of organosulfur compounds in anaerobic environments.

MATERIALS AND METHODS

Media and incubation conditions

Brackish medium designed for the cultivation of sulfate-reducing bacteria (17) was prepared using strict anaerobic technique. Resazurin (0.0002%) was included as a redox indicator and the medium was reduced with sodium sulfide (1 mM). The medium was amended with neutralized sterile anoxic solutions of sodium lactate (10 mM) and sodium sulfate (20 mM), as electron donor and acceptor respectively. The medium received

sodium nitrate (20 mM) instead of sulfate or was not amended with an electron acceptor other than carbon dioxide, to cultivate cells under nitrate-reducing or methanogenic conditions, respectively. The medium was dispensed into serum bottles (25 ml) inside an anaerobic chamber and each culture was inoculated with 0.5 ml of oily sludge unless otherwise indicated. The oily sludge used as inoculum was collected from a settling tank (Tank #63) from the US Navy Craney Island Fuel Depot in Portsmouth, Virginia, and stored in sealed glass bottles at room temperature. Serum bottles were sealed with composite stoppers (Mormile and Suflita 1996), removed from the chamber, and secured with aluminum crimp seals. The headspace of the serum bottles was adjusted to N_2:CO_2 (80:20). For each of the three electron-accepting conditions, duplicate cultures were prepared with varying amounts of the four compound mixtures. Positive controls received lactate but did not receive organosulfur compounds. Due to the limited aqueous solubility of the thiols, thiophenes, and aromatic sulfides, these compounds were added to cultures in an organic overlay of 2,2,4,4,6,8,8-heptamethylnonane (HMN). In contrast, the more polar thiophenic acids were added from aqueous stock solutions. Cultures were incubated at room temperature, in the dark, without shaking. At the start of the experiment and periodically thereafter, samples of the aqueous phase were withdrawn and stored frozen until analyzed. Prior to analysis, the samples were thawed and centrifuged (10 000 x g) for 5 min to separate particulate debris. Strict anaerobic technique was used at all times in the preparation and sampling of cultures.

Alicyclic, cyclic, and aromatic thiols

The "thiols" consisted of eight compounds, all containing a mercaptan group, including pentanethiol, hexanethiol, heptanethiol, octanethiol, cyclopentanethiol, cyclohexanethiol, phenyl mercaptan (benzenethiol), and benzyl mercaptan (toluenethiol). Stock solutions of thiols were prepared by adding pre-determined amounts of each compound to three serum bottles, adding HMN that had been thoroughly flushed with N_2

to expel oxygen, sealing the bottles with a composite stopper under a flow of N_2, and autoclaving the mixture. The N_2 was scrubbed of oxygen by passage over heated copper filings. The HMN mixtures were added to 15 ml cultures to give approximately 0, 0.5, 5, and 50 mmol of each compound per L aqueous culture. Duplicate sterile controls received lactate, sulfate, nitrate, as well as 50 mmol/L of each thiol and were autoclaved. For this experiment only, the lactate and sulfate concentrations were 4 mM and 10 mM, respectively.

Thiophenes

The "thiophenes" consisted of five compounds possessing a thiophene ring, including thiophene, 2-methylthiophene, 3-methylthiophene, 2,5-dimethylthiophene, and benzothiophene. Stock solutions of these compounds were prepared in HMN as for the thiols except that the solution of HMN was flushed with N_2 after the addition to the thiophenes. Thiophenes were added to 15 ml cultures to give 0, 3.3, 6.7, or 10 mmol of each compound per L aqueous culture. Duplicate sterile controls received lactate, sulfate, nitrate, as well as 10 mmol/L of each compound and were autoclaved.

Thiophenic acids

The "thiophenic acids" consisted of six compounds having a thiophene ring and either carboxyl or acetyl groups, including thiophene-2-carboxylic acid, thiophene-3-carboxylic acid, thiophene-2-acetic acid, thiophene-3-acetic acid, 3-methylthiophene-2-carboxylic acid, and 5-methylthiophene-2-carboxylic acid. A neutralized, sterile, anoxic, aqueous stock solution was prepared containing all six compounds, and added to duplicate 100 ml cultures to give 0, 0.05, 0.10, and 0.50 mmol/L of each compound. The organic HMN overlay was not used, and cultures were prepared with 20 ml sludge inoculum. Duplicate sterile controls established under each incubation condition contained 0.10 mmol/L of each thiophenic acid and were autoclaved.

Aromatic sulfides, sulfoxides, sulfones, and disulfides

The "aromatic sulfides" consisted of ten related compounds, including phenyl sulfide, phenyl sulfoxide, phenyl sulfone, phenyl disulfide, benzyl sulfide, benzyl sulfoxide, benzyl sulfone, and benzyl disulfide. Stock solutions of these compounds were prepared in HMN as for the thiophenes, and added to 10 ml cultures to give 0, 1, 5, and 10 mmol of each compound per L aqueous culture. Duplicate sterile controls established under each incubation condition received 2 mmol/L culture of aromatic sulfides and were autoclaved.

Analytical techniques

Sulfate and nitrate were analyzed using Dionex IC system with an AS4A-SC column (Dionex, Sunnyvale, CA) as previously described (Londry and Suflita, submitted), except for the experiments with the thiols and thiophenes in which a previously described high performance liquid chromatography (HPLC) system was used (Smolenski and Suflita, 1987). For experiments with thiophenic acids, sulfate, nitrate, and lactate were all analyzed using a Dionex IC system with an AS11 column as previously described (Londry and Suflita, submitted). Otherwise, lactate was analyzed using a HPLC system (Beckman Instruments, Berkley, CA) with an Aminex HPX-57H organic acid column (Bio-Rad Laboratories, Richmond, CA) with a 0.016N H_2SO_4 mobile phase at 0.9 ml/min and UV detection (214 nm). Methane was analyzed by gas chromatography as previously described (Mormile and Suflita 1996). The amount of methane in the headspace of cultures was calculated as percent of headspace by comparison with external standards analyzed the same day.

Hydrophobic substrates were analyzed by gas chromatography (GC) using a Hewlett Packard (Palo Alto, CA) 5890 Series II GC, with a Carbograph VOC capillary column (30 m, Alltech Associates, Inc., Deerfield, IL). The oven temperature programs

were as follows: thiols - an initial temperature of 50°C held for 2 min raised at 3°C/min to 140°C then raised to 200 °C at 25 °C/min and held for 3 min; thiophenes - initially 40°C for 5 min raised at 20°C/min to 240°C for 2 min; and aromatic sulfides - initially 160°C for 12 min raised at 15°C/min to 220°C for 4 min. Benzyl sulfoxide and benzyl disulfide were not separated by this procedure, and both phenyl and benzyl sulfones were only slightly soluble in the HMN and gave much smaller responses than the other compounds. Samples of the HMN organic overlay (1 μL) were removed from the cultures and injected directly without extraction or derivatization. Substrates were detected using a flame ionization detector. The amount of substrate was calculated from the ratio of the peak area of each compound to external standards analyzed the same day. Only one of the duplicate cultures of each concentration under each condition was analyzed for organosulfur compound loss.

Thiophenic acids were analyzed with a HPLC system (Beckman Instruments), equipped with an Econosphere reverse-phase C_{18} column (Alltech Associates), and a mobile phase consisting of 7.5% acetonitrile and 92.5% 50 mM sodium acetate (pH 4.5) at 1 ml/min. Compounds were detected using a variable wavelength detector at 240 nm, which was determined to be at or near the absorbance maximum for each compound in the class. The amount of substrate was calculated by comparison to a standard curve based on standards analyzed the same day. Only one of the duplicate cultures of each concentration under each condition was analyzed for substrate.

Chemicals

Organosulfur compounds were obtained from Aldrich Chemical Co. (Milwaukee, Wis.), were of at least 97% purity, and used without further purification. HMN was obtained from Sigma Chemical Co. (St. Louis, Mo).

RESULTS

Effects of thiols on microbial activity

A toxicity experiment was performed to determine whether mixtures of thiols would affect anaerobic activity, as determined by the rate and extent of lactate degradation, nitrate reduction, sulfate reduction, and methanogenesis. Although the organothiols were potential substrates under these conditions, analysis of the amount of thiols in the organic overlay indicated that they were not degraded in these cultures (data not shown) during the initial 35 day incubation period in which lactate metabolism occurred.

Addition of the thiol mixtures decreased the rate of lactate degradation compared to the positive controls without thiols, under all three electron-accepting conditions. Under methanogenic conditions, thiols caused a slight inhibition of lactate degradation at 0.5 mmol/L, moderate inhibition at 5 mmol/L, and almost complete inhibition at 50 mmol/L (Table 1). In contrast, under sulfate-reducing conditions, there was no apparent effect of thiols on lactate degradation at 0.5 or 5 mmol/L since lactate was completely consumed within one week. There was almost complete inhibition of lactate degradation when the highest amount of thiols was similarly evaluated (Table 1). Acetate accumulated to as much as 2 mM in 21 days in lactate-degrading cultures under methanogenic and sulfate-reducing conditions, but not under nitrate-reducing conditions. The rate of lactate degradation under nitrate-reducing conditions steadily decreased with increasing amounts of thiols (Table 1). Therefore, although the inhibitory effects were the least under sulfate-reducing conditions, 50 mmol/L of thiols was still inhibitory under all three conditions.

Nitrate reduction was also affected by the addition of thiols, and the inhibition was dependent on the amount of thiols added (Table 1). In contrast, sulfate reduction was not affected by the addition of thiols, as the rate of sulfate reduction was essentially equal in all non-sterile cultures. The inhibitory effect of the thiols on anaerobic activity were most

Table 1. Effects of thiols on lactate degradation and electron acceptor reduction under methanogenic, sulfate-reducing, and nitrate-reducing conditions. Average rates are compared for positive controls without thiols, cultures with three different amounts of thiols, and sterile controls.

Thiol	Lactate degradation (mM/day)			Sulfate	Nitrate
(mmol/L)[a]	Methanogenic	Sulfate-reducing	Nitrate-reducing	(mM/day)	(mM/day)
0	0.36	≥0.58[b]	0.36	0.58	1.23
0.5	0.34	≥0.54	0.29	0.44	1.10
5	0.19	≥0.59	0.17	0.55	0.63
50	0.02	0.00	0.05	0.64	0.00
sterile		(0.01)[c]		0.06	ND[d]

[a] Amount of each of eight thiol compounds added per L of aqueous culture

[b] Minimum rate of lactate degradation, as lactate depleted by first sampling time

[c] Sterile controls for whole experiment rather than each condition

[d] Not determined

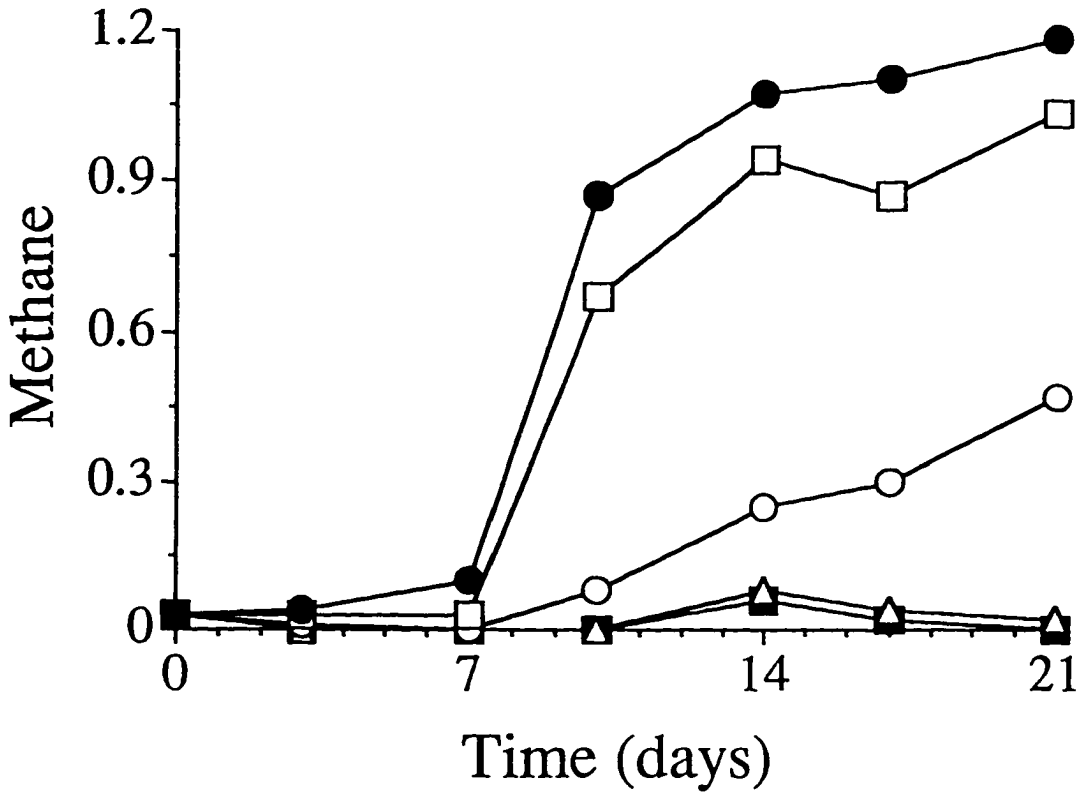

Fig. 2: Effects of thiols on methanogenesis in anaerobic cultures established with oily sludge inoculum and lactate as a substrate. Compared to positive controls without thiols (●), the addition of 0.5 mmol/L (□), 5 mmol/L (○), or 50 mmol/L (Δ) of each thiol resulted in decreased methanogenesis, almost to levels in sterile controls (■).

evident for methanogenesis. Positive controls in which lactate was degraded accumulated methane in the headspace, to up to 1.2% of the headspace volume in 21 days (Fig. 2). Increasing amounts of thiols resulted in slower rates of methanogenesis compared to thiol-free controls, and the amount of methane that accumulated in cultures with 50 mmol/L of thiols was no greater than the sterile controls (Fig. 2). As with lactate degradation, these results confirmed that microbial activity under sulfate-reducing conditions was the least affected by thiol addition.

Effects of thiophenes on microbial activity

Thiophenes inhibited lactate degradation under all three incubation conditions, and the effect was dependent on the amount of thiophenes added. Surprisingly, there was no lactate degradation in the methanogenic positive controls, but the rate of lactate degradation decreased with 6.7 mmol/L and 10 mmol/L of thiophenes relative to the incubation containing 3.3 mmol/L (Table 2). As much as 4 mM acetate accumulated in these cultures, and that corresponded with the amount of lactate degraded in 14 days. Under sulfate-reducing conditions, less lactate was degraded as the amount of thiophenes increased (Table 2). Acetate accumulated in sulfate-reducing cultures, reaching concentrations of 5 mM in 14 days. Lactate degradation under nitrate-reducing conditions progressed at decreasing rates with increasing amounts of thiophenes, with complete inhibition at 10 mmol/L (Table 2). Therefore, the effects of thiophenes on lactate degradation were most pronounced under nitrate-reducing conditions and least under sulfate-reducing conditions, but 10 mmol/L was the most inhibitory under all redox conditions.

Thiophenes also affected the rate of nitrate reduction and methanogenesis, but had only marginal effects on sulfate reduction. Nitrate reduction was inhibited completely at 10 mmol/L, whereas the rate of sulfate reduction was lowered only somewhat by the same amount (Table 2). Methanogenesis was inhibited by all test amounts of thiophenes, in a

Table 2. Effects of thiophenes on lactate degradation and electron acceptor reduction under methanogenic, sulfate-reducing, and nitrate-reducing conditions. Average rates are compared for positive controls without thiophenes, cultures with three different amounts of thiophenes, and sterile controls.

