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THE METHANOGENIC DEGRADATION OF m-CRESOL AND RELATED AROMATIC COMPOUNDS

BY

© KATHLEEN LONDRY

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled THE METHANOGENIC DEGRADATION OF m-CRESOL AND RELATED AROMATIC COMPOUNDS submitted by KATHLEEN LONDRY in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

Dr. Phillip M. Fedorak

Dr. Donald W.S. Westlake

Dr. Susan E. Jensen

Dr. Steve E. Hrudey

December 16, 1992

DEDICATION

I DEDICATE THIS THESIS TO MY FAMILY,

especially my husband Steve Londry.

Their love and support made this possible.

ABSTRACT

The methanogenic metabolism of m-cresol and related aromatic compounds was studied. Stable m-cresol-degrading methanogenic consortia were enriched from domestic sewage sludge and characteristics of these enrichment cultures were explored. Enrichment cultures were also established that could degrade 2-methylbenzoic acid, a metabolite originally detected in m-cresol-degrading cultures. The degradation of 2-methylbenzoic acid occurred only after long lag periods and was difficult to maintain. Factors affecting 2-methylbenzoic acid degradation including inocula, 2-methylbenzoic acid concentration, and reducing agents, were analyzed, and a metabolite tentatively identified as a hydroxy-methylbenzoic acid was detected.

The use of fluorophenols and 3-fluorobenzoic acid was an effective tool for studying the methanogenic degradation of phenol. The addition of 2-fluorophenol to phenol-degrading cultures caused the accumulation of the metabolites 4-hydroxybenzoic acid and benzoic acid as well as the fluorinated transformation products 3-fluoro-4-hydroxybenzoic acid and 3-fluorobenzoic acid. All three fluorophenol isomers inhibited phenol degradation at ≥100 mg/L, but not at ≤25 mg/L. At concentrations of ≤10 mg/L, 2-fluorophenol was mineralized to methane, carbon dioxide, and fluoride. The release of fluoride was also observed from 3-fluorophenol when it was initially present at ≤ 10 mg/L. 3-Fluorobenzoic acid was not transformed by phenol-degrading cultures, but facilitated the detection of 4-hydroxybenzoic acid and benzoic acid from phenol.

The addition of fluorinated m-cresol (6-fluoro-3-methylphenol) to m-cresol-degrading cultures caused the accumulation of the metabolites 4-hydroxy-2-methylbenzoic acid, 2-methylbenzoic acid, and the fluorinated transformation products 5-fluoro-4-hydroxy-2-methylbenzoic acid, 5-fluoro-2-methylbenzoic acid, and 3-fluoro-4-hydroxybenzoic acid. The addition of 50 mM BESA to cultures facilitated the detection of benzoic acid in m-cresol-degrading cultures, and a trace amount of a metabolite that was tentatively identified as 3-fluorobenzoic acid, in 6-fluoro-3-methylphenol-transforming cultures. 4-Hydroxybenzoic acid was degraded in an m-cresol-degrading enrichment culture as well as in subcultures, was

detected as a transient metabolite in cultures inhibited by the addition of 3-fluorobenzoic acid, and was detected in enrichment cultures inhibited with 6-fluoro-3-methylphenol and BESA. The intermediates detected were consistent with a pathway for m-cresol degradation involving an initial *para*-carboxylation followed by a reductive demethylation and finally a dehydroxylation to give benzoic acid, a common intermediate in the anaerobic degradation of aromatic compounds.

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LIST OF ABBREVIATIONS

ACS	Aqueous Counting Scintillant
BESA	Bromoethanesulfonic acid
BSA	N,O-bis-(trimethylsilyl)acetamide
CoA	Coenzyme-A
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
TLC	Thin-layer chromatography
TMS	Trimethylsilyl
UV	Ultraviolet

1 INTRODUCTION

1.1 Phenols in the Environment

The motivation behind studying the methanogenic degradation of m-cresol can be assessed in terms of two generalized applications: bioremediation of contaminated environments, and treatment of industrial wastewaters. Once released into the environment, deliberately, accidentally, or naturally, many aromatic compounds end up in anaerobic environments, including aquifers, sediments, soils, landfills, and sewage sludges. While subsurface communities have previously been able to degrade natural sources of phenols, the introduction of large quantities of xenobiotic compounds threatens to disturb the natural balance. Once aromatic compounds enter anaerobic environments, their fates depend on many factors, most of which are poorly understood at this time. Understanding the processes that affect the fate of organic contaminants is necessary in order to make sound decisions about their hazards to the public and environment, and to develop suitable mitigation techniques.

1.1.1 Natural sources of phenols

Plants produce simple phenols including hydroxyquinone, gallic acid, salicylic acid, protocatechuic acid and 4-hydroxybenzoic acid; more complex phenols such as the flavonoids; and complex phenolic polymers including lignins, catechol melanins and flavolans. Harborne (1980) reported that several thousand plant phenols have been described. These must be biodegraded to ensure that carbon will be recycled in the biosphere. Phenols are also produced in the intestinal tracts of many farm animals, and are components of the bad smell emitted by their swine (Beaudet et al. 1986).

1.1.2 Industrial sources of phenols

Phenols released into the environment from industrial processes are of greater concern than plant phenols because of their potential toxicity and recalcitrance. Petroleum refining and coal conversion processes such as coking, liquefaction, and gasification produce wastewater effluents containing up to gram quantities of phenols per litre (for review see

Fedorak and Hrudey 1988). Coal tar creosotes used for wood preservation consist of about 10% phenols which are much more water-soluble than the polycyclic aromatic hydrocarbons that account for about 85% of these materials (Mueller et al. 1989). Other industries or activities that result in phenol-containing wastewaters include the textiles industry, landfill leachates, and the production of chemicals and synthetics such as pesticides, insecticides, herbicides, detergents, solvents, fertilizers, dyes, and wood preservatives. A summary of individual constituents of wastewaters produced in the synthetic fuels industry indicated that cresols were found in almost all wastewaters from this industry, in quantities of up to grams per litre, often as the main constituents of these wastewaters (Neufeld 1984). Because of their relatively high solubilities, phenol, the cresols, and other alkyl phenols become the most abundant organic compounds dissolved in aquifers beneath soils contaminated with creosote (Mueller et al. 1989).

Pentachlorophenol is one of the most common biocides used in wood preserving preparations which often contain a variety of other chlorinated phenols (NRCC 1982) and these can be found in soils and waters near sawmills (Valo et al. 1984). Chlorinated phenols are also produced during the bleaching process in pulp and paper mills (Lindström and Nordin 1976; Kovacs et al. 1984). In addition, the agricultural use of some herbicides yields chlorophenol metabolites. For example, the microbial metabolism of the herbicides 2,4-D and 2,4,5-T produced 4-chlorophenol and 2,4,5-trichlorophenol, respectively (Mikesell and Boyd 1985).

1.1.3 Bioremediation

Understanding the fate of phenols in the environment has been the driving force behind much of this research. In addition, application of anaerobic microbiological processes for the removal of phenols from industrial wastewaters has been suggested and demonstrated on laboratory-scale (Cross et al. 1982; Suidan et al. 1983, 1987; Fedorak and Hrudey 1986a; Fox et al. 1988). Recently, the potential for using bioremediation as a means of removing organic compounds from contaminated soils and groundwaters (Thomas and Ward 1989) has fostered many investigations on the anaerobic metabolism of phenols and other aromatic compounds.

The use of bioremediation for treatment of subsurface regions is challenging because these regions are difficult to characterize and the

introduction of chemicals and microorganisms is not easy. Coupled with the limited understanding of factors controlling biotransformation pathways and reaction rates of many organic contaminants of concern, the utility of *in situ* bioremediation is an important scientific and engineering problem to establish. The advantages of biotransformation are that contaminants can be completely mineralized to innocuous products, and that microbially-mediated enzymatic reactions are generally faster than those of the same reactions in the absence of microorganisms (Bouwer 1992). An additional advantage of methanogenic processes is the production of methane, a valuable fuel source. This methane can be harvested from bioreactors and treatment plants, or recovered from *in situ* environments such as landfills. In comparison with aerobic microbiological treatment of the contaminated subsurface, anaerobic processes are significantly slower, but they are cheaper, and they create far less biofouling because anaerobic microbial communities produce less biomass for the same amount of substrate. Furthermore, for some pollutants, anaerobic biotransformation may be the only workable option (Grbic´-Galic´ 1990).

The content of phenols in wastewaters must be reduced prior to release into the environment to meet strict environmental guidelines. Wastewaters must also be treated to remove biological oxygen demand prior to introduction into receiving waters. Although methanogenic processes are not ready for use for the full-scale treatment of phenol- and cresol-contaminated wastewaters, studies with laboratory-scale bioreactors (Suidan et al. 1988; Fedorak and Hrudey 1988) are progressing towards that goal.

1.2 Anaerobic Environments

Although this research is concerned primarily with degradation under methanogenic conditions, it is important to understand the significance of the various electron acceptors, before comparing the degradation of various compounds in different anaerobic environments.

1.2.1 Environmental significance of electron acceptors

The equations below compare the free energies available when selected terminal electron acceptors are reduced with H_2 as the electron donor (Thauer et al. 1977). The greatest amount of energy is available when oxygen is used as the terminal electron acceptor.

$O_2 + 2H_2$	$\rightarrow$	$2H_2O$	$\Delta G^{\circ\prime} = -237$ kJ/H_2
$2Fe^{3+} + H_2$	$\rightarrow$	$2Fe^{2+} + 2H^+$	$\Delta G^{\circ\prime} = -228$ kJ/H_2
$NO_3^- + 2H^+ + 4H_2$	$\rightarrow$	$NH_4^+ + 3H_2O$	$\Delta G^{\circ\prime} = -150$ kJ/H_2
$SO_4^= + H^+ + 4H_2$	$\rightarrow$	$HS^- + 4H_2O$	$\Delta G^{\circ\prime} = -38$ kJ/H_2
$HCO_3^- + H^+ + 4H_2$	$\rightarrow$	$CH_4 + 3H_2O$	$\Delta G^{\circ\prime} = -34$ kJ/H_2

Anaerobic conditions prevail in an environment when the rate of consumption of molecular oxygen exceeds its rate of diffusion into that environment. Within a microbial community, that portion of the population which uses a terminal electron acceptor yielding the greatest amount of energy has a competitive advantage. This group of microbes directs the principal flow of electrons as long as there is an adequate supply of the required electron acceptor. For example, ferric iron-reducing microorganisms out-compete sulfate-reducing bacteria and methanogens when ferric iron is present as amorphic ferric oxyhydroxide (Lovley 1987). When soils and freshwater sediments become anaerobic, ferric iron is often the most abundant potential electron acceptor for microbial metabolism (Lovley and Lonergan 1990).

If nitrate is present in environments that are devoid of oxygen, this anion serves as terminal electron acceptor for many facultative anaerobes. Indeed, the addition of nitrate to groundwaters contaminated with hydrocarbons or creosote is an attractive method of providing both a nitrogen source and an electron acceptor for bioremediation (Thomas and Ward 1989; Hutchins et al. 1991). Sulfate is usually the predominant terminal electron acceptor in marine sediments, and the abilities of sulfate-reducing bacteria to out-compete methanogens in the presence of sulfate is well documented (Winfrey and Zeikus 1977; Abram and Nedwell 1978; Lovley and Klug 1983). Usually, methanogenesis coupled to fermentation will occur only after sulfate has been depleted, because sulfate-reducers can successfully out-compete methanogens for common substrates, such as

acetate and H_2. It has been shown that the catabolic enzymes of sulfate-reducing bacteria have higher affinities for acetate and hydrogen than those of methanogens (Kristjannsson et al. 1982). In the absence of other electron acceptors, methanogenic conditions exist wherein bicarbonate serves as the electron acceptor.

1.2.2 Associations in methanogenic consortia

The interactions between fermenters, obligate proton-reducers, and methanogens are indispensable to achieve mineralization of organic matter. As described here, mineralization within methanogenic systems refers to the complete degradation of a compound to CO_2 and CH_4. Fermentative bacteria can hydrolyze organic polymers such as cellulose, proteins, or lipids, and ferment the resulting monomers to aliphatic alcohols, acids, acetate, formate, molecular hydrogen, and CO_2. Fermentative bacteria can metabolize their substrates independently, but in the presence of hydrogen-scavenging methanogens, they can form different fermentation products and thereby obtain more energy. This is possible because the hydrogen-utilizers keep the partial pressure of H_2 exceedingly low, thereby shifting the overall reaction forward. Obligate proton-reducers, which are syntrophically dependent on a hydrogen-removing agent, degrade aliphatic alcohols and acids to acetate, H_2, and CO_2. Finally, methanogens use a limited range of organic compounds (methanol, formate, acetate, methylated amines) and H_2 plus CO_2. Methanogens benefit from the products formed by fermentative and syntrophic acetogenic bacteria, as well as from the low oxidation-reduction potential that is indispensable for methanogenic metabolism which is maintained by the associated microorganisms. These different physiological groups of microorganisms are interconnected through interspecies transfer of intermediates, formate, and hydrogen (Grbic´-Galic´ 1990). The ecological result of these microbial interactions is the complete breakdown of numerous organic molecules.

1.3 Anaerobic Degradation of Aromatic Compounds

Tarvin and Buswell (1934) were the first to demonstrate that aromatic compounds could be completely biodegraded to methane and

carbon dioxide under anaerobic conditions. Using anaerobic sewage sludge as an inoculum, they showed that benzoic, cinnamic, hydrocinnamic and phenylacetic acids were converted to methane and carbon dioxide and that phenol was produced as a transient intermediate in the methanogenic biodegradation of tyrosine. During the late 1970s and throughout the 1980s, there were numerous investigations to determine the susceptibilities of many aromatic compounds to anaerobic degradation via photometabolism or with nitrate, sulfate, or carbon dioxide serving as the terminal electron acceptor. Many of the findings of these studies have been reviewed (Evans 1977; Sleat and Robinson 1984; Young 1984; Berry et al. 1987; Fedorak and Hrudey 1988; Evans and Fuchs 1988; Grbic´-Galic´ 1989; Haddock and Ferry 1990; Grbic´-Galic´ 1990). The following discussion focuses on the degradation of oxidized aromatic compounds, almost to the exclusion of aromatic heterocycles or hydrocarbons, for the sake of brevity. However, it should be noted that the anaerobic degradation of many such compounds has been reported, and the initial steps in some of the pathways have been determined.

1.3.1 Anaerobic metabolism of phenol

Not only is phenol a frequent environmental contaminant, but it is also a common metabolite in the anaerobic metabolism of many compounds. For example, Grbic´-Galic´ and Vogel (1987) showed that ^{18}O-labelled water was incorporated into benzene to form labelled phenol. Phenol arising as an intermediate is subjected to further metabolism leading to mineralization. For example, Zhang and Wiegel (1990) demonstrated that 2,4-dichlorophenol was metabolized to methane and carbon dioxide via 4-chlorophenol, phenol, benzoic acid, acetate and hydrogen. Thus, the degradation of phenol is necessary to complete the metabolism of many other compounds.

1.3.1.1 Evidence for a reductive pathway

Bakker (1977) demonstrated that phenol could be degraded by a mixed culture using nitrate as the terminal electron acceptor. *n*-Caproic acid was the only intermediate identified and it was postulated that the aromatic ring was reduced to yield cyclohexanone prior to ring fission.

Balba and Evans (1980a) added [U-^{14}C]phenol to a catechol-adapted methanogenic consortium that metabolized phenol without a lag period. They found labelled cyclohexanone, 2-hydroxycyclohexanone, adipate, succinate, propionate, and acetate in the culture extracts. The presence of cyclohexanone confirmed the reductive pathway hypothesized by Bakker (1977). In cultures with non-radioactive substrate, Balba and Evans (1980a) detected cyclohexanol as an intermediate of phenol metabolism and proposed the reductive pathway shown in Figure1-1. Although this pathway has been widely cited in review articles (Evans 1977; Young 1984; Fedorak and Hrudey 1988; Evans and Fuchs 1988), there have been only a few reports of the early intermediates being found by other investigators: Williams and Evans (1975) detected cyclohexanone during the metabolism of benzoic acid by a *Moraxella* sp. via nitrate-respiration; Grbic´-Galic´ and Vogel (1987) observed cyclohexanone in a methanogenic consortium degrading benzene; and Grbic´-Galic´ and Young (1985) found cyclohexanone in a methanogenic consortium degrading ferulate and benzoic acid. Indeed, several investigators have added cyclohexanol or cyclohexanone to phenol-degrading anaerobic cultures and failed to detect utilization of these intermediates (Fedorak, unpublished data; Bak and Widdel 1986; Tschech and Fuchs 1987; Kobayashi et al. 1989; Béchard et al. 1990).

1.3.1.2 Carboxylation under methanogenic conditions

In their attempts to increase the excretion of intermediates by a phenol-degrading methanogenic consortium, Knoll and Winter (1987) used an atmosphere of 80% H_2 and 20% CO_2 to stimulate feed-back inhibition. This led to the accumulation of an intermediate that was identified as benzoic acid, implicating a different pathway for phenol degradation. Radiolabelled benzoic acid was detected when unlabelled phenol and $^{14}CO_2$ were added to the consortium (Knoll and Winter 1987). This clearly showed the involvement of CO_2 in the metabolism of phenol under methanogenic conditions and was consistent with the results of an earlier study that led Fedorak and Hrudey (1986b) to hypothesize that the phenol-degrading bacteria in a methanogenic consortium may require CO_2. In the earlier study, draw and feed cultures received an artificial wastewater that

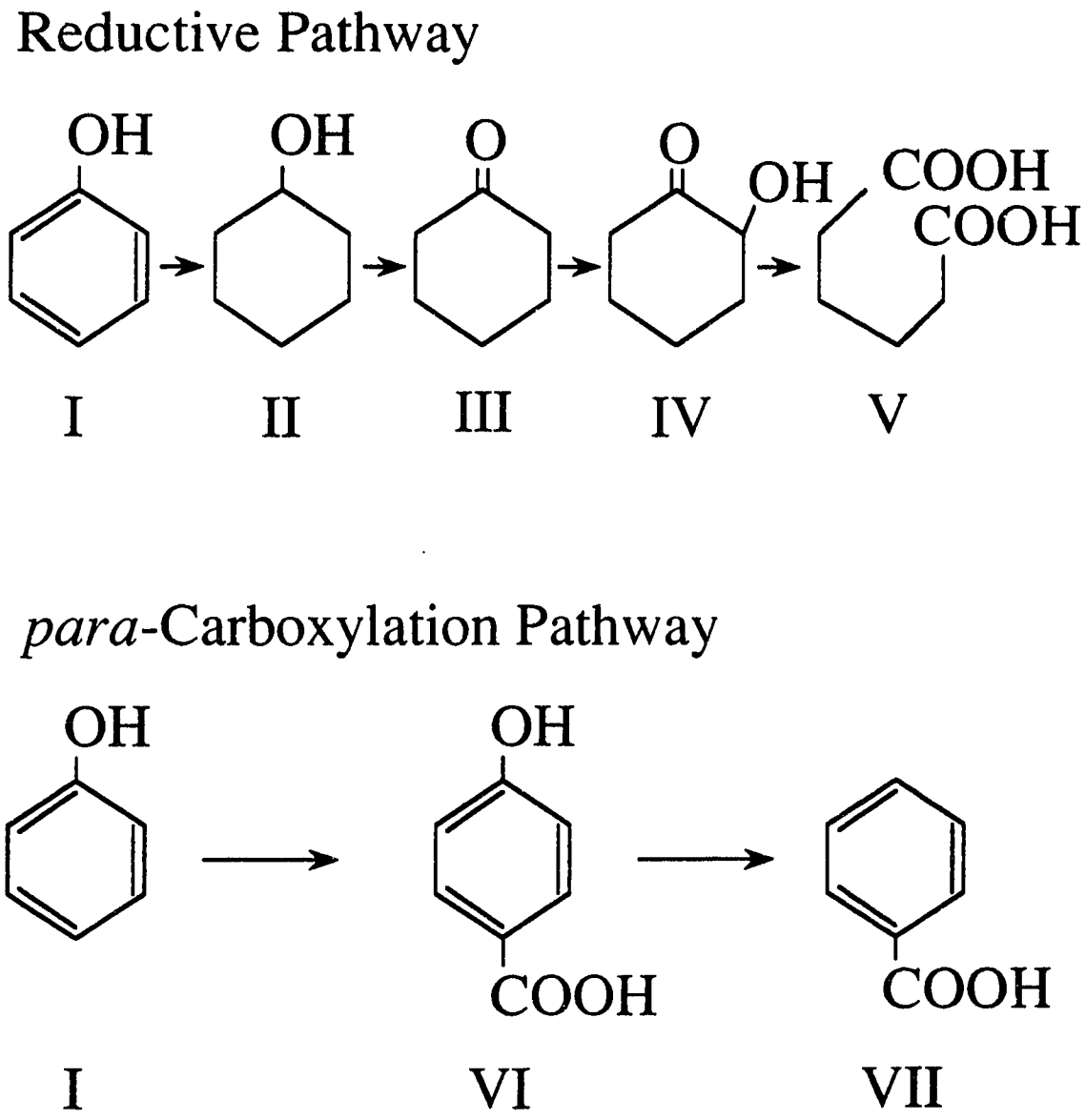


Figure 1-1: Comparison of the reductive and *para*-carboxylation pathways of phenol degradation under anaerobic conditions. Phenol (I), cyclohexanol (II), cyclohexanone (III), 2-hydroxycyclohexanone (IV), adipic acid (V), 4-hydroxybenzoic acid (VI), and benzoic acid (VII).

contained phenol as the major carbon source. The requirement for CO_2 was realized when methane production ceased in cultures that received a feed solution devoid of bicarbonate.

Kobayashi et al. (1989) established two methane-producing consortia; one enriched on phenol and the other enriched on benzoic acid. By changing the composition of the headspace gas in the phenol-degrading culture, they obtained essentially the same results as Knoll and Winter (1987). Under an atmosphere of 80% H_2 and 20% CO_2, phenol was depleted from the medium and benzoic acid accumulated whereas under an atmosphere of 80% N_2 and 20% CO_2, benzoic acid was observed as a transient intermediate during phenol degradation. Kobayashi et al. (1989) also tested the abilities of the phenol-degrading and benzoic acid-degrading consortia to produce methane from a variety of intermediates in the proposed pathways for the anaerobic metabolism of benzoic acid. Although the rate of methane production in the benzoic acid-degrading consortium was faster than in the phenol-degrading consortium, both consortia degraded benzoic acid, cyclohexane carboxylate, cyclohex-1-enecarboxylate, and caproate to methane. Little methane was produced from phenol by the benzoic acid-degrading consortium. These results indicated that the metabolism of phenol proceeded via benzoic acid rather than via the reductive pathway (Figure 1-1).

Béchard et al. (1990) studied a methanogenic consortium enriched from a mixture of swamp water, sewage sludge, swine waste, and soil (Beaudet et al. 1986) that transformed phenol to benzoic acid. They observed that proteose peptone was required in the medium for the carboxylation of phenol to occur. They hypothesized that the carboxylation was accomplished by co-metabolism and that proteose peptone or some of its degradation products served as carbon and energy sources for the growth of the carboxylating bacteria. In a subsequent study, Bisaillon et al. (1991a) found that yeast extract or a mixture of tryptophan and lysine could replace proteose peptone without affecting the carboxylating activity.

The carboxylation reaction was studied in more detail by Sharak Genthner et al. (1989c, 1990) using fluorinated analogues of phenol under methanogenic conditions. They concluded that carboxylation occurred at the position *para* to the hydroxyl group based on the observations that 2-fluorophenol was transformed to 3-fluorobenzoic acid, 3-fluorophenol was

transformed to 2-fluorobenzoic acid, and 4-fluorophenol was not transformed. In a subsequent study, Sharak Genthner et al. (1990) showed that when 3-fluoro-4-hydroxybenzoic acid, the proposed but undetected intermediate of 2-fluorophenol carboxylation, was added to an active phenol-degrading culture, two products were formed. One was 2-fluorophenol, resulting from a decarboxylation, and the other was 3-fluorobenzoic acid, resulting from a dehydroxylation. Upon further incubation, 2-fluorophenol was transformed to 3-fluorobenzoic acid.

Zhang et al. (1990) also provided evidence for the *para*-carboxylation mechanism under methanogenic conditions by adding [1-^{13}C]phenol to a phenol-degrading enrichment culture and isolating [4-^{13}C]benzoic acid. Later, Gallert et al. (1991) used deuterated phenols to study the transformation of phenol to benzoic acid, and revealed that the carboxylation occurs exclusively in the C4-position.

To date, Sharak Genthner et al. (1991) have published the only report of the actual detection of 4-hydroxybenzoic acid as an intermediate in the methanogenic transformation of phenol. To detect this metabolite, they inhibited their phenol-degrading culture with a high concentration of phenol, benzoic acid, or with the phenol analogue, 6-hydroxynicotinic acid.

1.3.1.3 Carboxylation under nitrate-reducing conditions

There have been few studies on the degradation of phenol under nitrate-reducing conditions. Whereas Bakker (1977) found that a mixed culture was required for rapid phenol degradation under these conditions, Tschech and Fuchs (1987) obtained two *Pseudomonas* isolates that individually degraded phenol and had an absolute requirement for bicarbonate. When either isolate was exposed to 4-hydroxybenzoic acid in the presence of [^{14}C]bicarbonate, [^{14}C]4-hydroxybenzoic acid was detected in the medium. It was the result of an exchange mechanism: a decarboxylation to phenol and a subsequent *para*-carboxylation. Tschech and Fuchs (1989) have examined some characteristics of a novel phenol carboxylase that catalyzes the *para*-carboxylation (discussed in section 1.3.3.2). Lack and Fuchs (1992) demonstrated that phenylphosphate, the phosphoric acid ester of phenol, is carboxylated by a phenol carboxylase, and suggested that the formation of an enzyme-bound phenolate anion from

the activated phenolic compound is the rate-determining step in the carboxylation reaction.

1.3.1.4 Carboxylation under ferric- and sulfate-reducing conditions

Recently, Lovley and Lonergan (1990) described the degradation of phenol to CO_2 by an isolate, designated GS-15, that used Fe^{3+} as its terminal electron acceptor. They also found evidence of *para*-carboxylation because 4-hydroxybenzoic acid was detected as a transient intermediate in the culture medium of GS-15.

There appear to be no reports of mixed cultures degrading phenol under sulfate-reducing conditions, although there have been a few reports of mixed cultures degrading benzoic acid under these conditions (Balba and Evans 1980c; Mountfort and Bryant 1982). Bak and Widdel (1986) described a new sulfate-reducer, *Desulfobacterium phenolicum*, that degraded phenol, 4-hydroxybenzoic acid, and several other aromatic compounds. Although the degradation pathway involved in phenol utilization by this organism was not studied, its ability to use 4-hydroxybenzoic acid suggests that phenol may be metabolized via *para*-carboxylation. Schnell and Schink (1991) demonstrated that the sulfate-reducer *Desulfobacterium anilini* required carbon dioxide to metabolize phenol or aniline. The intermediates of phenol metabolism were not studied; however, aniline was *para*-carboxylated to 4-aminobenzoic acid.

1.3.2 Degradation of cresols

1.3.2.1 Anaerobic metabolism of p-cresol

Like phenol, p-cresol has been shown to arise as a metabolite during the anaerobic biodegradation of other compounds. However, the list of such occurrences is short. p-Cresol was found as a metabolite of tyrosine (Balba and Evans 1980b) and toluene (Vogel and Grbic´-Galic´ 1986) degradation under methanogenic conditions. p-Cresol has also been suggested as an intermediate in toluene degradation under denitrifying conditions (Kuhn et al. 1988), as well as by a recently isolated iron-reducer (Lovley and Lonergan 1990).