Thiophene[a]	Lactate degradation (mM/day)			Sulfate	Nitrate
(mmol/L)	Methanogenic	Sulfate-reducing	Nitrate-reducing	(mM/day)	(mM/day)
0	0.00	1.06	1.08	0.27	0.75
3.3	0.39	1.07	0.87	0.32	1.32
6.7	0.25	0.92	0.55	0.31	0.77
10	0.05	0.17	0.01	0.21	0.09
sterile		(0.00)[b]		0.14	0.00

[a] Amount of each of five thiophene compounds added per L of aqueous culture

[b] Sterile controls for whole experiment rather than each condition

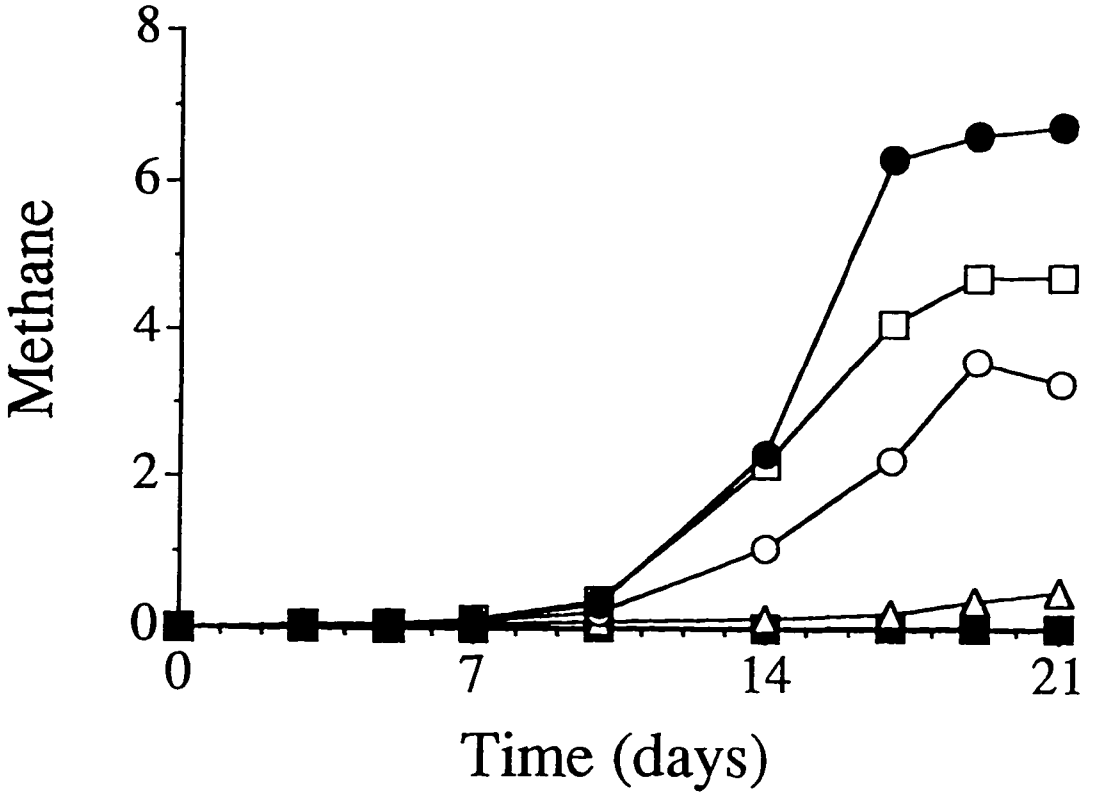

Fig. 3: Effects of thiophenes on methanogenesis in anaerobic cultures established with oily sludge inoculum and lactate as a substrate. Compared to positive controls without thiophenes (●), the addition of 3.3 mmol/L (□), 6.7 mmol/L (○), or 10 mmol/L (Δ) of each thiophene caused decreased methanogenesis. Sterile controls (■) are shown for comparison.

concentration-depenedent fashion. Methane production in the presence of 10 mmol/L was only slightly greater than sterile controls (Fig. 3). The thiophenes themselves were not transformed during 35 weeks incubation and therefore did not contribute to electron acceptor reduction in these cultures. Overall, the inhibitory effect of thiophenes was greatest on methanogenesis, intermediate for nitrate reduction, and minimal under sulfate-reducing conditions.

Effects of thiophenic acids on microbial activity

A second batch of oily sludge was obtained from the same source to use as inoculum for the toxicity experiments with the thiophenic acids. The microbial activity associated with this sludge was somewhat different than the first batch in that lactate was not completely degraded under methanogenic conditions even in the positive controls, and there was more methanogenesis, sulfate reduction, and nitrate reduction associated with endogenous substrates. Nevertheless, it was still possible to determine the effects of the thiophenic acids on the various microbial activities in these cultures. None of the thiophenic acids were transformed during 49 days of incubation, and therefore did not contribute to electron acceptor reduction in these cultures.

The addition of thiophenic acids affected the extent of lactate degradation under methanogenic conditions, the rate of lactate degradation under sulfate-reducing conditions, but not the extent and apparently not the rate of lactate degradation under nitrate-reducing conditions. Under methanogenic conditions, the amount of lactate transformed to acetate depended on the concentration of thiophenic acids added, with less conversion in the presence of increasing amounts of thiophenic acids (Table 3). Under sulfate-reducing conditions, lactate was completely degraded in all non-sterile cultures within 28 days, with no accumulation of acetate in these cultures. However, the rate of lactate degradation decreased slightly with increasing concentrations of thiophenic acids (Table 3). Insufficient data were obtained to determine the effects of thiophenic acids on the rate of lactate

degradation under nitrate-reducing conditions, although effects were minimal as lactate was completely degraded even in the presence of thiophenic acids within 3 weeks, and acetate did not accumulate in these cultures. Therefore, the thiophenic acids had only minimal inhibitory effects on lactate degradation at the concentrations employed, under any of the redox conditions.

In consistent fashion, thiophenic acids did not significantly affect methanogenesis or nitrate reduction, although the rate of sulfate reduction was lower in the presence of these compounds. The complete degradation of lactate would theoretically result in the reduction of 15 mM sulfate or 24 mM nitrate in these cultures. Nitrate (20 mM) was depleted in all non-sterile cultures within two weeks, suggesting that the thiophenic acids did not affect nitrate-reducing bacteria. Methanogenesis occurred readily in the presence of thiophenic acids (Fig. 4). The amount of methane produced (15% of headspace) in these cultures that only partially metabolized lactate suggested the presence of other substrates in the oily sludge amenable to anaerobic decay. Certainly the thiophenic acids did not preclude the methanogenic degradation of the endogenous substrates. In contrast, the reduction of sulfate was affected by this class of organosulfur compounds, as the rate of sulfate depletion decreased at the highest amount of thiophenic acids tested (Table 3). However, 20 mM sulfate was still depleted within 35 days in all cultures, again suggesting the presence of readily degradable substrates in the sludge. Overall, the results of the electron-accepting processes mirrored those of the lactate degradation results, and indicated that thiophenic acids had only minimal inhibitory effects under sulfate-reducing conditions at 0.5 mmol/L, but had minimal impact on microbial activity under methanogenic and nitrate-reducing conditions. Therefore, in contrast to the thiols and thiophenes, the thiophenic acids were relatively more inhibitory to biodegradation under sulfate-reducing conditions.

Table 3. Effects of thiophenic acids on lactate degradation and electron acceptor reduction under methanogenic, sulfate-reducing, and nitrate-reducing conditions. Average rates are compared for positive controls without thiophenic acids, cultures with three different amounts of thiophenic acids, and sterile controls.

Thiophenic	Methanogenic[b]		Lactate degradation (mM/day)		Sulfate	Nitrate
acid (mmol/L)[a]	Lactate	Acetate	Sulfate-reducing	Nitrate-reducing	(mM/day)	(mM/day)
0	3.48	4.02	0.52	≥0.58[c]	0.77	≥1.56[d]
0.05	2.77	2.74	0.52	≥0.58	0.78	≥1.58
0.10	2.53	1.61	0.47	≥0.61	0.73	≥1.67
0.50	2.49	0.49	0.41	≥0.06	0.59	≥1.63
sterile	0.00	0.00	0.01	0.00	0.00	0.00

[a] Concentration of each of six thiophenic acid compounds added to cultures

[b] Under methanogenic conditions, decrease in lactate (mM) and accumulation of acetate (mM) during the first 14 days of incubation

[c] Minimum rate of lactate degradation, as lactate was depleted by first sampling time

[d] Minimum rate of nitrate reduction, as nitrate was depleted by first sampling time

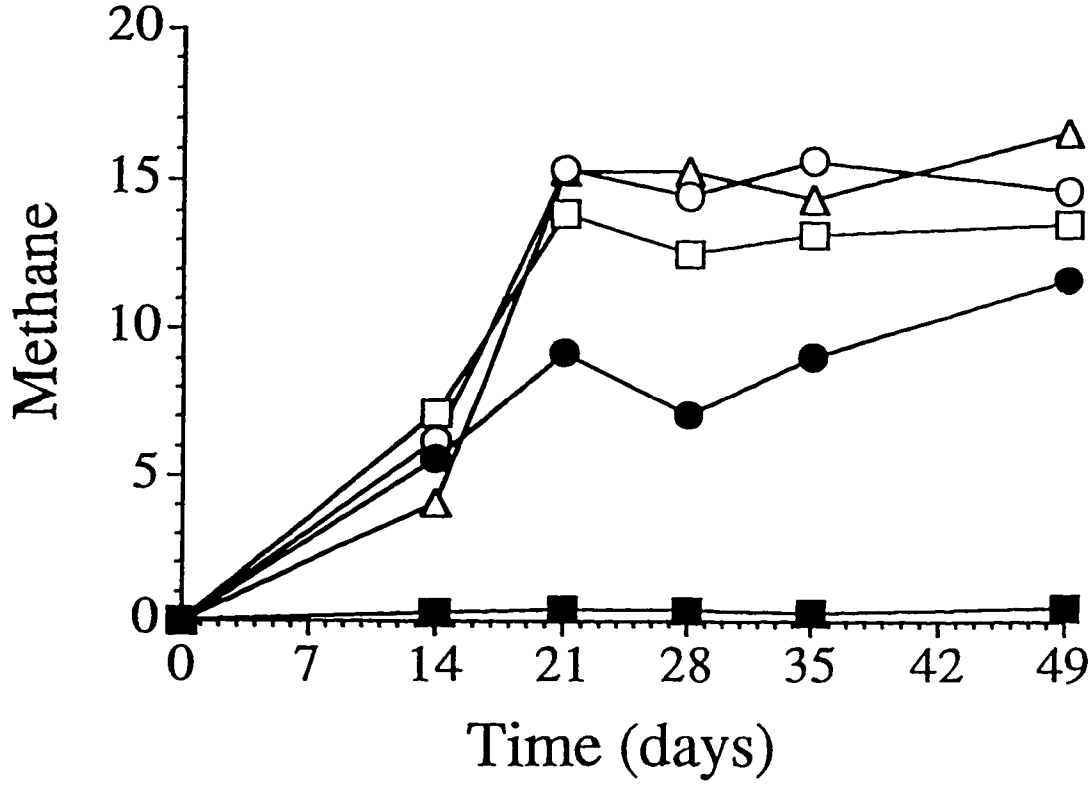

Fig. 4: Effects of thiophenic acids on methanogenesis in anaerobic cultures established with oily sludge as inoculum and lactate as a substrate. The addition of 0.05 mmol/L (□), 0.10 mmol/L (○), or 0.50 mmol/L (Δ) of each compound resulted in uniformely increased methanogenesis compared to positive controls without thiophenic acids (●) and sterile controls (■).

Effects of aromatic sulfides on microbial activity

The effect of aromatic sulfides on lactate degradation depended on both the amount of compounds added and the electron-accepting condition. Aromatic sulfides had only a slight inhibitory effect on lactate degradation under methanogenic or nitrate-reducing conditions, whereas lactate degradation was significantly inhibited under sulfate-reducing conditions at 5 or 10 mmol/L (Table 4). Acetate accumulated up to 2 mM in 28 days in methanogenic cultures, and as much as 5 mM in sulfate-reducing cultures. Lactate degradation did not occur in any of the sulfate-reducing cultures until after an initial 7 day lag period. The rates reported in Table 4 are calculated after the acclimation period was overcome. The decreased rates of microbial activity were also observed under methanogenic conditions, in which methane production started after a 14 day lag period. The aromatic sulfides, with the possible exception of phenyl and benzyl sulfoxides, were not transformed during the 42 days of incubation, and did not appear to contribute to electron acceptor reduction in these cultures. The addition of the amounts of aromatic sulfides tested had only slight inhibitory effects on lactate degradation under methanogenic and nitrate-reducing conditions at all but the highest concentrations, whereas the aromatic sulfides were more inhibitory under sulfate-reducing conditions.

Aromatic sulfides had a significant concentration-dependent inhibitory effect on sulfate reduction, nitrate reduction, and methanogenesis. Rates of sulfate reduction decreased in cultures containing 1, 5, or 10 mmol/L sulfides by 21%, 66%, and 84% respectively, compared to positive controls (Table 4). Similarly, rates of nitrate reduction decreased with increasing amount of sulfides, with very little activity at 10 mmol/L (Table 4). Methanogenesis was not affected by the addition of 1 mmol/L sulfides but larger amounts inhibited this process compared to positive controls (Figure 5). These compounds appear to affect the microbial populations responsible for electron accepting processes more

Table 4. Effects of aromatic sulfides on lactate degradation and electron acceptor reduction under methanogenic, sulfate-reducing, and nitrate-reducing conditions. Average rates are compared for positive controls without aromatic sulfides, cultures with three different amounts of aromatic sulfides, and sterile controls.