Although there have been numerous reports that p-cresol is biodegradable under methanogenic conditions (Chmielowski et al. 1965;

Boyd et al. 1983; Fedorak and Hrudey 1984; Young and Rivera 1985), there has been little work to elucidate the metabolic pathway. Senior and Balba (1984) observed 4-hydroxybenzoic acid and phenol in their chemostat culture; apparently, the methyl group was oxidized to a carboxylic acid and decarboxylated to yield phenol. Roberts et al. (1987) studied the fate of the methyl group using [*methyl*-^{14}C]p-cresol in methanogenic enrichment cultures. They found that about 92% of the label was transformed to carbon dioxide, while only about 2% was transformed to methane. These results were consistent with the methyl group oxidation and decarboxylation suggested by Senior and Balba (1984). Haggblom et al. (1990) compared the degradation of p-cresol by one sediment source under three reducing conditions: nitrate-reducing, sulfate-reducing, and methanogenic. p-Cresol was metabolized under all three conditions, proceeding through p-hydroxybenzaldehyde and p-hydroxybenzoic acid under denitrifying and methanogenic conditions. Benzoic acid was detected under methanogenic conditions only. These metabolites are consistent with the previous proposals for the sequential oxidation of the methyl group of p-cresol, followed by a dehydroxylation to yield benzoic acid (Figure 1-2).

The initial biotransformations of p-cresol under nitrate-reducing conditions have been studied in considerable detail. Bossert et al. (1986) isolated a two-member bacterial co-culture that utilized p-cresol as its growth substrate. One isolate, designated PC-07, oxidized p-cresol to 4-hydroxybenzoic acid, yielding energy but no carbon for growth. The other isolate, designated PB-04, could not grow on p-cresol but metabolized the 4-hydroxybenzoic acid produced by PC-07, thereby providing carbon for the growth of both members of the co-culture. However, the ring fission products were not identified. The oxidation of the methyl group of p-cresol by PC-07 was studied further by Bossert and Young (1986). They identified 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde as intermediates leading to 4-hydroxybenzoic acid. Further studies characterized the anaerobic oxidation of p-cresol to 4-hydroxybenzoic acid in cell extracts of the PC-07 isolate (Bossert et al. 1989). The PC-07 methylhydroxylase was partially purified by anion-exchange chromatography, and had a limited substrate range, requiring an alkyl-substituted phenolic ring with a hydroxyl group in the *para* position. The product, p-hydroxybenzaldehyde, was further oxidized to

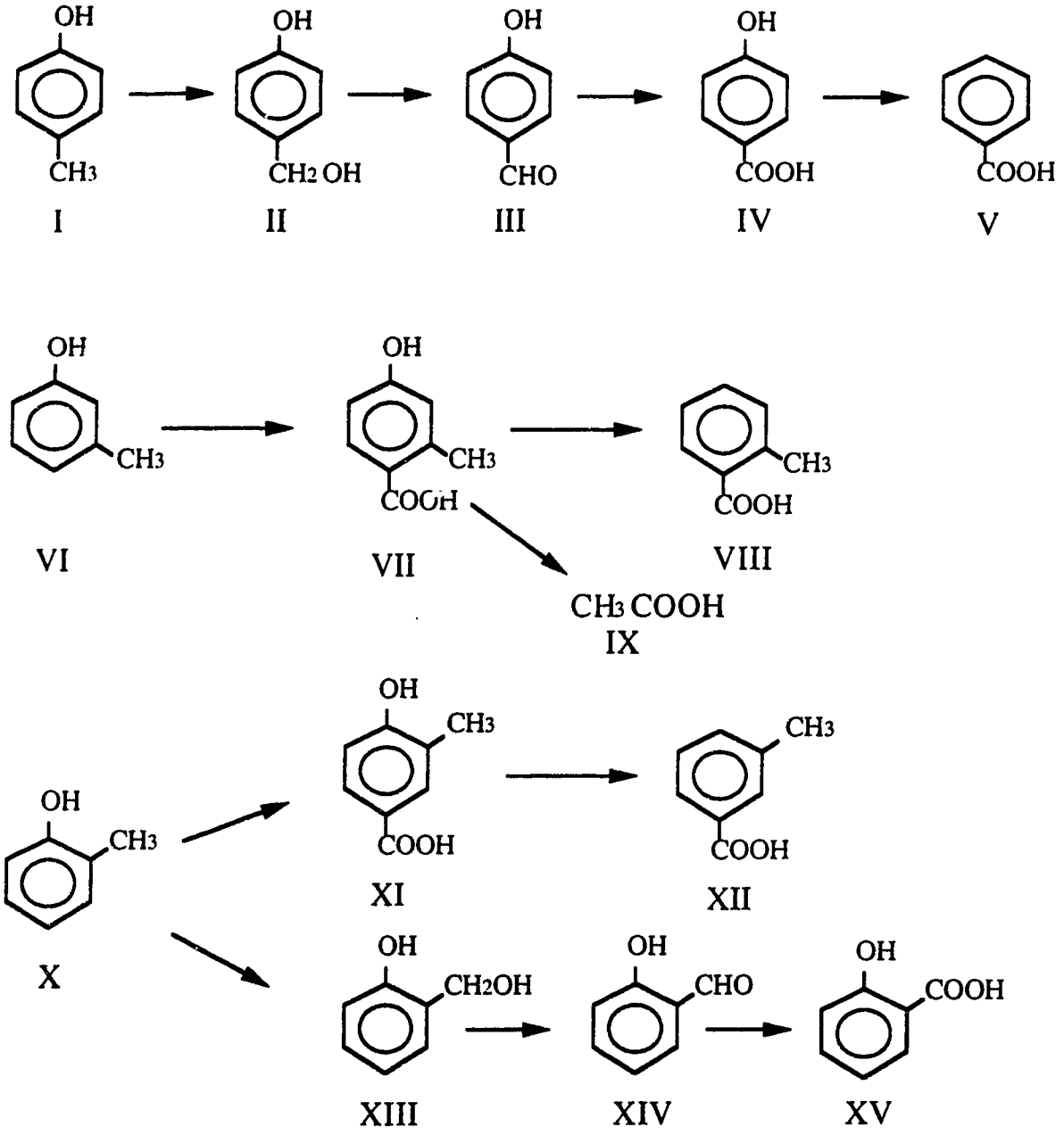


Figure 1-2: Simplified proposed pathways for the anaerobic degradation of p-cresol (I), m-cresol (VI), and o-cresol (X). The products of methyl group oxidation and *para*-carboxylation, leading to the formation of benzoic acids, are shown. 4-Hydroxybenzyl alcohol (II), 4-hydroxybenzaldehyde (III), 4-hydroxybenzoic acid (IV), benzoic acid (V), 4-hydroxy-2-methylbenzoic acid (VII), 2-methylbenzoic acid (VIII), acetate (IX), 4-hydroxy-3-methylbenzoic acid (XI), 3-methylbenzoic acid (XII), 2-hydroxybenzyl alcohol (XIII), 2-hydroxybenzaldehyde (XIV), and 2-hydroxybenzoic acid (XV).

4-hydroxybenzoic acid by an NAD^+-dependent dehydrogenase. The methylhydroxylase from strain PC-07, identified as an *Achromobacter* sp., was found to be a flavocytochrome that oxidized p-cresol to 4-hydroxybenzyl alcohol (Hopper et al. 1991). Rudolphi et al. (1991) isolated a nitrate-reducing *Paraccocus* sp. that completely oxidized p-cresol to CO_2. The oxidation of the methyl group was catalyzed by p-cresol methylhydroxylase, and after oxidation to 4-hydroxybenzoic acid, a 4-hydroxybenzoyl-coenzyme-A(CoA) synthetase formed 4-hydroxybenzoyl-CoA. This was then dehydroxylated to benzoyl-CoA by a reductase.

p-Cresol has also been shown to be biodegraded under sulfate-reducing conditions. Smolenski and Suflita (1987) enriched a consortium from a sand aquifer containing sulfate concentrations of 2 to 11 mM. When fed p-cresol, this mixed culture produced an intermediate that had the same retention time and the same ultraviolet (UV) spectrum as 4-hydroxybenzoic acid. Smolenski and Suflita (1987) postulated that the same intermediates of p-cresol degradation occurred under sulfate-reducing conditions as under nitrate-reducing conditions. To verify this hypothesis, 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, and 4-hydroxybenzoic acid were fed to the p-cresol-degrading, sulfate-reducing cultures in simultaneous adaptation experiments. The latter two compounds were metabolized without a lag period but the alcohol was not transformed during a 40-h incubation. When both p-cresol and 4-hydroxybenzoic acid were fed to a p-cresol-degrading culture, 4-hydroxybenzyl alcohol was detected. Later, Suflita et al. (1989) used gas chromatography-mass spectrometry (GC-MS) to identify the alcohol, aldehyde, and acid intermediates of the methyl group oxidation of p-cresol. In addition, they detected benzoic acid and phenol, which presumably originated from 4-hydroxybenzoic acid via dehydroxylation and decarboxylation reactions, respectively. Therefore, Suflita et al. (1989) postulated that the degradative pathway diverges after 4-hydroxybenzoic acid, and they provided several lines of evidence indicating that the benzoic acid pathway was likely the major route for further metabolism.

In their pure culture studies with strain GS-15 grown on p-cresol using Fe^{3+} as its terminal electron acceptor, Lovley and Lonergan (1990) detected 4-hydroxybenzoic acid as a transient intermediate. They also observed that GS-15 would grow on 4-hydroxybenzyl alcohol and 4-

hydroxybenzaldehyde, thus demonstrating the same series of oxidations of the methyl group as was observed by Bossert et al. (1986) and Suflita et al. (1989).

1.3.2.2 Anaerobic metabolism of m-cresol

Although the biodegradation of m-cresol has been demonstrated under nitrate-reducing (Bakker 1977; Tschech and Fuchs 1987), methanogenic (Boyd et al. 1983; Fedorak and Hrudey 1986a; Smolenski and Suflita 1987), and sulfate-reducing (Smolenski and Suflita 1987; Suflita et al. 1989) conditions, only recently have the initial steps in its metabolism been elucidated.

The study of the anaerobic degradation of m-cresol has been hampered by the relatively long acclimation time required to obtain active cultures (Boyd et al. 1983; Roberts et al. 1987; Smolenski and Suflita 1987), as well as the difficulty of maintaining m-cresol-degrading cultures because m-cresol degraders are inhibited by the 1 mM sulfide concentration typically used for methanogenic cultures (Roberts et al. 1988).

The metabolism of m-cresol differs from the methyl-group oxidation mechanism found for the anaerobic degradation of p-cresol. In metabolic studies, Roberts et al. (1987) used [methyl-^{14}C]m-cresol to determine the fate of the methyl carbon in methanogenic consortia. The majority of the label (85.9%) was converted to $^{14}CH_4$, whereas small percentages of the label were recovered as $^{14}CO_2$ (4.8%) and cell carbon (2.2%). This was in sharp contrast to their findings that 92% of the label from [methyl-^{14}C]p-cresol was recovered as carbon dioxide. Ramanand and Suflita (1991a) conducted simultaneous adaptation experiments with a consortium that degraded m-cresol under sulfate-reducing conditions. Based on the assumption that the methyl group of m-cresol was oxidized as was observed with p-cresol, the ability of the consortium to use the potential intermediates m-hydroxybenzyl alcohol or m-hydroxybenzoic acid was tested. However, neither was degraded in the absence or presence of equimolar concentrations of m-cresol. Furthermore, Rudolphi et al. (1991) found that their nitrate-reducing *Pseudomonas* strain S 100, did not exhibit any m-cresol-specific methyl group oxidation activity, although this organism was capable of oxidizing the methyl group of p-cresol. These

results suggest that m-cresol degradation does not arise from the oxidation of the methyl group.

Roberts et al. (1990) showed that 1 mol of bicarbonate was incorporated into m-cresol via a *para*-carboxylation to yield 4-hydroxy-2-methylbenzoic acid, that was usually metabolized to four units of acetate (Figure 1-2). ^{14}C-Labelled 4-hydroxy-2-methylbenzoic acid was recovered when cultures were incubated with either [methyl-^{14}C]m-cresol or [^{14}C]bicarbonate. Ramanand and Suflita (1991a) detected 4-hydroxy-2-methylbenzoic acid in their sulfate-reducing culture when bicarbonate was present in the medium. They also reported that their sulfate-reducing consortium could decarboxylate 4-hydroxybenzoic acid to near stoichiometric amounts of phenol in a non-sulfate-dependent reaction, and the presence of 4-hydroxybenzoic acid in the medium temporarily inhibited m-cresol metabolism such that the former compound was metabolized prior to the latter and phenol was degraded in a sequential manner. In contrast, the nitrate-reducing organism, *Pseudomonas* strain S 100, could not carboxylate m-cresol, nor could it degrade 4-hydroxy-2-methylbenzoic acid (Rudolphi et al. 1991).

Roberts et al. (1990) identified another aromatic metabolite as 2-methylbenzoic acid. This compound appeared to be a dead-end product which likely originated from the dehydroxylation of 4-hydroxy-2-methylbenzoic acid. Ramanand and Suflita (1991b) also detected small amounts of 2-methylbenzoic acid in their m-cresol-degrading, sulfate-reducing cultures. In contrast, Rudolphi et al. (1991) detected no intermediates in their m-cresol-degrading culture, although the involvement of, or degradation of, 2-methylbenzoic acid was not specifically addressed. There are no reports of the anaerobic biodegradation of 2-methylbenzoic acid.

1.3.2.3 Anaerobic metabolism of o-cresol

Of the three cresol isomers, o-cresol is the most resistant to anaerobic biodegradation. For example, Smolenski and Suflita (1987) incubated enrichment cultures under methanogenic and sulfate-reducing conditions for 90 and 100 days, respectively, with no evidence of degradation. Godsey and Goerlitz (1984) observed o-cresol degradation under methanogenic conditions after a lag time of 130 days. In contrast,

Kaminski et al. (1990) obtained an o-cresol-degrading methanogenic consortium within 40 days of inoculation with Saale River sediment.

Suflita et al. (1989) found two metabolites, o-hydroxybenzaldehyde and benzoic acid, in an o-cresol-degrading sulfate-reducing consortium. In subsequent simultaneous adaptation experiments, the consortium used the two metabolites as well as o-hydroxybenzyl alcohol and o-hydroxybenzoic acid, without a lag period. This study demonstrated the formation of a benzoic acid intermediate via oxidation of the methyl group of o-cresol under sulfate-reducing conditions (Figure 1-2).

A methanogenic culture that was known to carboxylate phenol to benzoic acid was fed o-cresol and incubated for 60 days (Bisaillon et al. 1991b). During that time, two metabolites formed. One was a transient intermediate and the other was not metabolized further. These were identified as 4-hydroxy-3-methylbenzoic acid and 3-methylbenzoic acid, respectively. The former compound would arise from a *para*-carboxylation of o-cresol and the latter would be the product of a subsequent dehydroxylation. These reactions are analogous to those observed with m-cresol under methanogenic conditions (Figure 1-2).

The first reported degradation of o-cresol under nitrate-reducing conditions was that of Bakker (1977), whose phenol-degrading mixed culture slowly metabolized this isomer at one-twelth the rate for phenol. However, no metabolites were reported. Rudolphi et al. (1991) reported that o-cresol was metabolized by a nitrate-reducing *Paracoccus* sp via 3-methylbenzoyl-CoA as the central intermediate. The o-cresol was first *para*-carboxylated to 4-hydroxy-3-methylbenzoic acid by a carboxylase, followed by thioester activation by a 4-hydroxy-3-methylbenzoyl-CoA synthetase, followed by a reductive dehydroxylation to 3-methylbenzoyl-CoA catalyzed by a dehydroxylase that used benzyl viologen as electron donor. This organism, which also possessed p-cresol methyl-oxidation capabilities, could also hydroxylate the *para*-methyl group only of 2,4-dimethylphenol and 3,4-dimethylphenol, although it could only metabolize the former to carbon dioxide. This organism could also use 3-methylbenzoic acid as a growth substrate, and had 3-methylbenzoyl-CoA synthetase activity.

1.3.3 Benzoic acids

Benzoic acids are metabolites in the degradation of many compounds, including complex natural polymers such as lignin. Lignin is a rich source of aromatic compounds that must be degraded as part of the carbon cycle. Alkaline hydrolysis leads to a variety of monocyclic and some bicyclic aromatic compounds (Hanselmann 1982). The products in the hydrolysate are quite susceptible to biodegradation and many of these have been considered as substrates for methanogenic consortia (Healy and Young 1979; Healy et al. 1980) and anaerobic photoheterotrophs (Harwood and Gibson 1988). Numerous substituted benzoic acids such as vanillic, protocatechuic, syringic, and 4-hydroxybenzoic acids are formed by this hydrolysis and these have been shown to be readily degraded by methanogenic consortia (Healy and Young 1979).

Several phenylacrylate derivatives such as ferulic, caffeic, and cinnamic acids are also released by hydrolysis, and Harwood and Gibson (1988) have studied the metabolism of ten of these compounds by *Rhodopseudomonas palustris* under anaerobic conditions and shown that the photometabolism of each compound yielded a benzoic acid. Similarly, the metabolism of ferulate (Healy et al. 1980; Grbić-Galić 1986) and phenylpropionate (Balba and Evans 1979) gave benzoic acid as an intermediate in methanogenic consortia.

The anaerobic degradation of benzoic acid and substituted benzoic acids are important, because these compounds often arise during the degradation of other compounds. For example, 4-hydroxybenzoic acid and benzoic acid are metabolites in phenol degradation under a variety of anaerobic conditions. As well, the formation of a benzoic acid often represents a detoxification of other compounds. Determining the fate of benzoic acids in anaerobic environments is a necessary step in accumulating the information necessary to understand the complete degradation of many aromatic compounds. More is known about the enzymology of degradation of benzoic acid than of other aromatic compounds in anaerobic environments. Furthermore, while many substituted benzoic acids are resistant to degradation, others are model compounds for probing the biochemical mechanisms of anaerobic organisms.

1.3.3.1 Anaerobic degradation of benzoic acid.

The anaerobic metabolism of benzoic acid has been studied more extensively than that of any other aromatic compound. Much of this work has been reviewed by Evans and Fuchs (1988). Figure 1-3 shows the intermediates detected and the proposed reductive pathways leading to ring cleavage of benzoic acid via photometabolism, nitrate-respiration, and under methanogenic conditions. The first two pathways were elucidated with pure cultures of *R. palustris* (Dutton and Evans 1969) and *Moraxella* sp. (Williams and Evans 1975), respectively, whereas the intermediates in the third pathway were found in a methanogenic consortium (Shlomi et al. 1978). Evans (1977) proposed the reduction of the carboxyl group in 2-oxocyclohexane under methanogenic conditions (Figure 1-3) to give the hypothetical intermediate 2-methylcyclohexanone (not shown) which would yield heptanoate upon ring cleavage. However, these transformations are not well documented.

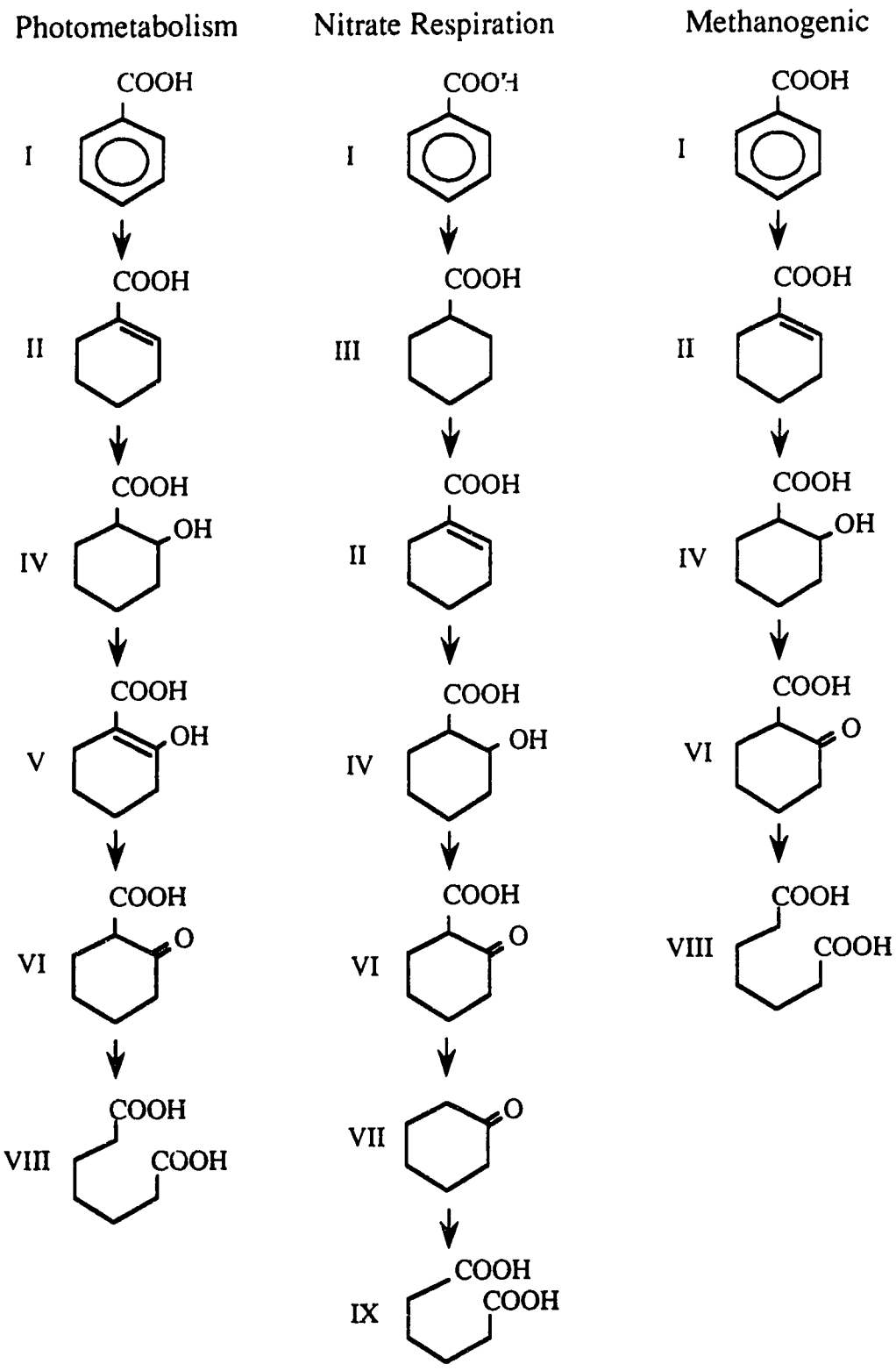
Although benzoic acid degradation under sulfate-reducing conditions has been observed (Balba and Evans 1980c; Mountfort and Bryant 1982; Cord-Ruwisch and Garcia 1985; Bak and Widdel 1986), no pathways are known to have been published. However, Balba and Evans (1980c) detected cyclohexanol and cyclohexanecarboxylate in a benzoic acid-degrading co-culture of *Pseudomonas aeruginosa* and *Desulfovibrio vulgaris* with sulfate serving as the terminal electron acceptor.

Clearly, the pathways observed under the three culture conditions shown in Figure 1-3 are very similar. They also have features that are analogous to the β-oxidation of fatty acids, including: the occurrence of an intermediate which has a double bond between the carbon atoms that are α and β to the carboxyl group (ie. cyclohex-1-enecarboxylate), the hydration of the double bond yielding a hydroxyl group on the β carbon, and the oxidation of this group to a keto group.

1.3.3.2 Anaerobic degradation of hydroxybenzoic acids

The anaerobic degradation of 4-hydroxybenzoic acid, either as an exogenous source or as a metabolite, has been reported under methanogenic (Healy and Young 1979; Tschech and Schink 1986; Kuhn et al. 1989; Knoll and Winter 1989; Kaminski et al. 1990), nitrate-reducing (Bakker 1977; Taylor 1983; Bossert et al. 1986; Tschech and Fuchs 1987;

Figure 1-3: Proposed pathways for the anaerobic degradation of benzoic acid (I) leading to ring cleavage by photometabolism (Dutton and Evans 1969), nitrate respiration (Williams and Evans 1975), and in a methanogenic consortium (Schlomi et al. 1979). Cyclohex-1-enecarboxylic acid (II), cyclohexanecarboxylic acid (III), 2-hydroxycyclohexanecarboxylic acid (IV), 2-hydroxycyclohex-1-enecarboxylic acid (V), 2-oxocyclohexanecarboxylic acid (VI), cyclohexanone (VII), pimelic acid (VIII), and adipic acid (IX).


Photometabolism
Nitrate Respiration
Methanogenic
COOH
I
II
III
IV
V
VI
VII
VIII
IX
OH
O

Kuhn et al. 1988; Merkel et al. 1989; Glockler et al. 1989; Seyfried et al. 1991; Rudolphi et al. 1991; Dangel et al. 1991), photoheterotrophic (Dutton and Evans 1969; Harwood and Gibson 1986, 1988; Geissler et al. 1988; Merkel et al. 1989), and sulfate-reducing conditions (Cord-Ruwisch and Garcia 1985; Bak and Widdel 1986; Smolenski and Suflita 1987, Suflita et al. 1989; Kuhn et al. 1989). However, the mechanism of degradation seems dependent on many factors that have not yet been elucidated. Reports indicate that a variety of transformations interrelate 4-hydroxybenzoic acid, benzoic acid, and phenol under different anaerobic conditions (Figure 1-4).

One such transformation is the dehydroxylation of 4-hydroxybenzoic acid to benzoic acid. Benzoic acid was detected as a subsequent metabolite after 4-hydroxybenzoic acid was formed from the oxidation of p-cresol under sulfate-reducing conditions (Suflita et al. 1989) or nitrate-reducing conditions (Rudolphi et al. 1991; Dangel et al. 1991), and from phenol under methanogenic (Haggblom et al. 1990; Sharak Genthner et al. 1991) or nitrate-reducing (Dangel et al. 1991) conditions. Under nitrate-reducing conditions, 4-hydroxybenzoic acid was activated to 4-hydroxybenzoyl-CoA which was then reductively dehydroxylated to benzoyl-CoA (Rudolphi et al. 1991; Dangel et al. 1991; Glockler et al. 1989).

Alternatively, 4-hydroxybenzoic acid can be decarboxylated to phenol. Phenol has been formed from 4-hydroxybenzoic acid under nitrate-reducing, sulfate-reducing, and methanogenic conditions, denoting that decarboxylation can occur just prior to ring fission (Krumholz et al. 1987; Tschech and Schink 1986; Kuhn et al. 1989; Zhang et al. 1990; Gallert et al. 1991). This is in spite of the fact that 4-hydroxybenzoic acid is formed from carboxylation of phenol or methyl-group oxidation of p-cresol under anaerobic conditions (see section 1.3.2.1). Knoll and Winter (1989) also observed the decarboxylation of 4-hydroxybenzoic acid, yielding phenol, and then its subsequent carboxylation, yielding 4-hydroxybenzoic acid, in their syntrophic mixed culture growing under methanogenic conditions. Tschech and Fuchs (1987) also reported the exchange of the carboxyl group of 4-hydroxybenzoic acid with $^{14}CO_2$, ascribed to the phenol carboxylase. Lack and Fuchs (1992), studying the carboxylation of phenol under nitrate-reducing conditions, elaborated on