Sulfide[a]	Lactate degradation (mM/day)			Sulfate	Nitrate
(mmol/L)	Methanogenic	Sulfate-reducing	Nitrate-reducing	(mM/day)	(mM/day)
0	0.34	0.71	0.23	0.80	0.87
1	0.31	0.64	0.27	0.63	0.33
5	0.24	0.28	0.21	0.27	0.18
10	0.24	0.22	0.02	0.13	0.09
sterile	0.04	0.02	0.00	0.00	0.05

[a] Amount of each of ten aromatic sulfide compounds added per L aqueous culture volume

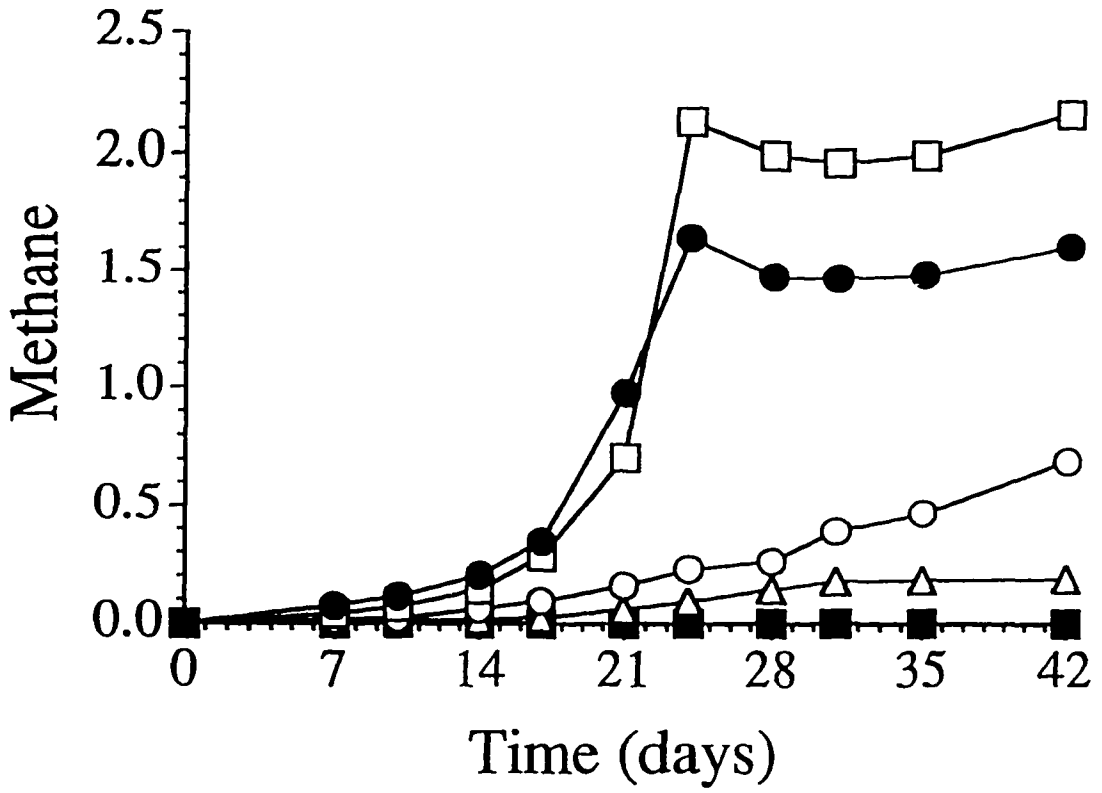

Fig. 5: Effects of aromatic sulfides on methanogenesis in anaerobic cultures established with oily sludge inoculum and lactate as a substrate. Compared to positive controls without sulfides (●), the addition of 1 mmol/L of each compound (□) increased methanogenesis, yet addition of 5 mmol/L (○), or 10 mmol/L (Δ) decreased methanogenesis, and there was no methanogenesis in sterile controls (■).

than those responsible for lactate degradation. In contrast with the thiols and thiophenes, the aromatic sulfides were most inhibitory under sulfate-reducing conditions, and least inhibitory under nitrate-reducing conditions.

Comparison of inhibitory effects

The inhibitory effects of the four classes of organosulfur compounds varied for each electron-accepting condition. Table 5 compares the effects of each group of compounds for each incubation condition. The star ratings were calculated by giving equal weight to effects of each mixture on lactate degradation and the corresponding electron acceptor reduction. Under methanogenic conditions, the thiols and thiophenes were relataively inhibitory, the aromatic sulfides were less so, but the thiophenic acids were the most inhibitory on a mole per volume basis. Under sulfate-reducing conditions, the aromatic sulfides were the most inhibitory, the thiophenes were somewhat inhibitory, the thiols had little effect except at the highest concentration, and the thiophenic acids were inhibitory only at the highest concentrations used. Under nitrate-reducing conditions, the effects of the thiophenic acids could not be compared to the other classes as no inhibition was observed, but of the other classes the aromatic sulfides were most inhibitory, the thiols were quite inhibitory, and the thiophenes had the least effect, although all three groups of compounds had similar effects overall on a mole per volume basis.

Although the four classes of compounds were evaluated in separate experiments at different amounts, a qualitative comparison of the toxicity of the various compounds was possible (Table 5). As previously discussed, the thiophenic acids had little effect under any condition at the low concentrations tested. All the other classes of compounds were tested at approximately 5 mmol of each compound per L of culture, and at these levels the aromatic sulfides had the greatest inhibitory effect, and the thiols and thiophenes were roughly similar.

Table 5. Concentration-dependent inhibitory effects of four classes of organosulfur compounds on microbial activity under three anaerobic conditions.

Compound class	Amount (mmol/L)[a]	Methanogenic	Sulfate-reducing	Nitrate-reducing
Thiols	0.5	☆☆☆☆☆☆☆[b]	☆☆☆☆☆☆☆	☆☆☆☆☆☆☆
	5	☆☆☆	☆☆☆☆☆☆☆	☆☆☆
	50	-	☆☆☆	-
Thiophenes	3.3	☆☆☆☆☆	☆☆☆☆☆☆☆	☆☆☆☆☆☆☆
	6.7	☆☆☆	☆☆☆☆☆	☆☆☆☆☆
	10	-	☆☆☆	-
Thiophenic	0.05	☆☆☆☆☆	☆☆☆☆☆☆☆	☆☆☆☆☆☆☆
acids	0.10	☆☆☆☆☆	☆☆☆☆☆☆☆	☆☆☆☆☆☆☆
	0.50	☆☆☆☆☆	☆☆☆☆	☆☆☆☆☆☆☆
Aromatic	1	☆☆☆☆☆☆☆	☆☆☆☆☆☆☆	☆☆☆☆
sulfides	5	☆☆☆	☆☆	☆☆☆
	10	☆☆	☆	-

[a] Amount of each organosulfur compound in each class per L of aqueous culture volume.

[b] Total effect equally weighted for lactate degradation and electron-acceptor reduction (3 stars each). ☆☆☆ = >75%, ☆☆ = 50-75%, ☆ = 25-50%, no stars (-) for <25% of rates of lactate degradation, methanogenesis, sulfate- or nitrate-reduction in positive controls. Methane production was compared to positive controls after 21, 21, 49, and 42 days for compound classes in order listed in table.

DISCUSSION

The toxicity studies indicated that three of the four groups of organosulfur compounds inhibited microbial activity, in terms of lactate degradation or electron acceptor reduction or both, under methanogenic, sulfate-reducing, and nitrate-reducing conditions. The four compound classes were investigated in separate experiments and microbial activities in the positive controls without organosulfur compounds were easily evident but slightly different in each case. The inhibitory effects were dependent on the amount of organosulfur compound added to cultures, so dose response relationships could be determined for all but the thiophenic acids.

The inhibitory effects of the organosulfur compounds on lactate degradation did not necessarily correlate with electron acceptor reduction. This is not surprising when one considers that the activities being measured were for interacting microbial populations, in which the organisms transforming lactate are not necessarily the same ones reducing sulfate or nitrate, or producing methane. The accumulation of acetate under methanogenic and sulfate-reducing conditions, but not nitrate-reducing conditions also indicated that several different organisms were active in these cultures. The accumulation of acetate observed in many cultures may reflect a greater inhibition of methanogens than the syntrophic partners by the organosulfur compounds, although further studies would be required to delineate such effects.

In general, the toxicity of organosulfur compounds indicated by our results were consistent with previous observations. The aromatic sulfides were quite inhibitory to anaerobic microbial processes when added from 10 - 50 mmol/L. This is consistent with a previous report that thianaphthalene, phenyl sulfoxide, and 2-aminophenyl disulfide inhibited growth of *Rhodococcus rhodochrous* AGTS8, and tolyldisulphide, thiamin and sulfanilamide were specific inhibitors of the desulfurization activity of this organism, when

these organosulfur compounds were added at 20 mM concentrations (21). Likewise, benzothiophene was partially toxic to a *Pseudomonas aeruginosa* isolate when incorporated into cultures in a light oil phase at 19 mmol/L of total culture (22). We observed partial inhibition of microbial activity at 35 mmol/L of total thiophenes and substantial inhibition at 50 mmol/L in our mixed anaerobic cultures. By contrast, the effects of these compounds on *Daphnia magna* or *Photobacterium phosphoreum* were much more pronounced, with LC50 and IC50 values for benzothiophene of 0.44 mM and 0.01 mM, respectively (9). In our experiments, thiophenic acids exhibited minor toxicity effects when a total of 3 mM were added to cultures. Thiophene-2-carboxylate has previously been reported as a potentially toxic substrate, as growth of a *Rhodococcus* was inhibited at 15 mM and prevented at 80 mM concentrations (23). In addition, this compound became strongly inhibitory at concentrations above 9 mM to growth of a *Vibrio* isolate (24). By comparison, our results suggest that either anaerobic microorganisms were more sensitive to these compounds than these aerobic isolates that degrade thiophene-2-carboxylate, or else that other compounds in the mixture may be more inhibitory than this representative compound.

The four groups of organosulfur compounds were generally more toxic than their counterparts that do not contain sulfur. In our multi-phase incubation system established with oily sludge, inhibition of anaerobic microbial activity occurred at total amounts of as little as 40 mmol/L for thiols. In separate studies, we determined the toxicity effects of *n*-alkanes, *n*-alkanols, and *n*-alkanoic acids under the same conditions as these experiments. We found no inhibition of microbial activity at 110 mmol/L total *n*-alkanes (C_{10}-C_{20}), or at 80 mmol/L of *n*-alkanols (C_{11}-C_{20}) (18). Therefore removal of the sulfur moiety from the alkylthiols, or substitution of the sulfur with oxygen, reduces the toxicity of these compounds considerably. The aromatic hydrocarbon benzene has been reported to inhibit gas production during alfalfa fermentation at 30 mmol/L (25), acetoclastic methanogenesis in granular sludge at 20 mmol/L (26), and methane production by a methanogen at 30

mmol/L (27). Similarly, cyclohexane was found to have slight inhibitory effects on glucose methanogenesis at 0.8 mmol/L (20). We can only speculate that the cyclic and aromatic thiols may have contributed to the toxicity of the organothiols more than the alkylthiols. The mechanisms of toxicity for the organosulfur compounds are unknown and would require further study, but could be related to the toxicity effects of related hydrocarbons, which have been reviewed recently by Sikkema and colleagues (28).

Regardless of the class of compound tested and which redox conditions were used, the toxicity effects were dependent on the amount of organosulfur compounds added to the cultures. This is an advantage for conducting laboratory-based microcosm or biodegradation studies, as the amounts of these compounds can be controlled and reduced to non-toxic levels, thereby not predisposing biodegradation experiments to failure (29). In the environment, the release of organosulfur compounds could seriously impact ground water reservoirs if they are transported to the terrestrial subsurface (15). However, these organosulfur compounds are found typically only in small amounts in the complex mixtures that make up petroleum, coal, and creosote. In addition, sulfur heterocyclic aromatic compounds such as dibenzothiophene are absorbed strongly to sediments, as the sulfur atom does not appear to significantly influence the sorptive behavior compared to polycyclic aromatic hydrocarbons (30). Therefore, their toxicity to humans and animals would normally be expected to be minimal due to limited exposure. Indeed, sulphoxides and sulphones are not usually chemically reactive, and therefore do not pose any toxicological problems (31). However, these compounds could seriously impact microbial communities in sediments and subsurface environments when these fossil fuels are released into sensitive habitats. Organosulfur compounds could inhibit the activities of anaerobic communities responsible for biodegradation of a wide variety of components in these wastes, thus limiting the potential for intrinsic bioremediation.

In general, the organosulfur compounds were not degraded or transformed during the course of these experiments. The amount of each compound was monitored by

appropriate analytical techniques and did not decrease over time, even in cultures in which no inhibitory effects on microbial activity were indicated. The possible exceptions were phenyl and benzyl sulfoxide, but further research is required to clarify and confirm this observation. These results do not necessarily indicate that all of these organosulfur compounds are recalcitrant under anaerobic conditions, as these cultures were monitored only for 5 to 7 weeks, whereas the lag period prior to anaerobic degradation of many compounds is typically much longer (29). These cultures will be monitored further for substrate loss, and additional experiments will be performed to determine whether they are susceptible to anaerobic biodegradation. This information is needed for understanding the fate of compounds associated with oily wastes in anaerobic environments and is necessary for predicting the potential for *in situ* bioremediation of components should they be released into the environment.

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Chapter 6.

ANAEROBIC BIODEGRADATION OF ORGANOTHIOLS

Abstract

Degradation of eight organothiol compounds was examined under nitrate-reducing, sulfate-reducing, and methanogenic conditions. A multi-phase incubation system was used that employed an organic carrier containing the hydrophobic substrates, overlaying anaerobic cultures inoculated with an oily sludge. Degradation of organothiols occurred under nitrate-reducing conditions, but not in sulfate-reducing or methanogenic cultures, or sterile controls. Biodegradation was observed for all eight thiols tested, whereas alkanes introduced from the oily sludge were not transformed in these cultures. Decrease of most of the thiols occurred after an initial lag period of 9 to 13 weeks, although benzenemethanethiol was metabolized without a lag period. Degradation of hexanethiol and cyclohexanethiol was accompanied by the stoichiometric reduction of nitrate. Nitrate reduction was associated with accumulation of nitrite and nitrous oxide in cultures, which appeared to inhibit microbial activity. The fate of the sulfur moiety of the thiols could not be accounted for as either sulfate or sulfide.

Introduction

Organosulfur compounds are naturally present in petroleum products, and can make up a significant proportion of the sulfur content of oils. These compounds are of environmental concern because their combustion to sulfur dioxide contributes to acid rain (Orr & Sinninghe-Damste´ 1990). In addition, oily wastes associated with petroleum processing, refining, utilization, transportation, and disposal are released into marine and subsurface environments every year. Organosulfur compounds are among the heterocyclic compounds that enter the environment from creosote and are known groundwater contaminants (Kuhn & Suflita 1989). Components of these heterogenous mixtures enter sediments or the subsurface, and as the readily degradable compounds are metabolized, oxygen reserves can be depleted and anaerobic conditions prevail. Depending on the availability of electron acceptors such as nitrate, iron, sulfate, or carbon dioxide, different anaerobic microbial processes will predominate. Understanding the fate of organosulfur compounds under anaerobic conditions is necessary to predict the potential for intrinsic bioremediation of fossil-fuel related components, as well as the feasibility of treatment processes for fossil fuels, including desulfurization.

Most reports of the biodegradation of organosulfur compounds involve the fate of the model compounds benzothiophene and dibenzothiophene under aerobic conditions. The degradation or transformation pathways for these compounds usually resemble those of structurally similar hydrocarbons or involve oxidation of the sulfur moiety (Fedorak 1990). A variety of organosulfur compounds are also degraded under anaerobic conditions. The transformation of volatile sulfides including methanethiol, dimethylsulfide has been studied extensively because of the roles these compounds play in sulfur release to the atmosphere in marine environments (Kelly & Smith 1990), and the anaerobic biodegradation of these compounds has been demonstrated (Visscher & Taylor 1993, Tanimoto & Bak 1994). There are also reports of benzothiophene degradation under methanogenic (Grbic'-Galic' 1990, Kurita et al. 1971), and sulfate-reducing (Kohler et al.

1984) conditions, as well as a report of dibenzothiophene degradation (Kohler et al. 1984). In addition, selective desulfurization of dibenzothiophene to biphenyl and hydrogen sulfide was catalyzed by *Desulfovibrio desulfuricans* (Kim et al. 1990), and by an uncharacterized Gram-positive isolate (Finnerty 1993), in solvent-based reaction media under a hydrogen atmospheres. A single report of thiophene degradation (Kurita et al. 1971), and a report of thiophene-2-carboxylate degradation under methanogenic conditions (Kuhn & Suflita 1989) also indicate that thiophene rings are susceptible to anaerobic attack.

The fate of alkyl and aromatic thiols, disulfides, and sulfides under anaerobic conditions has received limited attention. A sulfate-reducing bacterium transformed benzylsulfide to benzylmercaptan, toluene, and sulfide, but did not transform hexanethiol, cyclohexanethiol, or benzylmercaptan (Kohler et al. 1984). Similarly, a sulfate-reducing bacterium that degraded phenyl- and benzylsulfide did not degrade ethanethiol or butanethiol (Kim et al. 1990). Benzyldisulfide (Miller 1992) was transformed to benzylmercaptan in methanogenic consortia, and although recovery of products indicated that the carbon skeleton was not attacked, the detection of toluene from benzyldisulfide or benzylmercaptan indicated a potential for sulfur removal from aromatic thiols. Methane-, ethane-, *n*-propane-, and *n*-butanethiol were used by a variety of methanogenic bacteria as a sulfur source, but whether they also served as a carbon source was not determined (Rajagopal & Daniels 1986). By comparison, a nitrate-reducing *Thiobacillus* sp. grown on alkyl sulfides oxidized methane-, ethane-, and butanethiol under aerobic conditions, but these reactions did not support growth (Visscher & Taylor 1993).