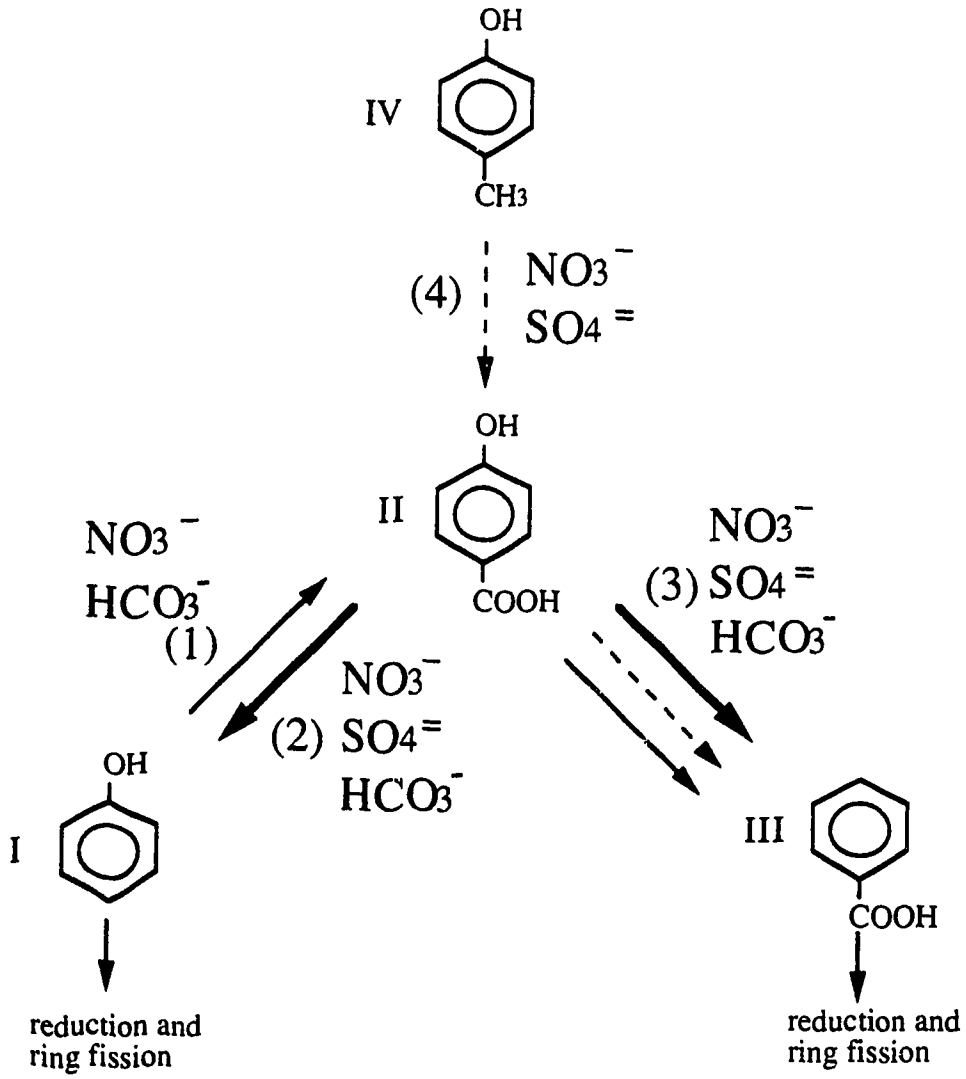


Figure 1-4: The interrelationships among phenol (1), 4-hydroxybenzoic acid (II), and benzoic acid (III) under different anaerobic conditions. The formation of 4-hydroxybenzoic acid from p-cresol (IV) is also shown, and reactions observed with p-cresol as substrate are indicated with the dashed arrows. The conditions under which *para*-carboxylation (1), decarboxylation (2), dehydroxylation (3), and methyl group oxidation (4) occur are indicated by the corresponding terminal electron acceptor. The thin arrows refer to the detection of both metabolites from phenol under the two conditions. See text for details.

the possible mechanism of the carboxylation and decarboxylation and factors that could determine the balance between the two reactions in their culture.

Thus, two alternate pathways have been proposed for 4-hydroxybenzoic acid degradation. One involves a dehydroxylation to benzoic acid, which is subsequently degraded, whereas the other involves a decarboxylation to phenol, which is sometimes converted to benzoic acid, with or without a 4-hydroxybenzoic acid intermediate, and eventually degraded. The involvement of CoA thioesters at both the 4-hydroxybenzoic and benzoic acid stages have been observed for both mechanisms. Neither mechanism is specific to a particular anaerobic environment, nor to the origin of the 4-hydroxybenzoic acid, whether added exogenously or when formed as a metabolite from p-cresol or phenol. Therefore, no absolute mechanism is indicated for 4-hydroxybenzoic acid degradation at this time.

There are several reports of 3-hydroxybenzoic acid degradation under methanogenic (Tschech and Schink 1986), nitrate-reducing (Brawn and Gibson 1984; Heising et al. 1991; Rudolphi et al. 1991), and sulfate-reducing (Schnell et al. 1989; Sembiring and Winter 1990) conditions. Heising et al. (1991) reported that the initial step in the degradation of 3-hydroxybenzoic acid was activation to 3-hydroxybenzoyl-CoA in an ATP-consuming reaction. 3-Hydroxybenzoyl-CoA synthetase activity was measured, but a reductive dehydroxylation of 3-hydroxybenzoyl-CoA could not be demonstrated due to rapid hydrolysis of chemically synthesized 3-hydroxybenzoyl-CoA by cell extracts. Kuhn et al. (1989) reported that benzoic acid was an intermediate of both 3- and 2-hydroxybenzoic acid degradation in methanogenic and sulfate-reducing incubations.

1.3.3.3 Anaerobic degradation of methyl-substituted benzoic acids

2-Methylbenzoic acid was identified in methanogenic m-cresol-degrading cultures (Roberts et al. 1990). This compound was a transformation product of m-cresol via a *para*-carboxylation to 4-hydroxy-2-methylbenzoic acid followed by a dehydroxylation to yield 2-methylbenzoic acid. While it was abundant in culture supernatants, 2-methylbenzoic acid did not appear to be readily metabolized by the m-

cresol-degrading culture. It was considered to be a dead-end product based on the observation that m-cresol-degrading cultures would not metabolize authentic 2-methylbenzoic acid, and 2-methylbenzoic acid accumulated in 2-L enrichment cultures.

3-Methylbenzoic acid was degraded by a nitrate-reducing *Paracoccus* sp. (Rudolphi et al. 1991). In addition to being used as a growth substrate, extracts grown on 3-methylbenzoic acid contained a 3-methylbenzoyl-CoA synthetase activity. These results were consistent with the finding that 3-methylbenzoyl-CoA was an intermediate in o-cresol degradation by this bacterium. These same extracts could degrade 2,4-dimethylphenol to CO_2 via an initial *para*-methyl group oxidation to 4-hydroxy-3-methylbenzoic acid, followed presumably by a dehydroxylation to 3-methylbenzoic acid. The mechanism of the degradation of 3-methylbenzoyl-CoA was not determined.

1.3.4 Halogenated phenols and benzoic acids

The degradation of halogenated aromatic compounds has become of increasing concern as the prevalence of chlorinated aromatic compounds in the environment, from sources ranging from pulp and paper mill effluents and chlorine bleaching to herbicide usage, has become more widely publicized.

1.3.4.1 Anaerobic degradation of chlorinated aromatic compounds

The degradation and reductive dehalogenation of monochlorobenzoic acids have been studied under most anaerobic conditions. Suflita et al. (1982) found direct evidence for the reductive dechlorination of chlorobenzoic acids in stable enrichments derived from sludge by following the fate of the parent substrates, the appearance of intermediates and products, and carbon mass balances. Horowitz et al. (1983) reported that the decomposition of both 3-chlorobenzoic acid and 3,5-dichlorobenzoic acid were linked with methane production. Sharak Genthner et al. (1989a, b) compared the anaerobic degradation of monochlorobenzoic acids under methanogenic, nitrate-reducing, and sulfate-reducing conditions. Degradation of chloroaromatic compounds was observed most often in methanogenic enrichments. The relative order

of biodegradability of the chlorobenzoic acids was *meta* > *ortho* > *para* substituted.

The most well-studied dechlorination of an aromatic compound under anaerobic conditions involves strain DCB-1, renamed *Desulfomonile tiedjei*, a strict anaerobe isolated in pure culture that reductively dechlorinates 3-chlorobenzoic acid. This organism was isolated from a methanogenic consortium by Shelton and Tiedje (1984). It has since been used to show: (1) that the dechlorination was unrelated to other aryl substituent removal reactions (DeWeerd et al. 1986); (2) that dehalogenation by DCB-1 was restricted to the *meta* substituted benzoic acids (Linkfield and Tiedje 1990); (3) that DCB-1 conserves energy for growth by coupling formate, and probably H_2 oxidation, to reductive dechlorination (Mohn and Tiedje 1990); (4) that the thermodynamically exergonic reductive dechlorination reaction yields biologically useful energy (Dolfing and Tiedje 1987; Dolfing 1990); and (5) that the presence of a *para*-amino or *para*-hydroxy group inhibited the rate of dechlorination, suggesting that the rate-limiting step is a nucleophilic attack on the negatively charged π electron cloud around the benzene nucleus (Dolfing and Tiedje 1991).

In studies with anaerobic sludge (Boyd et al. 1983; Boyd and Shelton 1984; Mikesell and Boyd 1985; Gibson and Suflita 1986; Hrudey et al. 1987b; Dietrich and Winter 1990; Haggblom and Young 1990; Madsen and Aamand 1992), and anaerobic freshwater sediments (Gibson and Suflita 1986; Kohring et al. 1989; Zhang and Wiegel 1990), preferential o-dechlorination of di- tri- and penta-chlorophenols was generally observed although m- and then p-chlorines were also eventually eliminated. With monochlorophenols, a similar relative order of degradability (o- > m- > p-chlorophenol) was observed (Boyd and Shelton 1984; Hrudey et al. 1987a; Sharak Genthner et al. 1989a; Dietrich and Winter 1990).

1.3.4.2 Anaerobic degradation of brominated and iodinated aromatic compounds

The fates of bromo- and iodo-substituted aromatic compounds in anaerobic environments have also been studied to a limited extent. Suflita et al. (1982) reported that all three bromobenzoic acid isomers were debrominated to benzoic acid, which was subsequently degraded by

methanogenic consortia to methane and carbon dioxide. Horowitz et al. (1983) reported the accumulation of benzoic acid from the metabolism of 2-bromobenzoic acid, which was subsequently mineralized to carbon dioxide and methane. Linkfield et al. (1989) demonstrated that the ring position affected acclimation periods, such that the *ortho* and *meta* forms always exhibited lag periods shorter than that of the *para* form. 2-Bromophenol was debrominated to stoichiometric amounts of phenol and HBr by a sewage sludge enrichment culture (Dietrich and Winter 1990). 3-Iodobenzoic acid (Horowitz et al. 1983; Linkfield et al. 1989) and 2- or 4-iodobenzoic acid (Suflita et al. 1982) have been shown to be degraded anaerobically. Benzoic acid was observed as a transient product of 3-iodobenzoic acid degradation (Suflita et al. 1982; Horowitz et al. 1983). Apart from these preliminary studies, little has been published about aromatic compounds substituted with these halogens under anaerobic conditions, as they have been overshadowed by the more environmentally predominant chlorinated aromatic compounds.

1.3.4.3 Fluorinated aromatic compounds in anaerobic cultures

Of the halogenated aromatic compounds studied to date, the fluorine-containing aromatic compounds have been perhaps the most interesting because they have the unusual property of being structurally similar to their unhalogenated analogues. Fluorinated analogues of aromatic compounds have been used frequently to study metabolism under aerobic conditions (Engesser et al. 1990; Gallert et al. 1991). Indeed, the use of fluorinated compounds has been a common technique for metabolic studies because the fluorine atom does not dramatically affect the physical shape and size of the compound. In some cases the substitution does not interfere with association of fluorinated analogues with enzymes that transform the natural substrate. Nevertheless, a replacement of a hydrogen with a fluorine does significantly affect the chemical characteristics of substrates and metabolites, allowing them to be differentiated analytically. In addition, because of the high carbon-fluorine bond energy, the fluorine atom is less likely to be removed than other halogens, and therefore metabolites would be fluorinated.

Although fluorobenzoic acids (DeWeerd et al. 1986; Linkfield and Tiedje 1990; Suflita et al. 1982) and fluorophenols (Dietrich and Winter

1990) are generally not degraded under anaerobic conditions, Schennen et al. (1985) reported the anaerobic degradation of 2-fluorobenzoic acid involving a stoichiometric release of fluoride, by a denitrifying *Pseudomonas*. sp. Taylor et al. (1979) reported that cell yields of *Pseudomonas* strain PN-1 increased during anaerobic growth on succinate or 4-hydroxybenzoic acid when 2- or 4-fluorobenzoic acid was present in the medium. Fluoride ions were released stoichiometrically by this nitrate-reducing culture. Linkfield et al. (1989) reported the degradation of 3-fluorobenzoic acid in less than 40 days at concentrations of 20 or 40 mM, but not within a year at 40 or 800 mM. One would expect the reductive dehalogenation of fluorinated aromatic compounds to be more difficult than the other halogens within a particular redox environment, based on the bond dissociation energies for aromatic carbon-halogen bonds.

Sharak Genthner et al. (1989c, 1990) used monofluorophenols to study the degradation of phenol under methanogenic conditions. They found a conversion of 5 or 25 mg/L 2-, and 3-fluorophenol to 3-, and 2-fluorobenzoic acids respectively, but no transformation of 5 or 50 mg/L 4-fluorophenol. The proposed 3-fluoro-4-hydroxybenzoic acid and 2-fluoro-4-hydroxybenzoic acid intermediates were not detected, but authentic 3-fluoro-4-hydroxybenzoic acid was transformed to 3-fluorobenzoic acid and 2-fluorophenol. None of the fluorinated analogues tested were defluorinated or mineralized to methane and carbon dioxide, but the detection of fluorine-labelled intermediates allowed them to elucidate the pathway for phenol degradation by their methanogenic consortium.

As a general rule, microbiologically-catalyzed reductive elimination of the aryl halogens occurs prior to the reduction and cleavage of the aromatic ring. The processes of reductive dechlorination and ring reduction and cleavage are likely to be independent, because they are catalyzed by different members of a methanogenic consortium (Shelton and Teije 1984; Dolfing and Tiedje 1987; DeWeerd et al. 1990). The environmental significance of reductive dehalogenation is the detoxification of chlorinated aromatic compounds; the products are generally less hazardous than the parent compounds (Grbic´-Galic´ 1990).

1.4 CoA Intermediates

CoA has recently been implicated in the anaerobic degradation of aromatic compounds. It is a relatively large molecule compared to benzoic acid, consisting of three moieties: pantetheine, adenosine, and phosphoric acid. Purified, it is a white powder with a characteristic thiol odor, soluble in water but practically insoluble in ethanol, ether, or acetone, with an absorption maximum of 260 nm (Mieyal et al. 1974). CoA plays a key role in the β-oxidation of fatty acids and has been shown to participate in the metabolism of benzoic acid and some substituted benzoic acids under anaerobic culture conditions. Approximately 65% of the molecule of benzoyl-CoA exists in a folded conformation near physiological temperature. Physical data have suggested a specific intramolecular complex, involving a ring stacking interaction between the adenosyl and benzoyl moieties with the charged and polar groups of the phosphopantetheine moiety facing the exterior, and the methyl and methylene groups forming a hydrophobic interior of the folded molecule (Mieyal et al. 1974). Activation to the CoA thioester facilitates the reduction of the aromatic ring, and the subsequent series of β-oxidation-like reactions that ultimately result in ring fission (Merkel et al. 1989).