We have investigated the anaerobic biodegradation of eight organothiols under nitrate-reducing, sulfate-reducing, and methanogenic conditions. This class of compounds included *n*-alkyl thiols five to eight carbons long, cyclic thiols, and aromatic thiols, as depicted in Figure 1. Each of these compounds has been detected in petroleum, and thiols

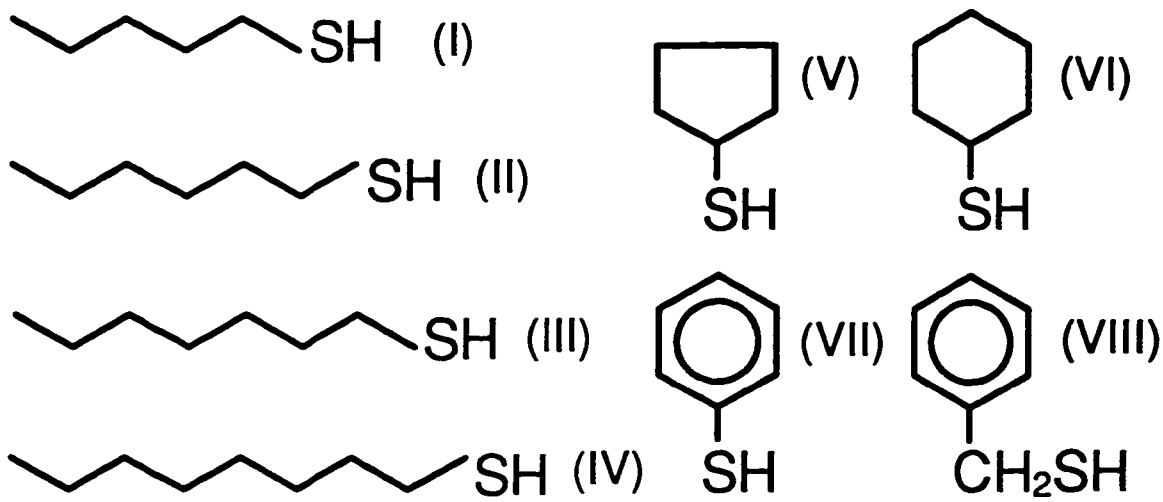

Figure 1: Structures of organothiols under investigation: (I) 1-pentanethiol, (II) 1-hexanethiol, (III) 1-heptanethiol, (IV) 1-octanethiol, (V) cyclopentanethiol, (VI) cyclohexanethiol, (VII) benzenethiol, (VIII) benzenemethanethiol.

can represent a large but variable proportion of the sulfur compounds present in vacuum distillates (Dean & Whitehead 1976, Orr & Sinninghe Damste´ 1990, Constantinides & Arch 1967). These substrates were combined and added as a mixture in an organic carrier to cultures established with oily sludge as an inoculum. We found that these compounds were susceptible to biodegradation under nitrate-reducing conditions, but were not attacked under sulfate-reducing or methanogenic conditions.

Materials and methods

Media and incubation conditions

A multi-phase incubation system was employed as previously described (Chapter 5). The oily sludge used as inoculum was collected from a settling tank (Tank #63) from the US Navy Craney Island Fuel Depot in Portsmouth, Virginia, and stored in sealed glass bottles at room temperature. Organothiols were added as a solution of 2,2,4,4,6,8,8-heptamethylnonane (HMN), negative controls received HMN only, and sterile controls received organosulfur compounds in HMN and were autoclaved. The thiols mixture included: pentanethiol, hexanethiol, heptanethiol, octanethiol, cyclopentanethiol, cyclohexanethiol, benzenethiol (phenyl mercaptan), and benzenemethanethiol (benzyl mercaptan). Stock solutions of thiols were prepared by adding pre-determined amounts of each compound to three serum bottles, adding HMN that had been thoroughly flushed with N_2 to expel oxygen, sealing the bottles with a composite stopper (Mormile and Suflita 1996) under a flow of O_2-free N_2, and autoclaving the mixture. Cultures were incubated at room temperature, in the dark, without shaking. At the start of the experiment and periodically thereafter, samples of the aqueous phase were withdrawn using strict anaerobic technique and stored frozen until analyzed. Prior to analysis, the samples were thawed and centrifuged (10 000 x g) for 5 min to separate particulate debris.

Thiols concentration experiment

As part of an initial toxicity study, mixtures of thiols were added to oily sludge incubations at different concentrations to determine their effect on anaerobic microbial activity (Chapter 5). The cultures were monitored for thiol loss even after the completion of the toxicity assessment. The organothiol in HMN solutions were added to duplicate 15 ml cultures to give approximately 0, 8, 80 or 800 µmol of each compound. These cultures also received an initial amendment of lactate. Negative controls were established without lactate and received HMN without thiols, and autoclaved sterile controls received lactate, sulfate, nitrate, as well as 800 µmol of each thiol.

Enrichment for thiols degradation

Subsequent to the initial toxicity assessment, separate oily sludge incubations were established with non-inhibitory amounts of thiols. The thiols were added as a HMN solution to each 15 ml culture to: pentanethiol 40.5 µmol, hexanethiol 35.7 µmol, heptanethiol 31.9 µmol, octanethiol 28.9 µmol, cyclopentanethiol 48.8 µmol, cyclohexanethiol 40.9 µmol, benzenethiol 50.8 µmol, benzenemethanethiol 42.7 µmol per culture. Cultures were amended with lactate (10 mM) to confirm microbial activity in the presence of the thiols. Cultures were established under methanogenic, sulfate-reducing, and nitrate-reducing conditions and amended with thiols; a sterile control received thiols and then autoclaved, a positive control received lactate but no thiols, and a substrate-unamended control received neither thiols nor lactate. Values for sterile controls under the three conditions were averaged.

Transfers were prepared using a nitrate-reducing thiol-degrading culture as an inoculum (0.5 ml) instead of oily sludge, for 15 ml cultures with 20 mM nitrate, no lactate, and 50 µmol hexanethiol or 49 µmol cyclohexanethiol per culture in HMN. Autoclaved incubations served as sterile controls.

Analytical techniques

Sulfate and nitrate were analyzed with an anion high performance liquid chromatography (HPLC) system as previously described (Smolenski & Suflita 1987) or using a Dionex IC system with an AS4A-SC column (Dionex, Sunnyvale, CA) as previously described (Chapter 5). Lactate was analyzed by HPLC and methane was analyzed by gas chromatography (GC) as previously described (Chapter 5). The amount of methane in the headspace of cultures was calculated as percent of headspace by comparison to external standards analyzed the same day.

Organothiols were analyzed by GC using a Hewlett Packard (Palo Alto, CA) 5890 Series II GC, with a Carbograph VOC capillary column (30 m, Alltech Associates, Inc., Deerfield, IL). The column temperature program was: an initial temperature of 50°C held for 2 min raised at 3°C/min to 140°C then raised at 25°C/min to 200°C and held for 3 min. Samples of the HMN organic overlay (1 µL) were removed from the cultures and injected directly without extraction or derivatization. Substrates were detected using a flame ionization detector. The amount of substrate was calculated from the ratio of the peak area of each compound from analysis of samples from cultures, to the peak area of external standards analyzed the same day. For the transfers, the peak areas for samples from cultures as well as external standards were expressed as the ratio relative to the peak area for the HMN to correct for injection volume variations. Since the aqueous solubilities of thiols and other organosulfur compounds are minimal, the amounts are expressed as µmoles per culture rather than aqueous concentration. Octanethiol could not be reproducibly detected due to interference from the HMN. For gas chromatography-mass spectrometry (GC-MS) analyses, the same GC was used but with a DB-5 capillary column (J&W Scientific, Folsom, CA), a Hewlett Packard 5970 mass selective detector, and a temperature program of 50°C for 2 min raised at 3°C/min to 100°C then raised at 30°C/min to 240°C and held for 2 min.

Chemicals

Organosulfur compounds were obtained from Aldrich Chemical Co. (Milwaukee, Wis.), were of at least 97% purity, and used without further purification. HMN was obtained from Sigma Chemical Co. (St. Louis, Mo).

Results

Initial survey for thiol degradation

The toxicity of organothiols to anaerobic microbial activity was reported previously, but the organothiols added to these cultures were not transformed during the initial period in which lactate was degraded (Chapter 5). However, upon further incubation, transformation of thiols occurred in nitrate-reducing cultures that contained intermediate amounts of thiols (80 μmol/culture). Degradation of benzenemethanthiol preceded that of the other substrates, which were removed at similar rates after a longer lag period of about 13 weeks (Figure 2). The amount of thiols did not change during 31 weeks incubation in cultures with larger amounts (800 μmol/culture) of thiols, under sulfate-reducing or methanogenic conditions, or in sterile controls (data not shown).

Thiols biodegradation experiment

Based on the results of the initial experiment, cultures were established under the three electron-accepting conditions with intermediate levels of thiols. Lactate was readily degraded within 7, 14, or 21 days under sulfate-reducing, nitrate-reducing, and methanogenic conditions respectively. There was no difference in the rate of lactate degradation in cultures containing thiols compared to thiol-free controls, indicating that the low amounts of thiols added (28-50 mmol/L) were not inhibitory. Likewise, there was no difference in the initial (5 weeks) rates of methanogenesis, sulfate reduction, or nitrate reduction in cultures with or without thiols. With extended incubation, methane

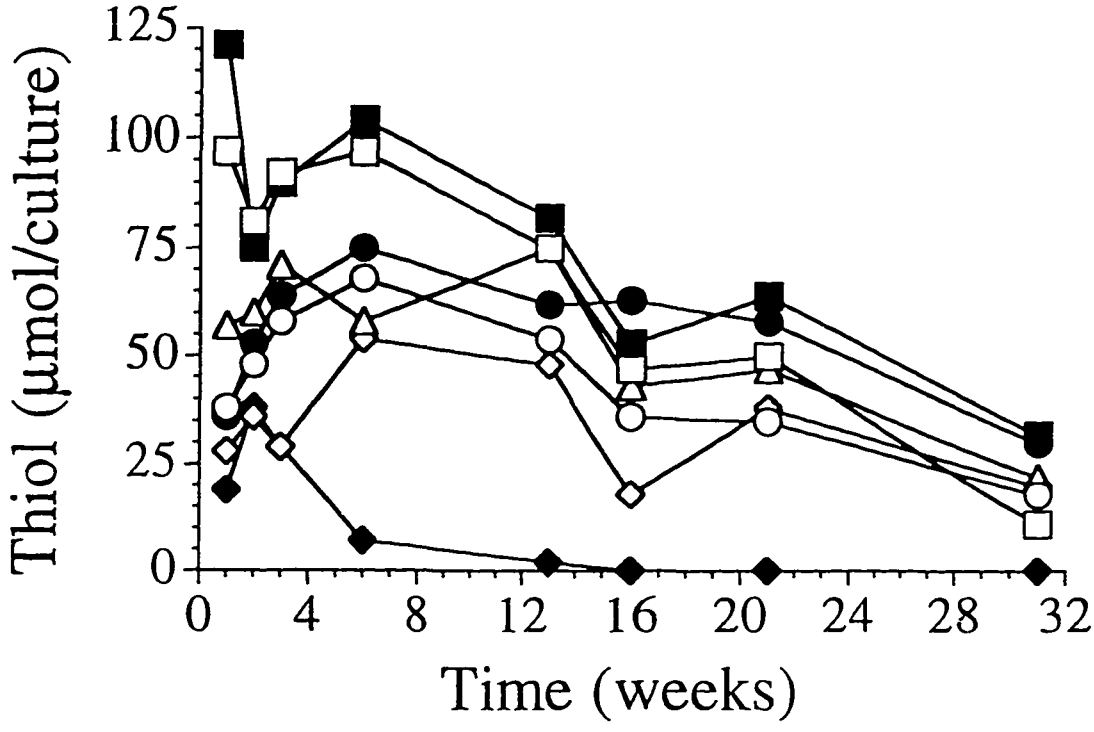

Figure 2: Degradation of individual thiols under nitrate-reducing conditions. Cultures were amended with HMN containing (□) pentanethiol, (■) cyclopentanethiol, (○) hexanethiol, (●) cyclohexanethiol, (◊) heptanethiol, (Δ) benzenethiol, and (♦) benzenemethanethiol. Octanethiol was also added but could not be quantified.

production in cultures with thiols (4.1% of headspace after 62 weeks) was greater than in thiol-free negative controls without lactate (1.8%), but less than the amount in positive controls that received lactate alone (7.6%). Thus, mineralization of thiols was not indicated under methanogenic conditions. After 39 weeks incubation, sulfate was almost depleted (1.4 mM) in sulfate-reducing cultures, so an additional 12 mM sulfate was added, and a subsequent reduction of an additional 3 mM occurred by 62 weeks total incubation. The amount of sulfate reduced in thiol-containing cultures was no greater than in negative controls without thiols, indicating that thiol degradation did not occur under sulfate-reducing conditions.

Thiol degradation occurred under nitrate-reducing conditions after an initial lag period of about 9 weeks, and all thiols were depleted within 39 weeks (Figure 3). All seven thiols were degraded at similar rates in these cultures, although benzenemethanethiol was removed after a somewhat shorter lag period (data not shown) as observed previously. Loss of thiols was associated with nitrate depletion, resazurin oxidation, and proliferation of multiple cell morphologies as observed microscopically. There was no loss of thiols under methanogenic or sulfate-reducing conditions, or in sterile controls (Figure 3). Since microbial activity was not inhibited, the lack of biodegradation under methanogenic and sulfate-reducing conditions was due to a limitation other than substrate toxicity.

The organic overlay from representative cultures under nitrate- and sulfate-reducing conditions were analyzed by GC-MS for the presence of thiols and potential metabolites after 33 weeks incubation. This analysis confirmed that the amount of thiols in the nitrate-reducing cultures had decreased relative to other cultures and relative to hydrocarbons in the organic overlay (Figure 4). The GC-MS analysis also confirmed that octanethiol, which could not be quantified by the GC-FID system, was also degraded in these cultures (Figure 4). No metabolites were detected in the organic overlay by GC-MS analyses, or by HPLC analysis of aqueous culture fluids.

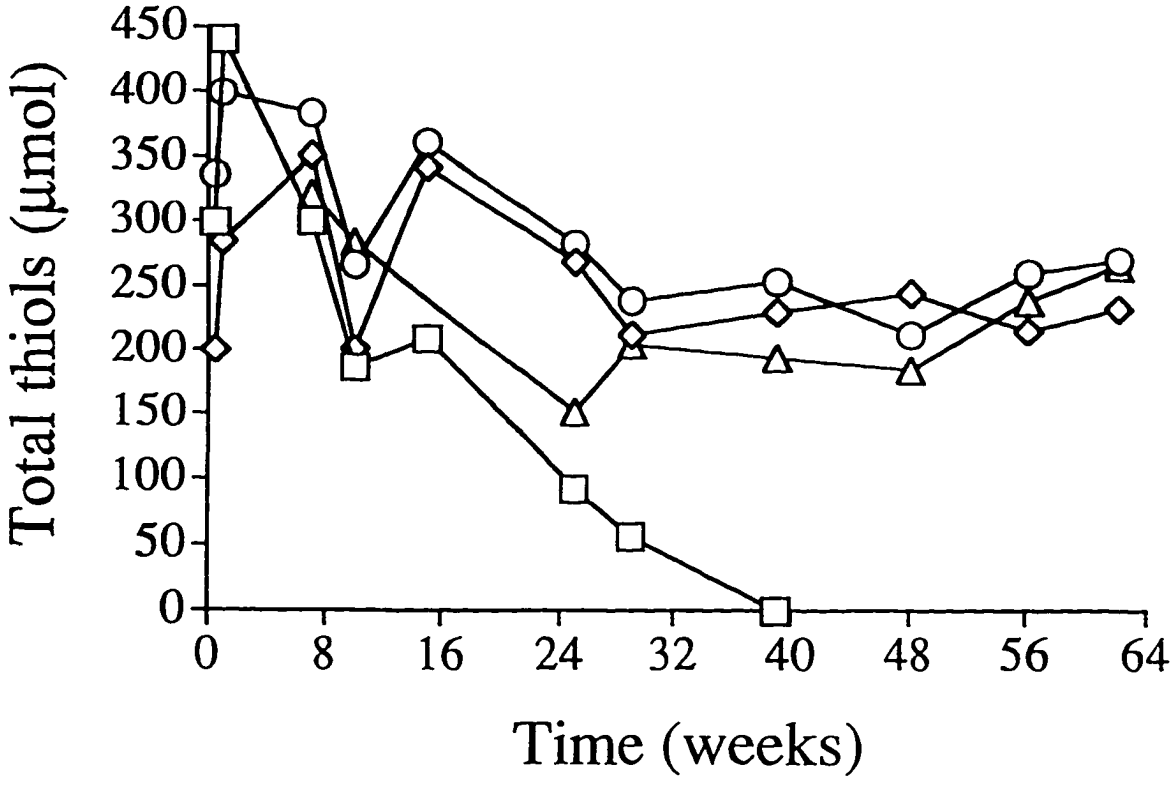

Figure 3: Degradation of thiols under nitrate-reducing conditions (□) but not under sulfate-reducing (◊), methanogenic conditions (○) or in sterile controls (Δ). Values shown are average total amounts of seven thiols per culture.

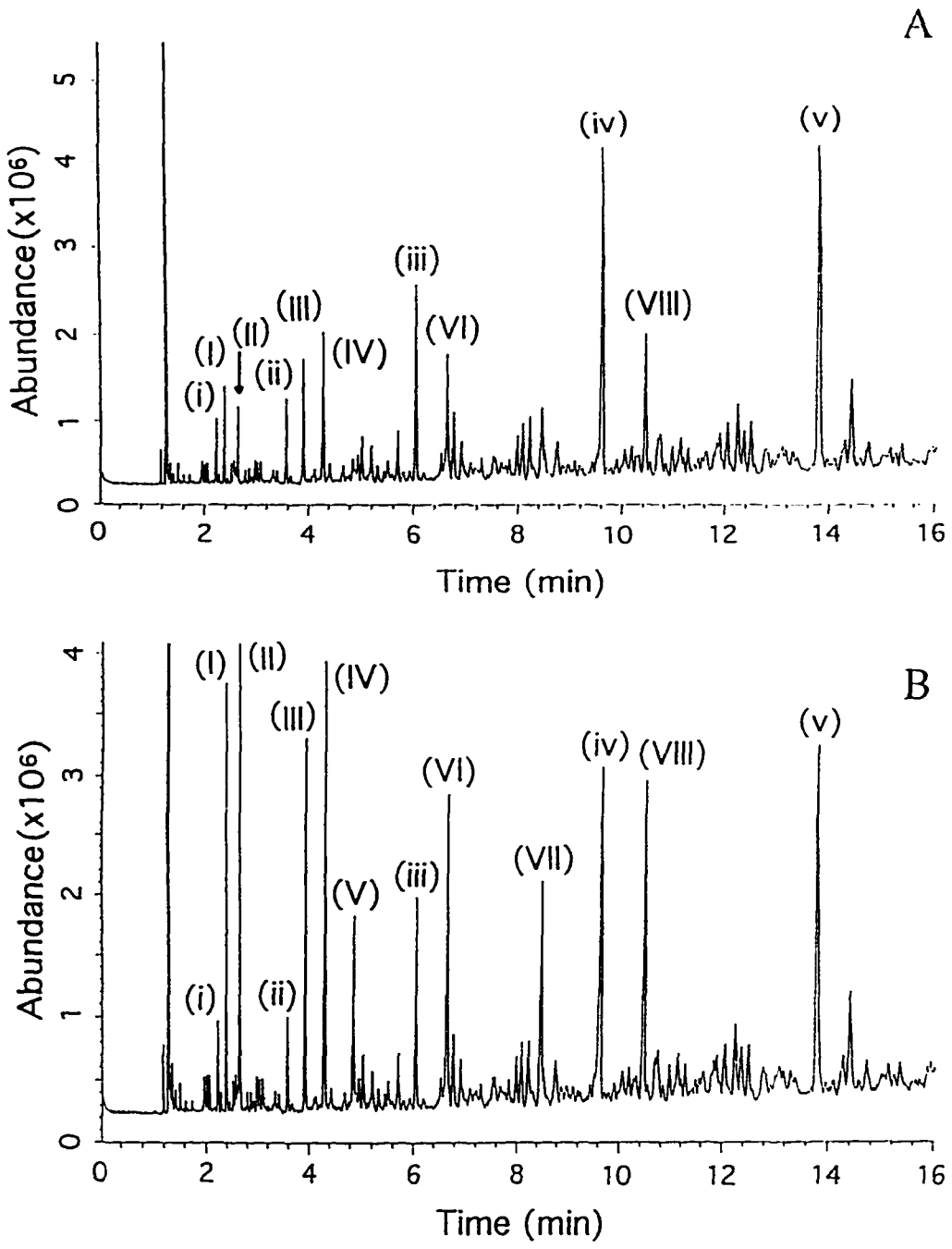

Figure 4: Total ion chromatograms from GC-MS analyses of a nitrate-reducing culture (A) and a sulfate-reducing culture (B) that was identical to methanogenic cultures and sterile controls. Hydrocarbons introduced with the oily sludge included: (i) octane, (ii) nonane, (iii) decane, (iv) undecane, and (v) dodecane. The organothiols amended to cultures include: (I) pentanethiol, (II) cyclopentanethiol, (III) hexanethiol, (IV) cyclohexanethiol, (V) benzenethiol, (VI) heptanethiol, (VII) benzenemethanethiol, and (VIII) octanethiol.

After 34 weeks incubation, cultures were re-amended with thiols and 10 mM nitrate. Each of the thiols added to the culture were slowly degraded, and the depletion of the total amount of thiols is depicted in Figure 5. Depletion of the thiols below 20 μmol each per culture after 70 weeks total incubation could not be confirmed due to analytical interference from hydrocarbons. As observed previously, the rates of degradation of individual thiols were similar except for benzenemethanethiol, which was removed after a shorter lag period than the other compounds (data not shown). Degradation of the additional thiols was associated with reduction of the amended electron acceptor. Nitrate was reduced to less than 1 mM by 47 weeks, and when an additional 15 mM nitrate was added to these cultures, this was again reduced to less than 2 mM by 60 weeks incubation (Figure 5). However, with each addition of nitrate, the nitrite concentration in these cultures increased. At the time of the second re-amendment, nitrite concentrations reached 9 mM, and by the time the third allotment of nitrate was reduced, nitrite had accumulated as high as 24 mM in these cultures (Figure 5).

Enrichment cultures for thiols degradation

To confirm the degradation of thiols, transfers were established under nitrate-reducing conditions using a thiol-degrading culture as inoculum with either hexanethiol (50 μmol/culture) or cyclohexanethiol (49 μmol/culture) as substrate. The average amount of hexanethiol decreased to <15 μmol per culture in 18 weeks, although further depletion of this substrate did not occur even after prolonged incubation (50 weeks). A similar decrease was observed with cyclohexanethiol, when corrected for abiotic losses, the average decrease of substrates in these cultures (after 28 weeks) were 43 μmol and 38 μmol, respectively (Table 1).

Substantial nitrate reduction occurred in enrichment cultures degrading hexanethiol and cyclohexanethiol as depicted in Figure 6. Reduction of nitrate was associated with

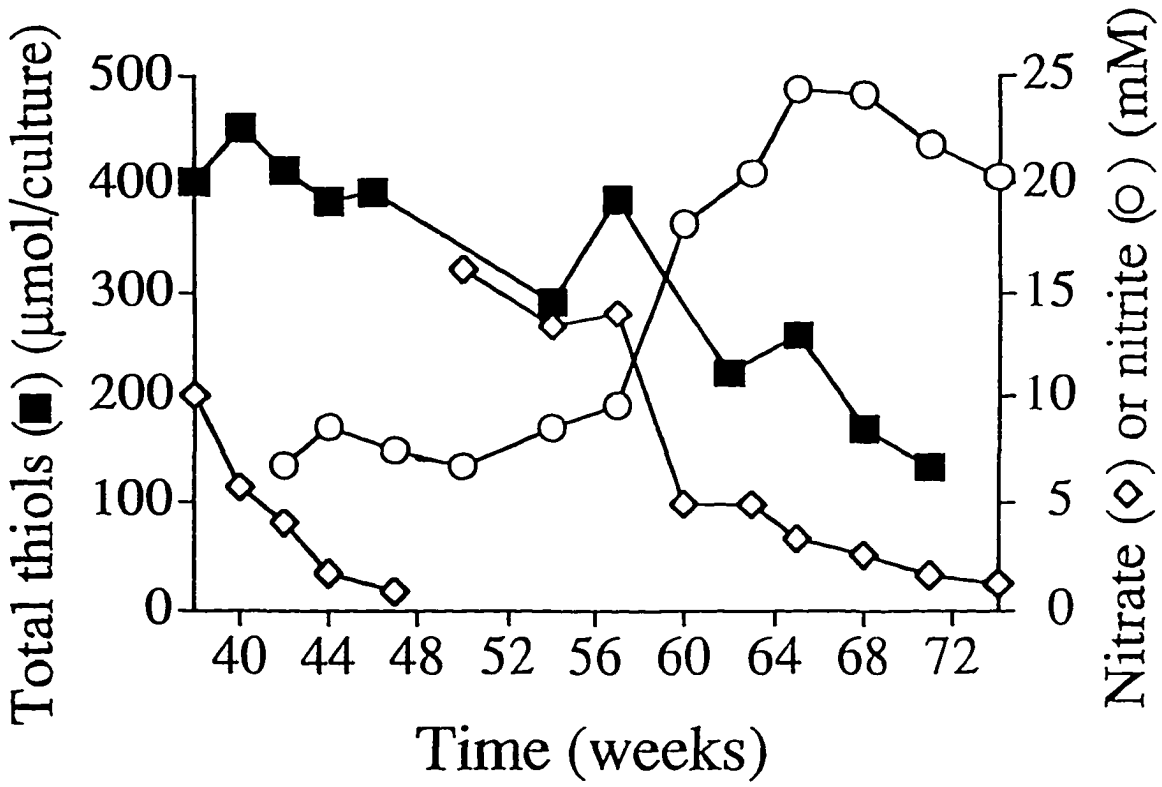

Figure 5: Total thiols decrease (■), nitrate reduction (◊), and nitrite accumulation (○) in nitrate-reducing cultures after re-amendment of thiols and nitrate. Additional nitrate was added after 50 weeks total incubation.

Table 1: Stoichiometry of nitrate reduction and sulfur release associated with the degradation of hexanethiol and cyclohexanethiol in enrichment cultures.

substrate	measured substrate decrease[a]	predicted nitrate decrease[b]	measured nitrate decrease[c]	predicted sulfide increase[b]	measured sulfide increase[d]	measured sulfate increase[d]
Hexanethiol	43 µmol	20.5 mM	18.8 mM	2.9 mM	<0.01 mM	<0.01 mM
Cyclohexanethiol	38 µmol	17.3 mM	17.2 mM	2.5 mM	≤0.01 mM	<0.01 mM

[a] Average difference between 4 and 28 week values

[b] Based on equations 1 and 2 and substrate decrease

[c] Average decrease in nitrate after 43 weeks

[d] Corrected for amount in sterile controls

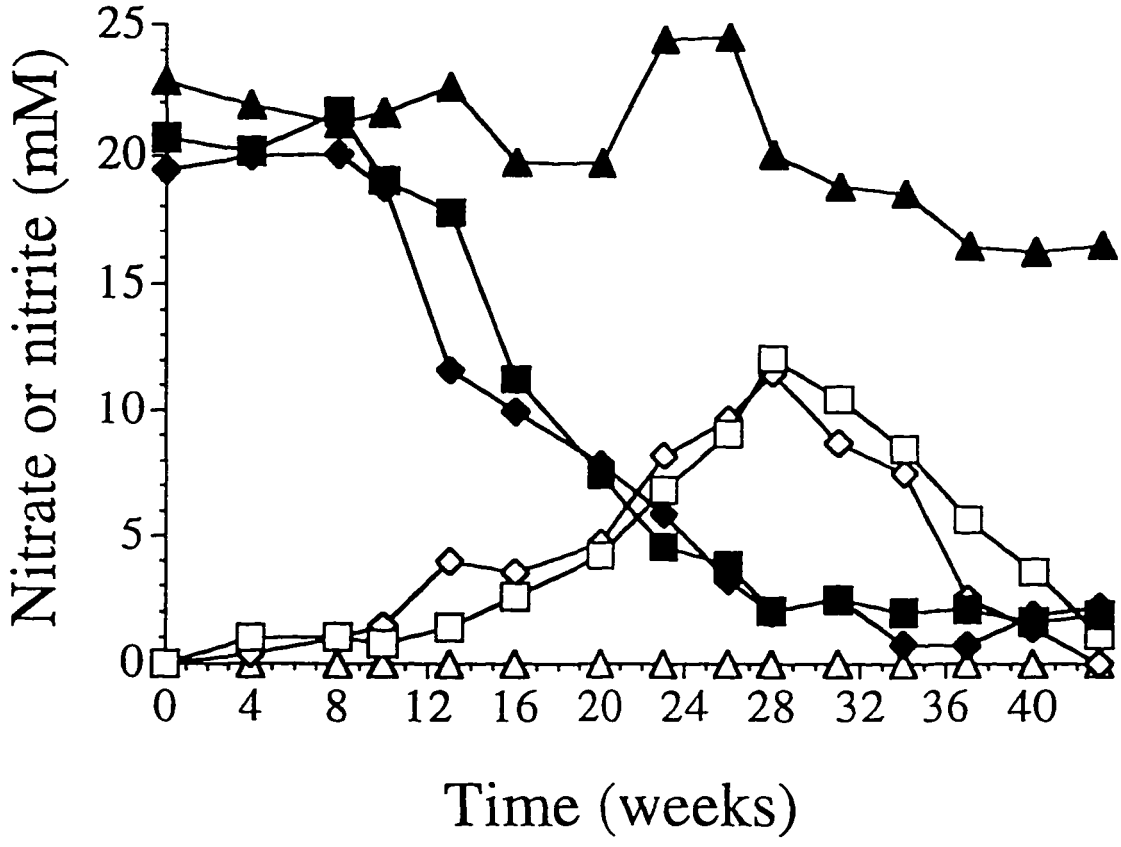

Figure 6: Nitrate reduction and nitrite accumulation associated with thiol metabolism in enrichment cultures. Nitrate was reduced (■) and nitrite (□) transiently accumulated in hexanethiol-degrading cultures and cyclohexanethiol-degrading cultures (♦ nitrate; ◊ nitrite), but not in sterile controls (▲ nitrate; Δ nitrite).

accumulation of nitrite, which was also removed with prolonged incubation (Figure 6). Nitrate reduction was also associated with the oxidation of resazurin, indicating nitrous oxide production. The pink color remained in the cultures throughout the incubation period. The stoichiometry of nitrate reduction associated with substrate mineralization was calculated based on equations 1 and 2 and the corrected amount of substrate loss in these cultures (Table 1). The nitrate reduced in cultures with hexanethiol and cyclohexanethiol were 92% and 99% of the theoretically expected amounts, respectively. The equations do not account for incomplete reduction of nitrate or for incorporation into biomass.