1.4.1 CoA and photometabolism of benzoic acids

The most thoroughly studied process of the photometabolism of an aromatic compound is that of benzoic acid by *R. palustris*. This organism does not actively transport benzoic acid (Harwood and Gibson 1986); instead, it is rapidly converted to benzoyl-CoA inside the cells by a benzoyl-CoA ligase. This leaves little free benzoic acid inside the cells, generating a concentration gradient across the cell membrane allowing the benzoic acid to diffuse into the cell. This ligase, from cells grown on benzoic acid, forms benzoyl-CoA from benzoic acid in a buffer supplemented with CoA, ATP, and Mg^{2+} (Whittle et al. 1976). Geissler et al. (1988) have purified and characterized the benzoate-CoA ligase.

Factors affecting the expression of these enzymes have been characterized by analyzing the effects of different substrates, including the proposed intermediates, on expression. Kim and Harwood (1991) reported that the benzoate-CoA ligase was induced by cyclohex-1-enecarboxylic acid and cyclohex-3-enecarboxylic acid, but not by cyclohexane-carboxylic acid or pimelate. The cell-free extract of *R. palustris* prepared by Whittle et

al. (1976) converted cyclohex-1-enecarboxylate to pimelate in a buffer containing CoA, ATP, Mg^{2+} and NAD^{+}. The enzymes needed for the β-oxidation of cyclohexanecarboxylate appeared to be constitutive in *R. palustris* (Hutber and Ribbons 1983). However, the acyl-CoA synthetase which used benzoic acid as its substrate was induced by anaerobic growth on benzoic acid. Gibson and Gibson (1992) showed that cyclohexa-2,5-diene-1-carboxylate and cyclohexa-1,4-diene-1-carboxylate, presumably as their CoA thioesters, are the first stable products in the reductive pathway of *R. palustris*. These intermediates correspond to the addition of two hydrogen atoms to the benzoyl group, which loses the equivalent of one double bond in the process. Dutton and Evans (1969) recovered radioactive cyclohex-1-ene-1-carboxylate, cyclohexanecarboxylate, and pimelate in anaerobic suspensions of *R. palustris* incubated with radioactive benzoic acid in the presence of potential metabolites.

In fact, *R. palustris* forms at least two CoA-ligases; one enzyme uses benzoic acid, and the other is equally active with benzoic acid and 4-hydroxybenzoic acid (Kim and Harwood 1991). Merkel et al. (1989) also reported the involvement of CoA thioesters in 4-hydroxybenzoic acid metabolism by *R. palustris*. Anaerobically-grown cells converted 4-hydroxybenzoic acid to its CoA-thioester by using a 4-hydroxybenzoate-CoA-ligase. This conversion appears to drive a high-affinity uptake of 4-hydroxybenzoic acid, and it also appears to initiate the anaerobic metabolism of this compound. The CoA-ligase that catalyzed this reaction was distinct from the benzoate-CoA ligase purified and characterized previously (Geissler et al. 1988). Merkel et al. (1989) found no evidence of benzoic acid or benzoyl-CoA formation from 4-hydroxybenzoic acid.

1.4.2 CoA under nitrate-reducing conditions

The degradation of benzoic acid under nitrate-reducing conditions also begins with activation to the CoA thioester. Schennen et al. (1985) reported the catabolism of both benzoic acid and 2-fluorobenzoic acid by a benzoic acid-degrading *Pseudomonas* sp. Both compounds were substrates for an inducible benzoate-CoA synthetase (in the presence of CoA, ATP, M^{2+}, and crude extracts of induced cells). Zeigler et al. (1987) also detected an aryl-CoA synthetase activity in cell-free extract of a benzoic acid-degrading denitrifying *Pseudomonas* strain. It had a broad substrate

specificity, producing CoA thioesters from benzoic acid, 2-aminobenzoic acid, and ten other monosubstituted benzoic acids. Lochmeyer et al. (1992) found that benzoyl-CoA was further converted to six products. The only two products identified were cyclohex-1-enecarboxyl-CoA and *trans*-2-hydroxycyclohexane-carboxyl-CoA.

The pathway for 2-aminobenzoic acid (anthranilic acid) degradation was further studied by Lochmeyer et al. (1992). As Zeigler et al. (1987) reported, the anaerobic degradation of 2-aminobenzoic acid begins with conversion to the CoA thioester. Lochmeyer et al. (1992) also reported that the organism contained 2-aminobenzoate-CoA ligase. 2-Aminobenzoyl-CoA is then reductively deaminated to benzoyl-CoA by an oxygen-sensitive enzyme, 2-aminobenzoyl-CoA reductase. The two enzymes were induced by the substrate. Incidentally, aryl-CoA synthetase activity was also observed in cell-free extracts of the sulfate-reducing bacterium *Desulfobacterium anilini* (Schnell and Schink 1991). This bacterium carboxylated aniline to 4-aminobenzoic acid. Extracts of aniline- or 4-aminobenzoic acid-grown cells activated 4-aminobenzoic acid to 4-aminobenzoyl-CoA, which was further metabolized via reductive deamination to benzoyl-CoA.

Other compounds degraded under nitrate-reducing conditions via CoA thioesters include 3-hydroxybenzoic acid and 4-hydroxybenzoic acid. The initial step in 3-hydroxybenzoic acid degradation by extracts of a nitrate-reducing *Pseudomonas*, Asl-3, was activation to 3-hydroxybenzoyl-CoA in an ATP-consuming reaction (Heising et al. 1991). Similarly, 4-hydroxybenzoic acid was activated to 4-hydroxybenzoyl-CoA by a CoA ligase from a phenol-degrading *Pseudomonas* species (Glockler et al. 1989). Cell extracts also catalyzed the reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA with reduced benzyl viologen as the electron donor. 4-Hydroxybenzoic acid derived from oxidation of phenol or p-cresol could undergo similar activation and dehydroxylation. Identical results were obtained by Dangel et al. (1991) with *Pseudomonas* strain K 172, and the 4-hydroxybenzoyl-CoA synthetase and 4-hydroxybenzoyl-CoA reductase appeared to be induced sequentially, probably by the 4-hydroxybenzoic acid and by its CoA thioester, respectively.

The detection of benzoyl-CoA and substituted benzoyl-CoA thioesters in anaerobic cultures has become increasingly common. Thus, the formation of these activated compounds likely plays a major role in the subsequent reduction of the aromatic ring. Evans and Fuchs (1988) discuss the thermodynamic problems associated with the reduction of the resonance-stabilized benzene ring. In essence, the system of bonding characterized by very extensive delocalization of the π-electrons must be converted to one in which little delocalization energy remains. The first step in the biochemical reduction of benzoic acid is its activation to benzoyl-CoA. The thioesterification of α-β unsaturated acids increases their reactivity towards reducing agents owing to the resonance effect between the double bond and the S-CO group (Lynen 1953). Evans and Fuch (1988) suggested that this phenomenon may also apply to the aromatic nucleus.

1.5 Trends in Anaerobic Degradative Pathways of Phenols

A number of trends have been identified regarding the metabolism of phenols in anaerobic environments. Most notable are *para*-carboxylation, methyl group oxidation, benzoic acid formation, and CoA activation. Considering the abundance of recent publications describing benzoic acid as an intermediate of phenol degradation and the scarcity of reports detecting metabolites of the reductive pathway (Figure 1-1), it appears that the benzoic acid pathway may be more common. The environmental or culture conditions that direct the anaerobic metabolism of phenol through the reductive pathway have not been elucidated.

The addition of a carboxyl group, derived from bicarbonate in the medium, *para* to the ring hydroxy group has been found to be the initial step in the biodegradation of phenol under most anaerobic conditions, and for o-cresol and m-cresol under methanogenic conditions (Figure 1-2). Carboxylation *para* to the hydroxy group is followed by a dehydroxylation in the case of phenol, although decarboxylation is also observed.

Oxidation of methyl substituents on aromatic rings has been observed under a variety of anaerobic conditions for p-cresol, and under sulfate-reducing conditions for o-cresol (Figure 1-2). The sequential oxidation of the methyl group through the alcohol and aldehyde to the carboxylic acid

results in the formation of a hydroxybenzoic acid. Thus both *para*-carboxylation and methyl-group oxidation lead to carboxylated metabolites.

Benzoic acid has recently been detected as an intermediate in the degradation of many aromatic compounds, most notably phenol and substituted phenols, including many chlorophenols. Benzoic acid has been formed as the result of the removal of other ring substituents such as the reductive dehalogenation of halobenzoic acids or dehydroxylation of hydroxybenzoic acids, or by oxidation of ring substituents. The prevalence of benzoic acid intermediates in the anaerobic degradation of many phenols, including phenol and cresol isomers, is clearly evident from the information summarized here. In the future, more detailed searches for intermediates formed during the anaerobic degradation of other phenols will likely find benzoic acids. These acids would facilitate activation by CoA thioesterification and subsequent ring reduction and cleavage. However, to date, the involvement of CoA has not been demonstrated under methanogenic conditions.

1.6 Objectives

This project focussed on the methanogenic degradation of m-cresol, and was an extension of the work done by Roberts (1990). The primary objectives were: (1) to investigate the pathway of m-cresol degradation, particularly to detect intermediates subsequent to 4-hydroxy-2-methylbenzoic acid; and (2) to determine whether 2-methylbenzoic acid could be degraded in a methanogenic consortium. A third area of investigation added later, involved the use of fluorinated analogues to study m-cresol degradation; the methods were first verified using fluorophenols and phenol. Secondary objectives included: (1) maintaining stable m-cresol-degrading methanogenic cultures for long periods using reduced sulfide concentrations; (2) determining whether intermediates of m-cresol metabolism were bound to CoA; and (3) investigating the metabolism of 2-methylbenzoic acid.

2 MATERIALS AND METHODS

2.1 General Culture Techniques

Domestic anaerobic sewage sludge from the Edmonton Gold Bar Wastewater Treatment Plant, Edmonton, Alberta, was used as the inoculum for all enrichment cultures unless otherwise stated. The sludge was collected from a 26-ft depth from Digestor 2.

All cultures were incubated in the dark without shaking at 37°C. Cultures were removed from the incubator for analyses, but were returned to the incubator within at least 4 h, usually much less. Feed solutions and substrates were stored at 4°C until needed.

2.1.1 Media preparation

The mineral feed solution contained 1 mL vitamin B solution, 1 mL mineral solution I, 10 mL of mineral solution II, 10 mL resazurin solution, and 5.7 g of $NaHCO_3$ per litre. The compositions of these solutions have been reported elsewhere (Fedorak and Hrudey 1984).

Strict anaerobic technique was used for all preparations. Oxygen-free gas, subsequently referred to as anaerobic gas, was scrubbed free of O_2 by passing a flow of 30% CO_2/70% N_2 through a heated copper column, that had first been purged with a flow of 5% H_2/95% N_2. "Deoxygenated" water was prepared by boiling double-distilled water for 5 min, then cooling with constant flushing with anaerobic gas.

To transfer solutions, serum bottles were first purged for 2 min with a steady flow of anaerobic gas. The pipette used to transfer medium was flushed with anaerobic gas in the headspace of the medium flask several times prior to use. The medium was added while purging the containers and the medium with anaerobic gas. Once the medium was transferred, a butyl rubber stopper was readied, and after an additional 30-60 sec flushing, the canula delivering the gas was removed and the stopper quickly sealed. All stoppers were secured by crimping with aluminum seals, and the containers were sterilized by autoclaving.

The feed solution was reduced to a final Na_2S concentration of 1 mM or 0.5 mM unless otherwise noted. Stock solutions of 2.5% Na_2S

were prepared by purging a Hungate tube containing 0.25 g $Na_2S \cdot 9H_2O$ with anaerobic gas for 2 min, adding 10 mL boiled water, flushing for an additional 30 sec, then sealing the tube.

To reduce the medium with iron nails, 1 in finishing nails were added at the time the medium was transferred to the serum bottle, and the sterilized medium was left for at least a week prior to use.

Titanium citrate reducing solution was prepared by adding 6.0 mL $TiCl_3$ in H_2O into 2.0 mL of 3 M HCl. This was added to 80 mL of 0.2 M sodium citrate, and then 103 mL saturated $NaHCO_3$ was added to give a neutral pH. The solution was extensively flushed with anaerobic gas but not sterilized. To reduce the medium, 3 mL titanium citrate solution was added per 100 mL to give a 1.5 mM final concentration.

2.1.2 Substrates and inocula

Substrates were prepared separately in 58-mL serum bottles using anaerobic technique. The appropriate organic compounds were added first, followed by water or feed solution, then the bottles were sealed and autoclaved. For the benzoic acid and substituted benzoic acids, 0.1 mL of 5 N NaOH was added prior to the medium to facilitate complete dissolution. Unless otherwise stated, the substrates were reduced with 1 mM or 0.5 mM Na_2S just prior to use. Substrates (and reducing agents) were added to cultures using sterile syringes.

Sewage-sludge based inocula were added to serum bottles using syringes attached with wide bore (18G x 1.5 in) needles. These syringes were first flushed with anaerobic gas, then an appropriate volume of inoculum was drawn up and expelled into the sterile, pre-reduced medium in a serum bottle. For the enrichment cultures, the sewage sludge in the collection container was stirred and anaerobic gas was bubbled through it during the inoculation procedure. For the subcultures, care was taken not to create a negative pressure within the enrichment culture from which the inoculum was taken. To ensure a positive pressure, an appropriate volume of anaerobic gas was added to the serum bottle to compensate for the withdrawn volume.

2.2 Enrichment Cultures

2.2.1 Enrichment cultures containing m-cresol

The enrichment cultures were 80-mL cultures prepared by adding 40 mL anaerobic sewage sludge to 39 mL boiled water and 1 mL resazurin redox indicator in sealed 158-mL capacity serum bottles. These were initially challenged with 250 mg/L m-cresol. When substrate analysis revealed that m-cresol-degrading enrichment cultures had depleted their substrate, a variation of the draw-and-feed procedure of Fedorak and Hrudey (1986a) was used to replenish the substrate. For this variation, the cultures were not inverted and allowed to settle, the serum bottles were instead carefully tipped so as not to disturb the sediment that settled during incubation. Ten percent, or 8-mL of the supernatant, was removed and replaced with an equal volume of reduced feed solution containing 2.5 mg/mL m-cresol. After feedings, the cultures were thoroughly mixed by shaking. The withdrawn supernatant was pooled, acidified with H_2SO_4, and stored for future use.