[1]: (hexanethiol) $C_6H_{14}S_1 + 7.2\ NO_3^- + 7.2\ H^+ \Rightarrow 6CO_2 + H_2S + 3.6\ N_2 + 9.6\ H_2O$

[2]: (cyclohexanethiol) $C_6H_{12}S_1 + 6.8\ NO_3^- + 6.8\ H^+ \Rightarrow 6CO_2 + H_2S + 3.4\ N_2 + 8.4\ H_2O$

The fate of the sulfur atom in these experiments was also addressed with the enrichment cultures. According to equations 1 and 2, the complete degradation of hexanethiol or cyclohexanethiol would be expected to release equimolar amounts of sulfide. However, sulfide did not accumulate in these cultures (Table 1). Under nitrate-reducing conditions, sulfide can be oxidized to sulfate, so the amount of this anion was also quantified. However, sulfate values in thiol-degrading cultures were no greater than sterile controls (Table 1). Intermediate oxidation states of sulfur such as thiosulfate and elemental sulfur have yet to be analyzed, so at present the fate of the sulfur moiety remains unknown.

Discussion

These results clearly indicate the susceptibility of organothiols to biodegradation under nitrate-reducing conditions. Depletion of thiols was observed in cultures from several experiments, and degradation of thiols was associated with nitrate reduction, nitrite and

nitrous oxide production, as well as cell growth. Degradation of thiols occurred only in cultures with the highest redox potential tested. Combined with the lack of toxicity of the organothiols to microbial acitivity at the concentrations employed, this observation suggests that anaerobic thiol decay may be influenced primarily by thermodynamic considerations. More energy would potentially be available to microorganisms by coupling the oxidation of the organothiols to the reduction of nitrate as opposed to sulfate or carbon dioxide. Such a relationship could be a determining factor for the fate of organothiols in anaerobic environments.

The products of thiol degradation and the mechanism by which these mercaptyl hydrocarbons were metabolized is not clear. The stoichiometry of nitrate reduction associated with the degradation of hexanethiol and cyclohexanethiol suggests that the carbon skeleton of these compounds was completely mineralized. The fate of the sulfur moiety is similarly unknown, but it may have been used as a source of sulfur for these microorganisms, as has been reported previously for other organosulfur compounds (Rajagopal & Daniels 1986, Miller 1992). The relatively rapid degradation of benzenemethanethiol (toluenethiol) in these cultures suggests that this compound was particularly susceptible to microbial attack. To our knowledge, this is the first report of the degradation of benzenemethanethiol under nitrate-reducing conditions, and the first report of the degradation of any of these alkyl-, cyclic-, and aromatic thiols under anaerobic conditions.

Determining the metabolic pathway by which these compounds are degraded would be greatly facilitated by the isolation and cultivation of the nitrate-reducing organism(s) responsible for their biodegradation. However, the accumulation of high concentrations of nitrite in these cultures apparently precluded the cultivation of the thiol-degrading organisms. The use of a nitrite-reducing organism in a defined consortium may facilitate the eventual enrichment and isolation of the requisite organism. In future studies, we will continue to enrich for thiol-degrading activity, isolate the microorganisms that carry out the

transformation, and probe the metabolic pathways by which these compounds are degraded.

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Appendix

REDUCTION OF NITROAROMATIC COMPOUNDS BY CELL-FREE EXTRACTS OF *DESULFOMONILE TIEDJEI.*

Abstract

Cell-free extracts of *D. tiedjei* reduced the nitro groups of a variety of nitroaromatic compounds to the corresponding aromatic amines, but did not transform them further. Nitro group reduction was non-specific in that it occurred for all nitroaromatic compounds and structural isomers examined. However, there were at least two separate activities; one that reduced primarily *meta-* or *para-* nitro groups, and a slower activity that reduced *ortho-* nitro groups. Similar reactions carried out by cell extracts of *Methanobacterium thermoautotrophicum* showed a preference for reduction of *para*-nitro groups but not to the same extent as *D. tiedjei*. Preliminary characterization of the nitroreductase activity in cell extracts with hydrogen as the electron donor revealed that the artificial electron carrier methyl viologen enhanced complete reduction of aromatic nitro groups. The physiological roles of the nitroreductases was uncertain. Copper inhibited nitroreductase activity, suggesting a role for hydrogenases; and reduction of intermediates prior to diaminotoluene was inhibited by nitrate and nitrite but not sulfate, indicating a possible relationship to nitrate utilization. Nitroaromatic reduction was expressed in fermentatively-grown cells and was not related to the dehalogenation activity of this organism.

Introduction

Due to their use in explosives, dyes, pesticides, polymers, and pharmaceuticals, nitroaromatic compounds have become widespread environmental contaminants. In particular, explosives such as 2,4,6-trinitrotoluene (TNT) were produced in large quantities, and contamination of soils and water supplies has resulted from the distribution of munitions as well as the storage and treatment of manufacturing wastewaters (Nay et al. 1974). Nitrotoluenes are mutagenic and are toxic to bacteria, yeasts, fungi, algae, tidepool copepods, oyster larvae, fish, and humans (reviewed in Marvin-Sikkema and de Bont 1994, and Funk et al. 1996). In addition, these largely anthropogenic chemicals have a tendency to persist in the environment. Incineration is the usual treatment option of TNT-contaminated soil, but the expense of this procedure has fostered the development of alternative treatment options including bioremediation. In the last decade, research has demonstrated biodegradation and biotransformation of nitroaromatic compounds. In addition to fundamental microbiological and biochemical studies on the potential for, and mechanisms of, nitroaromatic compound biotransformations, potential cleanup mechanisms are under development as recently reviewed by several authors (Rieger and Knackmuss 1995, Funk et al. 1996, and Nishino and Spain 1997).

The aerobic transformation of TNT and other nitroaromatic compounds can lead to the formation of recalcitrant polymers, although alternate fates, including mineralization, have also been demonstrated. Mechanisms of transformation and degradation include oxygenolytic removal of nitro groups, ring dioxygenation, reductive aromatic ring dehydrogenation, complete reductive removal of the nitro group, and degradation via partial reduction and replacement reactions (Marvin-Sikkema and de Bont 1994, Rieger and Knackmuss 1995, Spain 1995). In addition, a wide variety of organisms catalyze the reduction of nitroaromatic compounds to the corresponding amines without further transforming these products (Gorontzy et al. 1994, Marvin-Sikkema and de Bont 1994). Nitrotoluenes like TNT are particularly susceptible to reduction reactions due to their

chemical structure (Preuß and Rieger 1995) and reduction represents a primary fate process under a variety of anaerobic conditions (Krumholz et al. 1996). In fact, reduction of all three nitro groups of TNT occurs only under strict anaerobic conditions. Transformation of nitroaromatic compounds beyond nitro group reductions has also been observed under anaerobic conditions. A sulfate-reducing *Desulfovibrio* species (strain B) transformed dinitrotoluenes to the corresponding amines then reductively deaminated the aminoaromatic products and used the resulting ammonium as a nitrogen source (Boopathy and Kulpa 1993). The complete reduction of TNT to triaminotoluene has been postulated by other authors, as has further metabolism through toluene, methylphloroglucinol, methylpyrogallol, and *p*-cresol intermediates (Funk et al. 1995, Lewis et al. 1996), but conclusive evidence for the pathways of complete anaerobic TNT mineralization is lacking.

The reduction of nitrotoluenes can also be catalyzed by cell-free extracts of many organisms and is primarily a function of the redox potential. Under anaerobic conditions, crude extracts of *Veillonella alkalescens* catalyzed the complete reduction of the nitro groups of TNT, 2,4- and 2,6-dinitrotoluene to the corresponding amino groups with molecular hydrogen as the electron source (McCormick et al. 1976). Similarly, both whole cells and crude extracts of a *Desulfovibrio* sp. reduced TNT to triaminotoluene (Preuss et al. 1992). The initial reduction was catalyzed by enzymes reducing ferredoxin or methyl viologen, whereas the reduction of 2,4-diamino-6-hydroxylaminotoluene to triaminotoluene was mediated by a separate enzyme purported to be a sulfite reductase (Preuss et al. 1992). A mechanism for the reduction of aromatic nitro-compounds catalyzed by hydrogenase and ferredoxins, or by flavodoxin was also proposed for a *Clostridium* (Angermaier and Simon 1983) but other enzymes like pyruvate:ferredoxin oxidoreductases, sulfite reductases, nitrite reductases, cytochrome reductases, and quinone reductases are able to transform nitroaromatic compounds as well (Kinoushi and Ohnishi 1983, Bryant and DeLuca 1991). Therefore, a wide spectrum of enzymes with diverse physiological roles can catalyze the reduction of nitroaromatic compounds.

A variety of nitroreductases have been purified (Villanueva 1962, McCormick et al. 1976, Angermaier and Simon 1983, Bryant and Deluca 1991, Blasco and Castillo 1993, Rafii and Cerniglia 1993), and from these studies the mechanism of nitro group reduction has been generally elucidated. The enzymatic reduction of aromatic nitro groups involves the addition of three pairs of reducing equivalents, possibly through the involvement of single electron transfers via nitro radicals. The first reactions yield a nitroso derivative, which is subsequently reduced to a hydroxylamine, followed by reduction to the amine. In most systems, a single nitroreductase enzyme is responsible for all three reactions and many are pyridine nucleotide-dependent. However, reduction of nitro compounds does not seem to be the physiological function of the enzymes that catalyze these reactions. Diaphorases, ferredoxin-NADPH reductase, and a variety of other enzymes from prokaryotes and eukaryotes have been shown to catalyze the fortuitous reduction of aromatic nitro groups (Spain 1995, Preuß and Rieger 1995).

We have used the model organism *Desulfomonile tiedjei* to investigate the potential for sulfate-reducing bacteria to catalyze the biotransformation of nitroaromatic compounds, and to determine the biochemical mechanisms of nitro group reductions. This organism is known to reduce a wide variety of electron acceptors including aryl- and alkyl-chlorinated compounds which it reductively dehalogenates (DeWeerd and Suflita 1990, Townsend and Suflita 1996). We found that cell extracts of *D. tiedjei* rapidly reduce the nitro groups of a variety of nitroaromatic compounds to the corresponding amines, but further transformation of these compounds does not occur. Our results indicate that at least two apparent enzymatic activities were responsible for the reduction of aromatic nitro groups, and the rates of these activities depended upon substituent position and the availability of an artificial electron carrier.

Materials and Methods

Growth of microorganisms and preparation of cell-free extracts

D. tiedjei was grown fermentatively on pyruvate as previously described (DeWeerd et al. 1990). Cell-free extracts of *D. tiedjei* and dehalogenation assays were prepared as previously described (Townsend and Suflita 1996). *M. thermoautotrophicum* was grown and cell-free extracts prepared as previously described (Wolin et al. 1963).

Nitroreductase assays

Assays for reduction of nitroaromatic compounds were performed at room temperature in an anaerobic chamber with an atmosphere of N_2 (95%) and H_2 (5%) unless otherwise indicated. Assays were conducted in 10 ml serum vials in anoxic TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (50 mM, pH 8.0), with 5.4 mM methyl viologen, 1 mM nitroaromatic compound from ethanolic stock solutions, and 10% (v/v) cell-free extract of *D. tiedjei* or *M. thermoautotrophicum*. Samples (250 µL) were removed periodically, transferred to microcentrifuge tubes and removed from the anaerobic chamber. Saturated NaCl (100 µL) and acetonitrile (1 ml) were added to the tubes, which were then mixed and centrifuged (5 min; 16 000 x g) to precipitate protein and to separate into two layers. Aliquots of the acetonitrile layer were analyzed by high pressure liquid chromatography (HPLC) for nitroaromatic compounds. Alternatively, the samples were extracted with solvent procedure as previously described (DeWeerd and Suflita 1990) and then analyzed by HPLC.

Abiotic controls were prepared by substituting anoxic water for cell-free extract. To test the effects of alternate headspace atmospheres on the reactions, the serum vials were sealed with Teflon-lined stoppers, removed from the anoxic chamber, and secured with aluminum crimp seals. The headspace gas was then replaced with O_2-free N_2, H_2, or the vials were opened to an air atmosphere. The latter served as an oxidized control. Samples were removed periodically using syringes and strict anaerobic technique.

Analytical methods

Nitroaromatic compounds were analyzed with a reversed phase C_{18} column (Alltech Associates, Deerfield, IL) and a HPLC (Beckman Instruments Inc., Berkley, CA) equipped with a variable wavelength detector set at 230 nm. The mobile phase was 40% acetonitrile and 60% filtered water at 1 ml/min. Compounds were quantified by comparison of peak areas with external standards prepared identically and analyzed the same day. Protein was measured colorimetrically by the bicinchoninic acid assay (Smith et al., 1985) with bovine serum albumin as the standard.

Chemicals

Hydroxylamine, TES, and methyl viologen were obtained from Sigma Chemical Co. (St. Louis, Mo). All nitro- and amino-aromatic compounds were obtained in the highest available purity (>95%) from Aldrich Chemical Co., (Milwaukee, WI). Reagents for the protein assay were from Pierce Chemical Co.(Rockford, IL.).

Results

Reduction of nitroaromatic compounds by cell-free extracts of *D. tiedjei*

Cell-free extracts of *D. tiedjei* catalyzed a rapid reduction of the nitro groups of a variety of nitroaromatic compounds as listed in Table 1. Disappearance of the parent substrate was coupled with a stoichiometric increase in the reduced amino-aromatic product, but no further transformation of the latter occurred. No reduction of nitroaromatic substrates nor accumulation of aminoaromatic products were observed in controls without cell extracts. Reduction rates for the first nitro group reduced were similar regardless of other functional groups on the aromatic ring (data not shown). Complete reduction occurred regardless of whether one or two nitro or amino groups were substituted on toluene.

Table 1: Nitroaromatic compounds reduced to aminoaromatic compounds in assays with cell-free extracts of *D. tiedjei*

mono-nitroaromatic compounds
nitrobenzene
3-nitrophenol
4-nitrobenzoate
2-nitrotoluene
3-nitrotoluene
4-nitrotoluene
2-amino-4-nitrotoluene
4-amino-2-nitrotoluene
2-amino-6-nitrotoluene

di-nitroaromatic compounds
2,4 dinitrotoluene
2,6 dinitrotoluene

tri-nitroaromatic compounds
2,4,6 trinitrotoluene

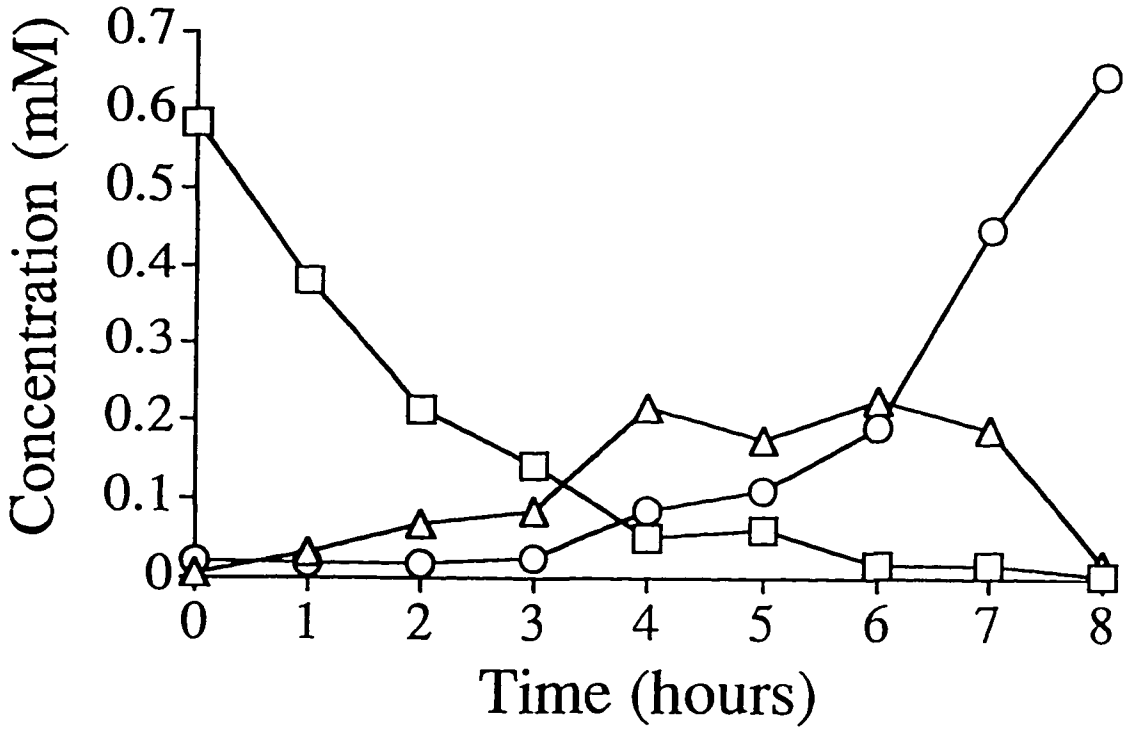

Figure 1: 2,6-Dinitrotoluene transformation in assays with cell-free extracts of *D. tiedjei*. Depletion of 2,6-dinitrotoluene (□) was followed by transient appearance of 2-amino-6-nitrotoluene (Δ) and eventual accumulation of 2,6-diaminotoluene (○).

In assays with cell-free extracts of *D. tiedjei*, both nitro groups of 2,6-dinitrotoluene were reduced to form 2,6-diaminotoluene. A transient intermediate was observed that corresponded to a single reduction product, 2-amino-6-nitrotoluene (Figure 1). Reduction of the second nitro group was subsequent to, but still concurrent with, reduction of the first nitro group of dinitrotoluene. Other intermediates were detected during the transformation that could be nitroso- or hydroxylamino- aminotoluenes, but their identity was not confirmed and they appeared to be present in only minor amounts in the assay mixtures.

Another isomer, 2,4-dinitrotoluene was likewise reduced to 2,4-diaminotoluene. Of the two possible nitro-aminotoluene isomers (Figure 2), only 4-amino-2-nitrotoluene was detected as a transient intermediate in the reduction. This indicated the preferential reduction of the *para*-nitro group of dinitrotoluene by extracts of *D. tiedjei*. Both 4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene were tested as substrates for reduction by cell-free extracts of *D. tiedjei*, and both compounds were reduced to 2,4-diaminotoluene at equal rates, indicating that reduction of a second nitro group in either position was not a limiting factor for the rate of complete reduction to diaminotoluene.

Cell extracts of *D. tiedjei* also transformed trinitrotoluene. TNT was rapidly depleted in these assays, and reduction of the substrate was followed by the consecutive appearance of three pairs of intermediates. Accumulation of intermediates was transient and the final product could not be detected. The intermediates were not identified, but based on their chromatographic characteristics likely correspond to reduced or partially reduced nitroso- or hydroxylamino- intermediates of isomers of dinitro-aminotoluene and diamino-nitrotoluene.

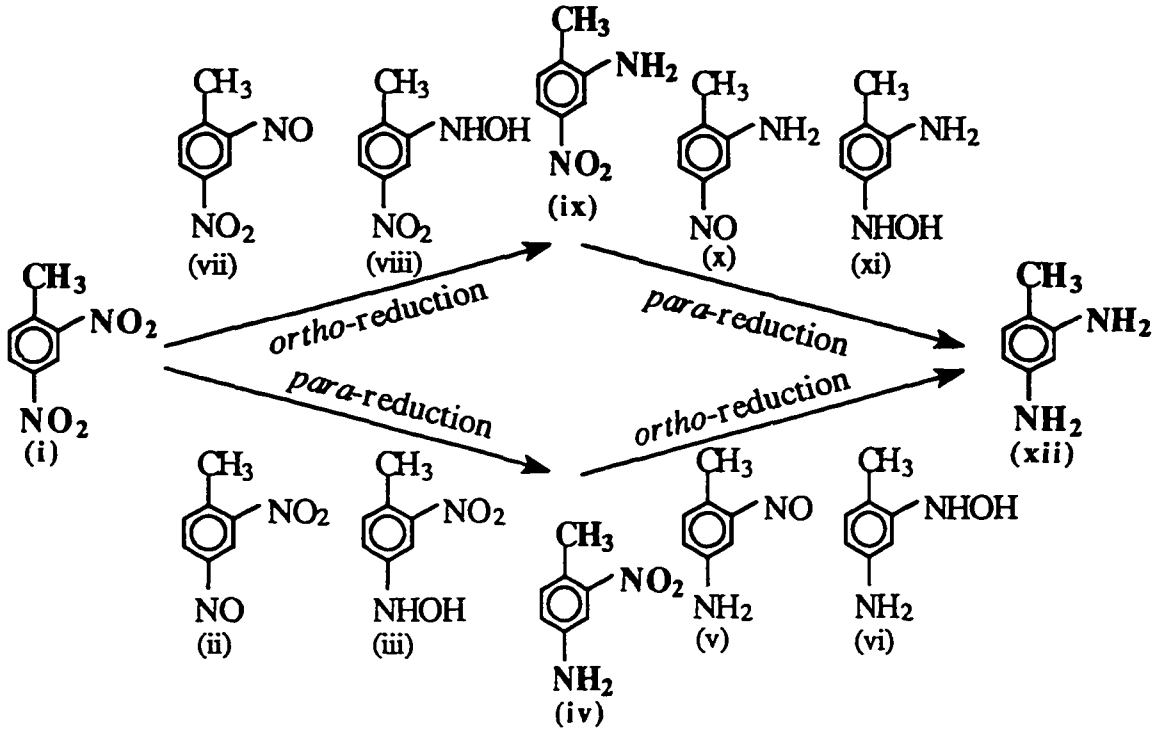

Figure 2: Proposed pathways for the transformation of 2,4-dinitrotoluene (i) to 2,4-diaminotoluene (xii). Initial reduction of the *para*-nitro group results in 4-nitroso-2-nitrotoluene (ii), 4-hydroxylamino-2-nitrotoluene (iii), 4-amino-2-nitrotoluene (iv), 4-amino-2-nitrosotoluene (v), and 4-amino-2-hydroxylaminotoluene (vi). Initial reduction of the *ortho*-nitro group results in 2-nitroso-4-nitrotoluene (vii), 2-hydroxylamino-4-nitrotoluene (viii), 2-amino-4-nitrotoluene (ix), 2-amino-4-nitrosotoluene (x), and 2-amino-4-hydroxylaminotoluene (xi).

Table 2: Comparison of rates of reduction of nitrotoluenes by cell-free extracts of *D. tiedjei*: Effects of the position of nitro groups on rates of reduction indicate two separate enzymatic activities.

	o-nitrotoluene	*m*-nitrotoluene	*p*-nitrotoluene
single substrate:	74 μM/hr	98 μM/hr	101 μM/hr
dual substrates:			
ortho & para	74 μM/hr	-	116 μM/hr
meta & para	-	69 μM/hr	61 μM/hr
ortho & meta	50 μM/hr	109 μM/hr	-

Reduction of dinitrotoluenes by cell-free extracts of *M. thermoautotrophicum*

In assays with cell-free extract of *M. thermoautotrophicum*, various nitrotoluenes were completely reduced to the corresponding aminoaromatic compounds but were not transformed further. 2,6-Dinitrotoluene was transformed to 2,6-diaminotoluene after a transient accumulation of 2-amino-6-nitrotoluene. 2,4-Dinitrotoluene was transformed to 2,4-diaminotoluene, and in contrast to experiments with cell extracts of *D. tiedjei*, both 2-amino-4-nitrotoluene and 4-amino-2-nitrotoluene were detected as transient intermediates, with the latter accumulating to twice the concentration of the former. The proportions of the two intermediates indicated that either of the two nitro substituents could be reduced first, but preferential reduction of the nitro group in the *para* position occurred. All three nitro-aminotoluene isomers were reduced to the corresponding diaminotoluenes, confirming that complete reduction occurred regardless of substituent position.

Effects of nitro-group position on reduction by cell-free extracts of *D. tiedjei*

To further investigate the preferential reduction of the *para*-nitro group by extracts of *D. tiedjei*, we conducted experiments with mono-nitrotoluene isomers. We used the isomers in competition assays where we compared the rates of reduction in assays containing two nitrotoluenes relative to the rates of compound reduction for individual substrates (Table 2). Each of the nitrotoluenes were reduced at similar rates when added as the sole substrates in these assays, although the rate for *o*-nitrotoluene (74 μM/hr) was slightly less than for the *m*-nitrotoluene (98 μM/hr) and *p*-nitrotoluene (101 μM/hr). When the *ortho*- and *para*- nitrotoluenes were added together, there appeared to be no competition between them for the enzymatic activity: *o*-nitrotoluene was reduced at the same rate (74 μM/hr) as with single substrate, and *p*-nitrotoluene was reduced at 116 μM/hr, slightly higher than when added as a single substrate. This is in contrast to the results when the *meta*- and *para*-nitrotoluenes were added together: *m*-nitrotoluene was reduced at only 69 μM/hr compared to 98 μM/hr, and *p*-nitrotoluene was reduced at 61 μM/hr compared to

101 μM/hr. The rates were decreased to about 65% of activity with single substrates, suggesting a competition for enzymatic activity. When the *ortho-* and *meta-* nitrotoluenes were added together, the reduction of the *m*-nitrotoluene was not affected (109 μM/hr compared to 98 μM/hr), although that of the *o*-nitrotoluene was reduced somewhat (50 μM/hr compared to 74 μM/hr). Overall, these findings suggest that there are at least two aromatic nitro-group reducing activities in cell-free extracts of *D. tiedjei*, one that reduces both *meta* and *para* groups, and another that reduces *ortho* groups. Furthermore, the *ortho*-nitro group reduction activity was somewhat slower than the other. This explains the pattern of reduction of nitroaromatic compounds with multiple nitro groups including the dinitrotoluenes mentioned previously as well as trinitrotoluene, for which two nitro groups are *ortho* to the aromatic methyl group.

Methyl viologen is an artificial electron carrier that was initially incorporated into the nitroreductase assay to facilitate transfer of low-potential electrons. During the nitroreductase assays, the reaction mixture turned blue from the presence of reduced methyl viologen after all the nitro group substituents were reduced, and thus indicated completion of the reaction. Methyl viologen addition did not substantially affect the rate of disappearance of 2,4-dinitrotoluene, but did affect the rate of accumulation of 4-amino-2-nitrotoluene and 2,4-diaminotoluene (Figure 3). This could reflect an effect on the reduction of the second nitro group, or an effect on an intermediate step during the reduction of each nitro group to the amino products. The addition of methyl viologen increased the rate of reduction of 4-amino-2-nitrotoluene as well as the accumulation of 2,4-diaminotoluene (data not shown) compared to assays without an electron carrier. Therefore, it appeared that methyl viologen specifically facilitated the *ortho-* nitroreductase activity, which corroborates our contention that the *ortho*-nitroreductase activity is distinct from the *para*-nitroreductase activity.

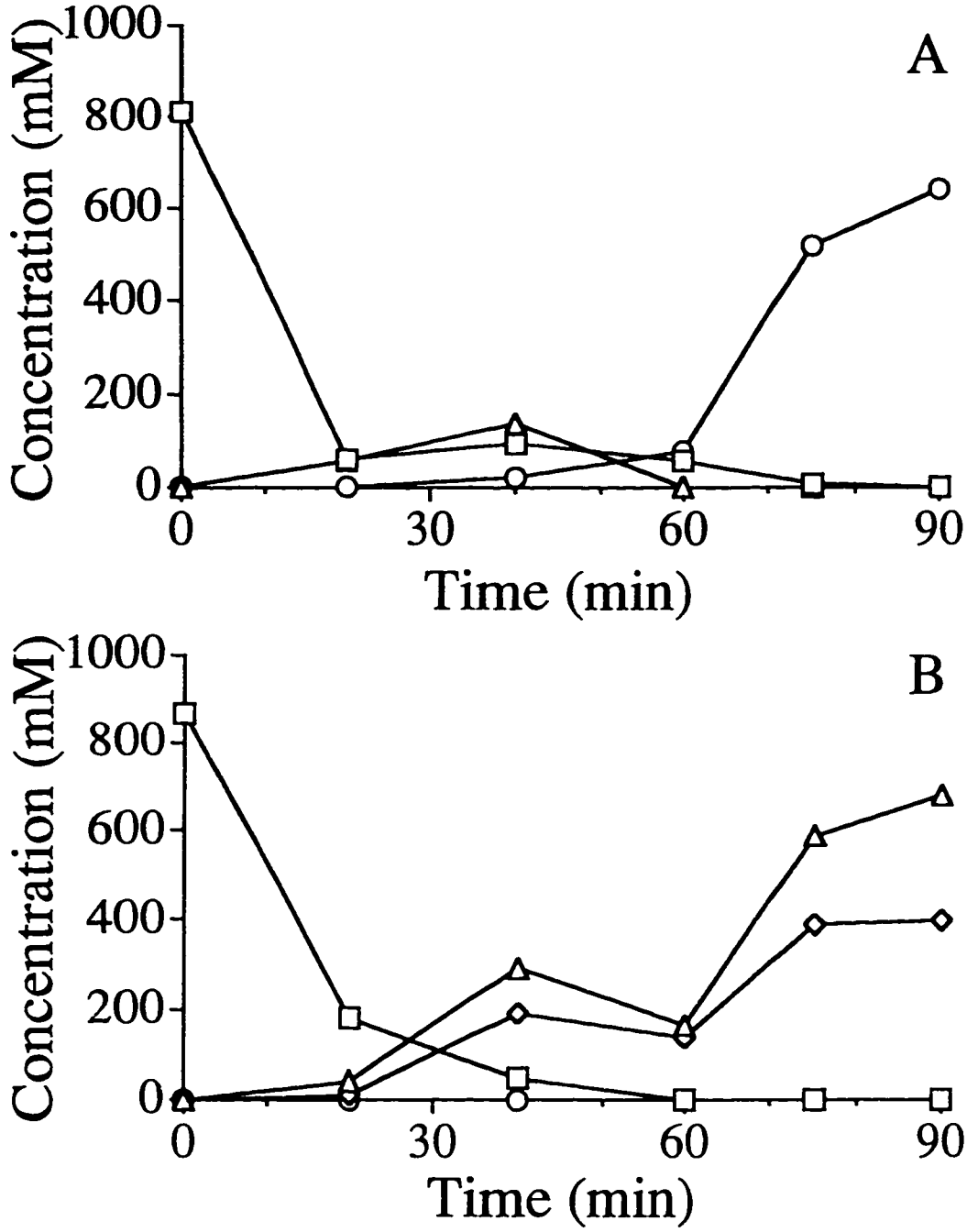

Figure 3: Effects of methyl viologen on complete reduction of 2,4-dinitrotoluene to 2,4-diaminotoluene by cell-free extracts of *D. tiedjei*. 2,4-Dinitrotoluene (□) depletion, 4-amino-2-nitrotoluene accumulation (Δ), and 2,4-diaminotoluene accumulation (○) in the presence (A) and absence (B) of 5.4 mM methyl viologen. The accumulation of an intermediate thought to be 4-amino-2-hydroxylaminotoluene is shown (◊) in peak area units x 10^{-4}. All values are averages of three assays with H_2 headspace.