The first set of enrichment cultures with m-cresol were established July 27, 1990. Two different anaerobic sewage sludge sources were used; three replicates plus a control with Gold Bar anaerobic sludge, and three replicates plus a control with Red Deer anaerobic sludge. The former was collected July 10, 1990 from the Gold Bar Wastewater Treatment Plant in Edmonton; the latter was anaerobic sludge from the wastewater treatment plant in Red Deer, Alberta, collected July 18, 1990.

A second set of m-cresol-degrading cultures was set up March 10, 1991 and maintained for over 20 months. A total of 25 cultures were set up: two controls that received no substrate, and 23 cultures that received 250 mg/L m-cresol. The sludge for the inoculum was collected March 9, 1991.

2.2.2 Enrichment cultures containing 2-methylbenzoic acid

Six replicate cultures, plus a control that received no substrate, were established at each of three different inocula levels: 25% by volume, 50% by volume, or 94% by volume. These cultures were prepared by adding 20, 40, or 75 mL sludge respectively to resazurin-containing deoxygenated water to a total volume of 80 mL. Unless otherwise stated, the initial 2-methylbenzoic acid concentration added was 200 mg/L. The depletion of 2-methylbenzoic acid was monitored by HPLC analysis of weekly 0.5-mL

samples of the supernatant of cultures. These enrichment cultures were also maintained by the draw-and-feed procedure with feed solutions containing 1 mg/mL 2-methylbenzoic acid unless otherwise stated, and the individual withdrawn portions were stored in vials until analyzed.

Three sets of enrichment cultures were established over the course of the study. The first set was established July 11, 1990, using sludge collected July 10. This set of cultures was monitored regularly for 40 weeks. The second set was inoculated April 15, 1991, using sludge collected April 14. This set of cultures, identical to the first, was monitored for 64 weeks. The third batch of cultures was inoculated December 10, 1991, but with only 100 mg/L rather than 200 mg/L 2-methylbenzoic acid, and were monitored for 47 weeks.

The headspaces of some cultures were flushed to reduce the high concentrations of methane prior to the onset of 2-methylbenzoic acid degradation. To do this, a vacuum was applied for 20 sec and then the gas was replaced with 50 mL anaerobic gas, and the cultures were shaken. This procedure was repeated a total of six times for each culture. Methane analysis revealed that this reduced the methane concentration in the headspace to below 1%, yet the resazurin indicator confirmed that the anaerobic environment was not compromised.

2.2.3 Cultures inoculated with rumen content

A total of 14 cultures were established containing rumen content as inoculum, 6 replicates with 250 mg/L m-cresol, 6 replicates with 200 mg/L 2-methylbenzoic acid, and 2 controls that received no substrate. The rumen content was collected October 8, 1990. On October 9, 40 mL rumen fluid was added to serum bottles containing 40 mL medium and appropriate substrates. After 7 weeks incubation, the large amount of gas pressure in the cultures was reduced by expelling gas with a syringe connected via rubber tubing to a pasteur pipet immersed in water, so that the expelled gas could be monitored. A positive pressure was maintained in the cultures.

2.2.4 Enrichment cultures containing phenol

A large phenol-degrading enrichment culture was maintained in a Magnaferm Fermentor Model Ma-114 (New Brunswick Scientific Co. Inc.,

New Brunswick, NJ.) This fermentor had a capacity to hold 12 L anaerobic sludge. The temperature was maintained at 35°C, and an internal stirring mechanism was in place. Gas produced by fermentation was expelled in a flask of water via a length of tubing.

The original 10-L phenol-degrading culture was maintained by tri-weekly additions of 3 mL liquified phenol. After a period when no gas evolution was observed, the phenol content of the culture was determined by sampling through the feeding hole and analyzing by GC. The phenol content was in excess of 1000 mg/L. The culture was discarded and the apparatus was washed.

The second culture was established November 28, 1990 using 10 L of undiluted sewage sludge. After the sludge was transferred to the fermentor, 100 mL resazurin solution, 50 mL phosphate solution (5%) KH_2PO_4, 5 mL vitamin B solution, 5 mL mineral II solution, 50 mL mineral I solution (Fedorak and Hrudey 1984), 40 mL distilled water, and 2.5 g phenol (to give 250 mg/L) were added. For the first 20 weeks of incubation, 1-mL samples were taken after mixing and analyzed for phenol content prior to phenol addition. Thus, the acclimation of the culture during this time was monitored. After 20 weeks, the amount of phenol added was increased to 3 g, and then again after 27 weeks it was increased to 4 g. The culture was maintained by almost daily additions of 4 g phenol; although the culture could degrade more than this, 4 g successfully maintained the activity in the culture. The culture was able to withstand at least five days without phenol addition.

2.3 Subcultures

2.3.1 Phenol-degrading subcultures

Almost all the subcultures that used the phenol-degrading culture as inoculum were of 100-mL size. Unless otherwise indicated, these were prepared by adding 10 mL of the phenol-degrading inoculum to 90 mL of pre-reduced medium in 158-mL serum bottles. All cultures within a series were inoculated at the same time using phenol-degrading sludge from the well-mixed 10-L enrichment culture. This culture was used as a inoculum only after the previous addition of phenol had been degraded. Substrates were added after inoculation of the subcultures, and if samples were

required, 1 mL was removed immediately after substrate addition. Gas volumes were adjusted to 5 mL more than atmospheric pressure using the apparatus of Fedorak and Hrudey (1983), after samples were taken, immediately prior to incubation.

2.3.2 Small subcultures

For studies with m-cresol-degrading and 2-methylbenzoic acid-degrading cultures, 10-mL size subcultures were used. To establish these, 5 mL of an enrichment culture was added as inoculum to each 58-mL or 61-mL capacity serum bottle containing 5-mL portions of pre-reduced anaerobic medium. For each test condition, triplicate cultures were prepared, and all cultures within a batch experiment were inoculated from a single enrichment culture. Substrates were generally added prior to inoculation. After inoculation, 0.5-mL samples were taken for substrate analysis, and if necessary, the gas volumes were then adjusted to 4 or 5 mL above atmospheric pressure prior to incubation.

2.4 Analytical Methods

2.4.1 Gas chromatography (GC)

2.4.1.1 Methane

Methane in the headspaces of serum bottle cultures was routinely analyzed using the method of Fedorak and Hrudey (1983). Either a Microtek GC equipped with a 2 m x 2 mm glass column packed with GP 10% SP1000/1% H_3PO_4 on 100/120 chromosorb WAW (Supelco), or a Hewlett Packard 5700A GC equipped with a 6 ft x 0.125 in OD Chromosorb 104 80/100 mesh column, was used to quantitate methane. The carrier gas was nitrogen (25 mL/min), and the flame ionization detector (FID) was supplied with 300 mL/min air and 30 mL/min hydrogen.

Methane standards were prepared by injecting known volumes of methane into sealed 158-mL serum bottles. Samples (0.1-mL) of standards and cultures were withdrawn using Lo-dose gas-tight syringes (Becton Dickinson, Rochelle Park, N.J.) then injected into the GC. The syringe was rinsed with carbon dioxide between samples to prevent carry-over between injections or oxygenation of cultures. The peak areas were recorded on a

HP model 3392A integrator. The methane concentration in the headspace of the cultures was calculated by comparison to a linear regression analysis of the standards, using the APL computer application DUNNETT on the Apple Macintosh Computer, based on the method of Dunnett (1955). Statistical comparison of methane in cultures also involved the student's t-test application.

Gas volumes were monitored using a pressure transducer (Microswitch 142 PC 30G; Honeywell, Freeport, IL) as described by Roberts et al. (1987). Volume standards were prepared at the time of inoculation for each set of cultures, by adding known volumes of methane to sealed serum bottles containing volumes of water equal to the total culture volumes. These standards were incubated with the cultures and analyzed with the pressure transducer at the time of each analysis. The methane volumes were calculated from the methane concentrations from GC analysis and the volumes from the pressure transducer using the TRANSDRYGAS2 program on the Macintosh computer.

2.4.1.2 Aqueous phenols and cresols

The concentrations of phenols or cresols in cultures were determined by analyzing the aqueous supernatants of cultures by GC. A Hewlett Packard 5790A GC with a 2 m x 2 mm stainless steel column packed with 5% polyphenylether coated on Tenax-GC (Bartle et al. 1977) was used. The column was maintained at 200°C, with injector at 200°C, the FID at 250°C, and a carrier gas (N_2) flow of 30 mL/min. Peak areas were recorded with a HP model 3390A integrator. Samples of 2 µL were injected using a 10-µL Hamilton syringe. Standards were analyzed the same day for quantitation. When a series of standards was used, each standard was prepared individually and the peak areas of the standards were converted by a simple linear regression to the equation of the line, used to calculate the concentrations of the samples. If a single standard was used, it was analyzed at least twice, and the average area was used to calculate the concentration in samples by a simple ratio comparison.

A Hewlett Packard 5700A GC was equipped with a 2 m x 2 mm glass column packed with Fluorad FC-431 (10% Fluorad FC-431 + 1% H_3PO_4 on Chromosorb W-HP 80/100 per 25 g) for aqueous analysis of benzoic acids. The oven was maintained at 170°C, the injection port at 200°C, and

the FID at 200°C. Injections of 2 µL were used, and the carrier gas was N_2. Peak areas were recorded on a HP 3392A integrator. Replicate injections of 50-150 mg/L 2-methylbenzoic acid samples demonstrated a linear relationship between peak area and concentration.

2.4.1.3 Capillary GC and GC-MS

To detect metabolites, ether-extracts of cultures were analyzed by GC. Samples were injected into a Hewlett Packard 5890A GC equipped with a 30-m DB-5 fused silica capillary column (J & W Scientific, Folsom, CA). The temperature was initially held at 90°C for 4 min then raised at 8°C/min to 250°C and held for 6 min. Helium was used as a carrier gas (linear flow rate 30 cm/sec), and the FID was supplied with 300 mL/min air and 30 mL/min hydrogen.

To identify unknown metabolites, the samples were analyzed by GC-MS. A Hewlett Packard 5890 series II GC equipped with a 30-m DB-5 fused silica capillary column and a Hewlett Packard 5970 mass selective detector were used to analyze samples using the same temperature program and conditions described above. The mass spectra obtained were compared to spectra within the National Bureau of Standards library, NBS43K.1, as well as to prepared standards.

2.4.2 High performance liquid chromatography (HPLC)

2.4.2.1 HPLC with a reverse-phase column

Most HPLC analyses used a Hewlett Packard series 1050 HPLC, with a variable wavelength detector set at 254 or 277 nm, and a HP 3396A integrator. Isocratic separations were performed with a reverse-phase column (LiChrospher 100 RP-18; 5 mm, 125 mm x 4 mm; Hewlett Packard Co., Palo Alto, CA). The mobile phase consisted of methanol, water purified through a Milli-Q system, and phosphoric acid, the proportions of which depended upon the goal of the analysis. All mobile phases and solvents were filtered under vacuum to purify and degas them just prior to use. All samples were centrifuged for 10 min to deposit solids, thereby prolonging column life. All sample injections were 100 µl, in order to rinse the sample loop prior to injection of 20-µL onto the column. The flow rate was 1 mL/min, and at the end of each day the

system was rinsed with water for 5 min and then methanol for at least 20 min, to wash away salts and help maintain the column.

For routine quantitation of 2-methylbenzoic acid, a mobile phase of 70:30:0.5 CH_3OH:H_2O:H_3PO_4 was used. This mobile phase was developed with the goal of finding a system that would effectively separate the 2-methylbenzoic acid, yet allow for rapid quantitation. The acetonitrile-based mobile phase used by Roberts et al. (1990) was not suitable for this. Variations of a method that used methanol, water and phosphoric acid were compared and the 70:30:0.5 ratio was found to be optimum for routine analyses. The optimum wavelength for detection of 2-methylbenzoic acid was determined by analyzing the UV-visible spectrum of a solution of 2-methylbenzoic acid in mobile phase by spectrometry, and the maximum was found to be 277 nm.

For analysis of metabolites or other substrates, a mobile phase of 40:60:0.5 CH_3OH:H_2O:H_3PO_4 was typically used. As the proportion of water in the mobile phase increased, the retention times of the compounds increased dramatically, therefore a variety of proportions were used to separate compounds in extracts or aqueous samples of cultures. UV spectra were obtained by stopping the flow of mobile phase when the compound of interest was in the detector cell and performing a UV scan using the detector. The UV scans obtained this way were used to determine the optimum wavelength for detection for each compound.

2.4.2.2 HPLC with an Aminex column

Metabolites in aqueous samples were separated using a Waters M-45 HPLC (Waters Associates, Inc.; Milford, MA) and a Lambda-Max Model 480 LC Spectrophotometer (Waters) set at 214 nm. Sample volumes of 50- or 100-µl were injected. The mobile phase of 0.010 M H_2SO_4 was run through an Aminex HPX-57H column (Bio-Rad Laboratories Ltd., Mississauga, Ont.) at 0.8 or 0.9 mL/min. Aminex HPLC columns are packed with a polymer-based matrix. The fundamental partition process responsible for separation is moderated by the ionic group bound to the resin, and by the choice of counterion. HPLC separations on Aminex resins use the mechanisms of ion exclusion, ion exchange, size exclusion, reversed phase, and normal phase partitioning. The HPX-87H column primarily uses ion exclusion and reverse phase mechanisms and is

optimized for analysis of carboxylic acids, volatile fatty acids, short chain fatty acids, alcohols, ketones, and many neutral metabolic by-products.