Biochemical characterization of nitroreductase activity of *D. tiedjei*

Preliminary studies characterizing the nitroreductase activities of *D. tiedjei* provide insight into the nature of the catalytic activity. The reduction of aromatic nitro groups was likely catalyzed by heat-labile enzyme, as no reduction of nitroaromatic compounds occurred when boiled extract was added to incubations. This finding makes it unlikely that cofactors in the extracts were responsible for the activity. The abiotic loss of substrate in the absence of cell-free extract, but with methyl viologen and hydrogen, was sometimes observed. For example, 2,4-dinitrotoluene decreased in such incubations at ≤20 μM/hr (not shown), but formation of intermediates or products was not observed. The source of electrons for the reduction of the nitro groups in our experiments was hydrogen. Increasing the availability of hydrogen greatly increased the rate of 2,4-dinitrotoluene reduction from 81 μM/hr (5% H_2) to 2260 μM/hr (100% H_2) until the mass transfer of H_2 became limiting. A nitrogen headspace precluded any 2,4-dinitrotoluene reduction. Similarly, no nitroreductase activity was observed under aerobic conditions, but the extracts could be initially exposed to aerobiosis and the activity recovered under anoxic conditions with a hydrogen headspace.

Physiology of nitroreductase activities

In these studies, *D. tiedjei* was grown in the absence of any nitroaromatic compounds. This suggests that the enzymes responsible for the nitroreductase activity participate in other catalytic reactions and have a separate physiological role for this microorganism. *D. tiedjei* performs several reductive transformations which were considered possibly related to the nitroreductase activity, including the reductive dehalogenation of 3-chlorobenzoate, sulfate reduction, and the use of hydrogen as a source of electrons for reduction of electron acceptors.

The nitroreductase activity was not related to the dehalogenation activity of *D. tiedjei*. The addition of 2,6-dinitrotoluene did not affect the reduction of 3-chlorobenzoate

to benzoate, and *visa versa*, although the rates of the two reactions were sufficiently different that any effect of the dinitrotoluene on 3-chlorobenzoate dehalogenation would be difficult to detect. Furthermore, the nitroreductase activity was found to be a soluble activity whereas the dehalogenase was membrane-associated (Townsend and Suflita 1996). After ultracentrifugation of cell-free extracts, 72% of the initial nitroreductase activity was recovered in the soluble fraction, and only 6% was recovered in the insoluble fraction. These observations demonstrated that nitroaromatic compound reduction was not biochemically related to reductive dehalogenation for this microorganism.

Hydroxylamine and carbon monoxide have previously been reported to cause inhibition of nitroreductase activity: During the reduction of 2,4-diamino-6-nitrotoluene to triaminotoluene, they inhibited the final reduction and cause the accumulation of the hydroxylamino intermediate (Preuss et al. 1993). Hydroxylamine was only somewhat inhibitory to nitroaromatic reduction with extracts of *D. tiedjei*. At 1 mM, hydroxylamine had no effect on nitroreductase activity, whereas the rate of 2,4-dinitrotoluene reduction decreased from 150 μM/hr to 66 μM/hr in the presence of 10 mM hydroxylamine. Furthermore, the formation of intermediates and accumulation of the final diaminotoluene product were both inhibited (data not shown). This indicated that hydroxylamine affected several steps of the reduction of dinitrotoluene to diaminotoluene. Thus, inhibition of the enzyme that reduces the final *ortho*-hydroxylamino group as observed by Preuss et al. (1993) was not indicated.

D. tiedjei is versatile in its ability to use a variety of terminal electron acceptors, and it was postulated that nitroreductase activity could be related to reduction of anions by this organism. The effects of the potential alternate electron acceptors sulfate, nitrate, and nitrite on reduction of 2,4-diaminotoluene to 2,4-diaminotoluene were determined, as illustrated in Figure 4. None of these anions affected the rate of depletion of 2,4-dinitrotoluene, and

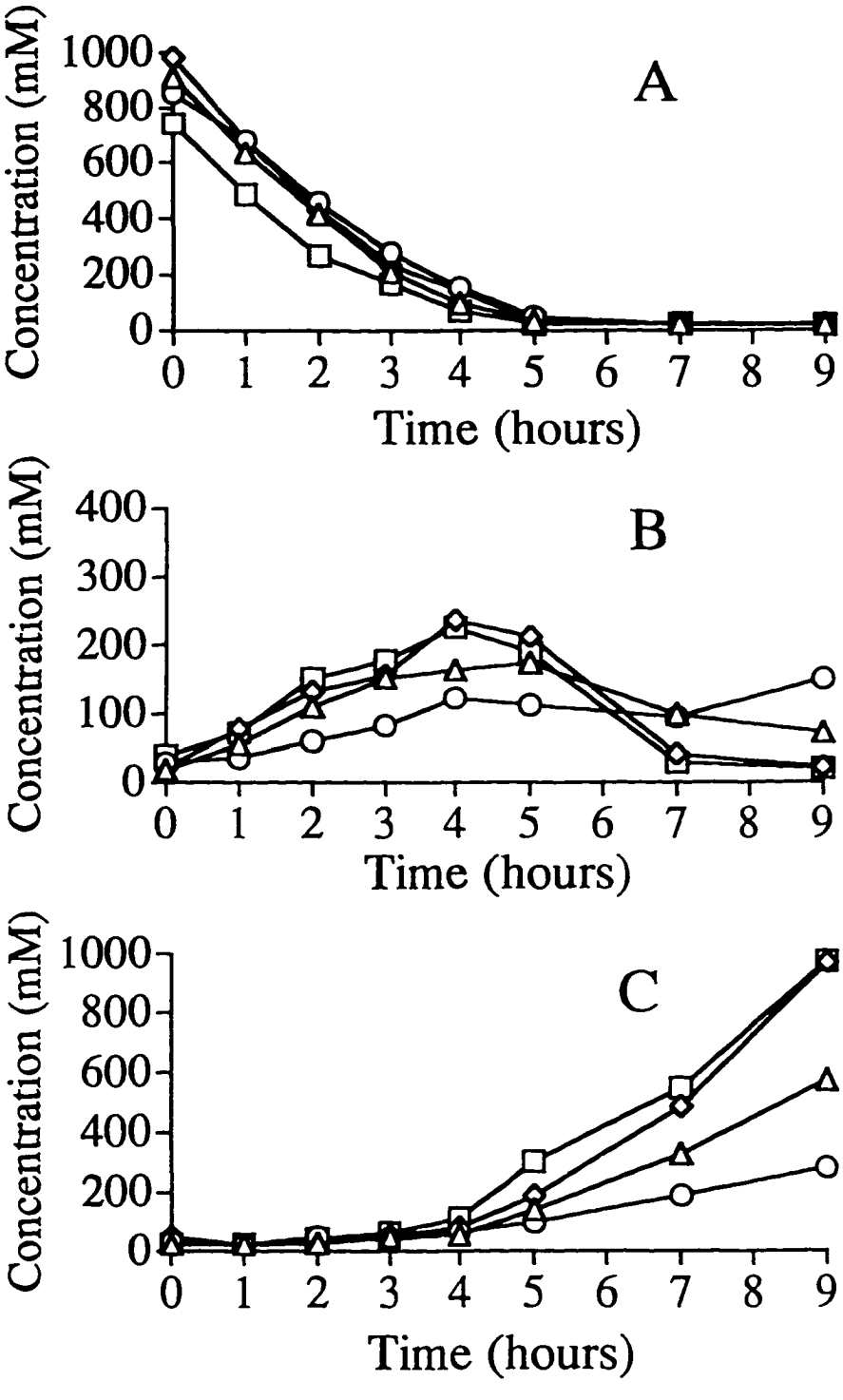

Figure 4: Effects of anions on reduction of 2,4-dinitrotoluene by cell-free extracts of *D. tiedjei*. Depletion of 2,4-dinitrotoluene (A), transient accumulation of 4-amino-2-nitrotoluene (B), and accumulation of 2,4-diaminotoluene (C) are shown in the presence of 10 mM of nitrate (Δ), nitrite (○), sulfate (◊), or controls without anions (□).

sulfate had no effect on the accumulation of either the intermediate (4-amino-2-nitrotoluene) or the product (2,4-diaminotoluene). The effects of sulfite on nitroreductase activity were not tested. Nitrate, and especially nitrite, inhibited the production of the intermediate as well as the accumulation of the final product (Figure 4). This indicated that these anions affect the reduction of the nitroso or hydroxylamino intermediates for both of the nitro groups on the aromatic ring. When the experiment was repeated with 2,6-dinitrotoluene, identical results were obtained (data not shown). Therefore, the inhibition of nitroaromatic compound reduction by nitrate and nitrite was not dependent upon the position of the nitro group, and was not an inhibition of the initial reduction of the nitro groups, but rather an inhibition of reduction of nitroso- or hydroxylamino intermediates that precede the aminoaromatic products.

During preliminary attempts to purify the nitroreductase enzyme(s) using ammonium sulfate precipitation or column chromatography, the fractions that contained nitroreductase activity invariably also reduced methyl viologen, suggesting that the activity was associated with hydrogenases. In fact, a known inhibitor of hydrogenases, copper, was found to inhibit nitroreductase activity. In the presence of 1 mM $CuCl_2$, nitroreductase activity decreased from 150 μM/hr to 55 μM/hr; with 10 mM $CuCl_2$ the activity decreased to 29 μM/hr and was identical to the abiotic losses observed in negative controls. This evidence supports the supposition that hydrogenases are involved in the original electron transfer to the nitroreductase enzyme(s) in *D. tiedjei*.

Discussion

D. tiedjei is similar to other anaerobic microorganisms (Gorontzy et al. 1993) in that it possesses enzymes that catalyze the non-specific reduction of a variety of nitroaromatic compounds. Similar reductions were observed with cell-free extracts of *M. thermoautotrophicum*, which has previously been shown to reduce 2,4-dinitrophenol (Gorontzy et al. 1993). Whole cells of both these organisms catalyze the reduction of 2,4-

dinitrotoluene in cell suspensions (Davidova, personal communication). Therefore, our results are consistent with the general observation that anaerobic microorganisms have an inherent ability to reduce nitroaromatic compounds provided that suitable electron donors are available. However, the reduction of dinitrotoluene isomers under methanogenic and sulfate-reducing conditions was not observed in aquifer slurries (Krumholz et al. 1996), so other factors may preclude the reduction of nitrotoluenes in anaerobic environments.

The complete reduction of 2,4-dinitrotoluene to 2,4-diaminotoluene involved conversion of nitro groups to amino groups, presumably via nitroso- and hydroxylamine-intermediates, in both the *para* and *ortho* positions. Cell extracts of *D. tiedjei* reduced the *para*-nitro group of 2,4-dinitrotoluene first to the exclusion of the *ortho*-nitro group, whereas extracts of *M. thermoautotrophicum* reduced both groups, but still favored reduction of the *para*-nitro group. Preferential reduction of nitro groups at the *para*-position for nitrotoluenes has been reported previously (McCormick et al. 1976) and can be explained in part by the positive field effect of the methyl group, which mainly affects the *ortho*-position (Preuß and Rieger 1995).

The physiological role of the enzymes catalyzing nitroreductase activities remain uncertain for *D. tiedjei* as with other organisms. Hydroxylamine and carbon monoxide are known to affect dissimilatory sulfite reductase - hydroxylamine as an alternative substrate and carbon monoxide as an inhibitor. Hydroxylamine caused inhibition of nitroreductase activity in a sulfate-reducing bacterium, which together with the observed inhibition of sulfide formation from sulfite by the addition of nitroaromatic compounds, led Preuss et al. (1993) to suggest that the nitroreductase activity was related to sulfite reductase. The results of our experiments with hydroxylamine and sulfate provided no evidence to this effect for *D. tiedjei*. The effects of carbon monoxide on nitroreductase activity, either as an inhibitor or as a source of electrons, were not determined. The nature of the inhibition of *D. tiedjei* nitroreductase by nitrate and nitrite was not determined. Boopathy et al. (1993) proposed that TNT reduction by *Desulfovibrio* sp. (strain B) was carried out by the enzyme

nitrite reductase, which is involved in the reduction of nitrate to ammonia, and is found in most *Desulfovibrio* sp. Nitrite has been reported to inactivate clostridial nitro-group-reducing ferredoxin (Angermaier and Simon, 1983). Angermaier and Simon (1983) addressed whether there was a relationship between clostridial enzymes which reduce sulfite, sulfate, nitrite, nitrate, or hydroxylamine and the reduction of nitro groups. In the presence of these anions or hydroxylamine (48 or 100 mM respectively), the hydrogen uptake catalyzed by crude extracts of *C. kluyveri* was less than 5% of that observed in the presence of *p*-nitrobenzoate, indicating no relationship between nitroreductase activity and anion reduction, although the effects of anions or hydroxylamine on hydrogen uptake in the presence of *p*-nitrobenzoate was not reported. Further investigation would be required to determine the relationship between nitroreductase activities and the reduction of nitrate and other potential electron acceptors for *D. tiedjei*.

Our preliminary evidence suggested that the complete reduction of 2,4-dinitrotoluene to 2,4-diaminotoluene in *D. tiedjei* was catalyzed by soluble methyl-viologen-utilizing enzyme complexes probably containing hydrogenases. Previous reports have also indicated that nitroreductase activity is related to hydrogenases. Hydrogenase purified from *Clostridium pasteurianum* and carbon monoxide dehydrogenase partially purified from *Clostridium thermoaceticum* catalyzed the partial reduction of 2,4-diamino-6-nitrotoluene to 2,4-diamino-6-hydroxylaminotoluene in the presence of methyl viologen or ferredoxin (Preuss et al. 1993). In addition, during the nitroreductase purification procedure, the ratio of hydrogenase and nitroreductase activities remained essentially constant. These authors concluded that the reduction of 2,4-diamino-6-nitrotoluene to 2,4-diamino-6-hydroxylaminotoluene was an non-specific reaction probably mediated by all enzymes which are able to reduce ferredoxin and/or viologen dyes, whereas the second step is probably mediated by sulfite reductase (Press et al. 1993). However, our results all agree with those of McCormick et al. (1976) who reported that the nitroreductase activity of

V. alkalescens extracts was associated with two protein fractions, one having some ferredoxin-like properties and the other possessing hydrogenase activity.

Future investigations and purification of enzymes responsible for nitroreductase activity could clarify the detailed mechanism by which the enzymes catalyzed the complete reduction of nitroaromatic compounds to the corresponding amines. In addition, further research with whole cells of *D. tiedjei* and *M. thermoautotrophicum* in progress are expected to demonstrate the ability of these organisms to reduce nitroaromatic compounds. Additional studies with cell extracts and whole cells of several anaerobes will be required to delineate the physiological basis of nitroreductase activity, and the ecological significance of reduction of nitroaromatic compounds by anaerobic microorganisms.

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