2.4.2.3 Fraction collecting and liquid scintillation counting

For experiments in which [^{14}C]m-cresol was added to enrichment cultures, samples were separated using the Aminex column and the outflow of the detector was attached to a fraction collector. A Gilson model 201 fraction collector was used to collect 5-min (4-mL) fractions from the outflow of the detector. The fractions were collected in 13 mm x 100 mm test tubes, and then the contents were transferred to liquid scintillation vials containing 10 mL aqueous counting scintillant (ACS)(Amersham Corp., Arlington Heights, IL), prior to liquid scintillation counting. All samples were dark-adapted for 15 min then counted for 2 min (program 2) using a Beckman LS 3801 Analytical Liquid Scintillation Counter (Beckman Instruments, Fullerton, CA). The disintegrations per minute (DPM) were determined by the external standard method automatically. Background samples consisted of 4 mL water in ACS.

2.5 Other Methods

2.5.1 Extraction of cultures

A variety of extraction procedures were compared. The variables compared included: methylene chloride vs ether; sonication and centrifugation, and dilution of sludge. These techniques were evaluated by GC analysis. One of the main problems that had to be overcome was the large background arising from the extraction of large amounts of sludge. As well, ether-extractions of sludge resulted in emulsions that clogged filters containing the sodium-sulfate drying agent.

The best procedure developed was used throughout, regardless of culture size or content. Cultures were acidified to pH < 2 with 10 M H_2SO_4. The acidified cultures were extracted with 3 x 25-mL diethyl ether. The ether was filtered through glass wool and anhydrous sodium sulfate to remove particulate matter and water. The extracts were pooled and concentrated using a rotory evaporator. The residues were dissolved in ether and transferred to dram vials with Teflon liners in the caps.

2.5.2 Derivatization of standards and metabolites

2.5.2.1 Methyl ester derivatives

Methyl esters of authentic standards and carboxylic acids in sample extracts were prepared by a variation of the method of Fedorak and Westlake (1983). Ether-extracts were transferred to 25-mL round bottom flasks and the ether evaporated under a slow stream of nitrogen. Then, 2 mL HPLC-grade methanol, one drop concentrated H_2SO_4, and a boiling chip were added. The sample was heated in a sand bath and refluxed for 2 h. The sample was then cooled, diluted with 3 mL distilled water and extracted with hexanes (3 x 5 mL). The hexanes were washed three times with 0.2 M potassium bicarbonate and then water. The sample was concentrated by evaporation under a slow stream of nitrogen.

This procedure was evaluated for the formation of the methyl esters of hydroxybenzoic acids. 4-Hydroxybenzoic acid was used as a model compound because of its availability and because it was one of the metabolites being sought. Two solutions containing 100 mg/L benzoic acid and 4-hydroxybenzoic acid were prepared and the methyl esters were prepared as described. Samples were removed from one of the preparations after the hexanes extractions, and again after all the washes. The other preparation was sampled after the hexanes extraction and again after each of the washes with the bicarbonate solution. Analysis of these samples clearly demonstrated that the 4-hydroxybenzoic acid-methyl esters were removed by the bicarbonate washing procedure, whereas the washing had no effect on the benzoic acid methyl esters. Thus, the bicarbonate and water washing procedures were omitted.

Methyl esters were also prepared using ethereal diazomethane prepared by the method of Fales and Jaouni (1973) in a diazomethane-generating apparatus (Wheaton Industries, Millville, N.J.). The reaction was allowed to proceed for 45 min to achieve a maximum yield of diazomethane. The ethereal diazomethane was then transferred with a flame-rounded pasteur pipette to solutions of authentic standards and ether-extracts of cultures in dram vials. These were allowed to react for 15 min, and then the ether and any remaining diazomethane were volatalized under forced nitrogen. The samples were dissolved in fresh ether and analyzed.

2.5.2.2 Trimethylsilyl (TMS) derivatives

Derivatives of compounds in culture extracts were made by silylating with N,O-bis(trimethylsilyl)acetamide (BSA) in acetonitrile according to the manufacturer's instructions (Pierce, Rockford, IL; method 5). Specifically, ether-extracts were transferred to dram vials and the ether removed with a slow flow of nitrogen. The residues were dissolved in 0.5 mL acetonitrile and then 0.25 mL BSA was added. The vials were sealed and shaken for 30 sec then heated in a 70°C water bath for 15 min and cooled. A wide variety of pure standards were prepared simultaneously by derivatizing small quantities of pure chemicals dissolved in acetonitrile.

2.5.3 Detection of CoA

A variety of methods were used to detect either CoA or CoA-bound derivatives. The method of Harwood and Gibson (1986) was used to attempt to separate authentic benzoyl-CoA, CoA, and benzoic acid by thin-layer chromatography (TLC). However, the components could not be detected using the visualization technique described, and the *n*-butanol and water formed two phases that did not appear to migrate well on the cellulose plates. Next, silica gel plates were used with a variety of mobile phases, with visualization under UV light. Benzoic acid had to be applied in large amounts in order to be detected, but some separation was achieved with a mobile phase of 70% methanol, 30% water.

An authentic standard of benzoyl-CoA was analyzed by HPLC to determine its retention time, UV spectrum, and other characteristics. A mobile phase of 70:30:05 H_2O:CH_3OH:H_3PO_4 was used with the detector set at a wavelength of 277 nm. Solutions of benzoyl-CoA were prepared (100 mg/L) in water and in ether. The benzoyl-CoA eluted with the same retention time as benzoic acid but had a significantly different UV spectrum. CoA eluted immediately after the solvent peak when an aqueous sample was analyzed, but could not be detected from the ethereal solution.

Ellman's reagent was prepared by adding 39.6 mg 5,5'-dithiobis(2-nitrobenzoic acid) to 10 mL 1 M phosphate buffer (pH 7.2). Samples were adjusted to a neutral pH using NaOH, and then a 3-mL sample was added to 5 mL water and 2 mL phosphate buffer in a large test tube. Three millilitres of this mixture was added to 0.02 mL of reagent in a small test tube and allowed to react for 5 min. The reaction of Ellman's reagent with

reduced CoA resulted in a brilliant yellow color that could be quantitated by spectrophotometry.

A Phillips PU 8740 UV/VIS scanning spectrophotometer from Pye Unicam Ltd. (Cambridge, England) was used for all spectrophotometric analyses. For the analysis of samples treated with Ellman's reagent, a wavelength of 412 nm was used, and the instrument was first calibrated with water as a blank. For the direct analysis of CoA and hydrolyzed benzoyl-CoA, the scanning function was used with the background subtraction of the absorbance of a water blank, with a range of 200 - 300 nm. Solutions were analyzed by diluting the test solutions, 0.5 mL per cuvette with the remaining volume filled with water. Scans of alkali-treated benzoyl-CoA were compared to scans of authentic standards of CoA and benzoic acid.

2.5.4 Alkali-treatment of cultures to release free acids from CoA thioesters

The alkali-treatment of culture supernatants consisted of a straightforward procedure. Culture supernatants that had been previously acidified to stop microbial activity were pooled to a known volume and divided into two equal portions. The treated portion was made alkaline by the addition of 5 N NaOH until the pH was 12. The solution was kept at room temperature and mixed often over the 15-20 min hydrolysis period. It was then acidified to pH <2 by the addition of H_2SO_4. The ratios of detected compounds to the internal standard were compared using Duncan's multiple range test.

To treat small (1-mL) samples of subcultures containing m-cresol and 6-fluoro-3-methylphenol, a 10-µL Hamilton syringe was used to deliver 10 µL 5 M NaOH to the eppendorf tube, increasing the pH to 12. This was mixed by shaking for 15 min, then acidified with 2.5 µl 10 M H_2SO_4, to neutralize the pH. Samples were centrifuged prior to HPLC analysis on the Aminex column.

2.5.5 Analysis of fluoride

As part of the study of the defluorination of fluorophenols, fluoride analyses were performed using the specific electrode method described in Standard Methods (APHA 1989), with an Orion Model 409 fluoride

electrode and an Orion 90-01 single junction reference electrode (Orion Research Inc., Boston, MA). The electrode was standardized with fluoride solutions of 1 mg/L and 10 mg/L. For each analysis, 25-mL portions of cultures were used.

To compensate for background fluoride present in the inoculum, the fluoride concentrations in the three replicate controls were measured. Then, volumes of a standard fluoride solution of 100 mg/L were added to individual portions of each control and measured to account for interferences in the fluoride analysis due to the sludge. The measured fluoride concentrations in triplicate analyses of the controls containing 0, 1, or 2 mg/L fluoride were averaged, plotted against the expected fluoride concentration, and by simple linear regression a calibration curve was obtained. This was used to calculate the actual fluoride concentrations in the cultures.

2.6 Chemical Syntheses

2.6.1 4-Hydroxy-2-methylbenzoyl-CoA synthesis

No published method was available for the synthesis of the CoA thioester of 4-hydroxy-2-methylbenzoic acid, at least in part because this acid is not commercially available. However, a method for synthesizing hydroxybenzoyl-CoA esters (Webster and Killenberg 1981) was available. This procedure involved the initial conversion of the carboxyl group to a benzoyl-chloride, and then the addition of reduced CoA under the right conditions would result in the CoA thioester, which could be purified by column chromatography. Due to the limited quantity of 4-hydroxy-2-methylbenzoic acid available (about 1 g), the method had to be altered for smaller quantities of materials, so only one-tenth of the quantities specified by Webster and Killenberg (1981) were used. To conserve 4-hydroxy-2-methylbenzoic acid, the method was tested using 2-hydroxy-4-methylbenzoic acid, also called 4-methylsalicylic acid.

Initially, 25 g of oxalyl chloride was refluxed with 10 g 4-methylsalicylic acid in 250 benzene for 2 h to prepare the benzoyl chloride. This was recovered by fractional distillation under reduced pressure at about 80°C. An estimated 2-3 fold excess of the benzoyl chloride was added dropwise to a 0°C solution of 200 mg CoA rapidly stirring in 4 mL

water adjusted to pH 8.0 with 2 M LiOH and maintained at pH 7-8 by LiOH additions. The reaction was monitored for CoA disappearance by analyzing samples with Ellman's reagent. The solution was adjusted to pH 3 with 5 N HCl, and the precipitate removed by centrifugation. The aqueous phase was evaporated to dryness with a rotary evaporator, extracted 8-10 times with 8 mL acetone:methanol (10:1) until the extract had no chloride as determined by precipitation with 1% $AgNO_3$. The residue was dissolved in 2 mL water, and was ready for purification. However, the yields of products from each step, particularly the vacuum distillation, were low and impure as determined by GC and HPLC analysis of samples from various stages of the procedure. Thus, the final amount of product was insufficient for the column chromatography purification.

2.6.2 3-Fluoro-4-hydroxybenzoic acid synthesis

3-Fluoro-4-hydroxybenzoic acid was synthesized by the method of Ferguson et al. (1946). 2-Fluorophenol (32.4 g), NaOH (77.2 g) and 260 mL water were combined and brought to 55°C. Chloroform (76.8 g) was added over 30 min with efficient stirring. After 70 min, the temperature was raised to 65°C for an additional 60 min. The color of the solution progressed from colorless, to yellow, orange, red and finally burgundy over the course of the reaction. The solution was then acidified with sulfuric acid and steam distilled. Chloroform and phenol were the first compounds to distill from the reaction mixure, followed by the fluorinated aldehydes (a side reaction produces 3-fluorosalicylaldehyde). After several attempts at the distillation, crystals of fairly pure 3-fluoro-4-hydroxybenzaldehyde were obtained. The aldehyde was converted to the corresponding acid by preparing the oxime by adding 30 mL ethanol and 4.1 g hydroxylamine hydrochloride to the aldehyde, making the solution basic with sodium carbonate and NaOH, and allowing to stand overnight. The oxime was collected by filtration and a small amount was recrystallized from water. The oxime was converted to the acid by refluxing in 25 mL acetic anhydride with 5 drops concentrated H_2SO_4 for 6 h. The acetic anhydride was removed by distillation and the product was recovered by ether-extraction of the acidified residue. HPLC analysis of the final ether-extracted product revealed impurities. GC-MS analysis

confirmed the presence of other compounds, several of which were consistent with the structures of unreacted oximes.

The product was purified by a combination of ether-extraction and bicarbonate washes of the ether-extracts to remove unreacted oximes. Two sequential extractions of the ether with 5% $NaHCO_3$, followed by acidification and re-extraction into ether, successfully removed much of the contaminating material. However, the final preparation contained some unidentified contaminants.

2.6.3 6-Fluoro-3-methylphenol synthesis

6-Fluoro-3-methylphenol (6-fluoro-m-cresol) was synthesized by formation of a diazonium salt followed by replacement of the diazo group with a hydroxy group. The method was an adaptation of the procedure for synthesizing p-cresol from 4-aminotoluene (Furniss et al. 1989). 2-Fluoro-5-methylaniline was dissolved in hot H_2SO_4 then cooled and reacted with a solution of sodium nitrite and urea. After addition of copper(II)nitrate and copper(I)oxide the final product was extracted into ether and dried. The final brown liquid product was virtually pure as determined by HPLC, GC, and GC-MS. The ^{1}H-NMR characteristics agreed with those of Claudi et al. (1990) and showed that trace amounts of ether remained in the preparation.

2.7 Chemicals

Phenol and benzoic acid were obtained from Fisher Scientific Co. (Fair Lawn, NJ). m-Cresol, o-cresol and p-cresol were purchased from Aldrich Chemical Co., Inc. (Milwalke, WI).

2-Methylbenzoic acid (o-toluic acid) was obtained from Matheson Coleman & Bell (Norwood, OH). 3-Methylbenzoic acid and 4-methylbenzoic acid were obtained from Pfaltz and Bauer Inc. (Stamford, CT).

4-Hydroxybenzoic acid was purchased from Eastman Organic Chemicals (Rochester, NY). 2-Hydroxy-4-methylbenzoic acid was from Aldrich. 4-Hydroxy-2-methylbenzoic acid was synthesized by the method of Cox (1927). The final product contained a small amount of benzoic acid.

3-Fluorobenzoic acid, 2-, 3-, and 4-fluorophenol, 4-fluoro-3-methylphenol, 2-fluoro-3-methylaniline and 3-fluoro-2-methylbenzoic acid were purchased from Aldrich. 3-Chlorobenzoic acid was purchased from Sigma Chemical Company (St. Louis, MO), and 3-chlorophenol was from Aldrich.

[Ring-^{14}C]m-cresol was obtained from Amersham Canada Ltd. (Oakville, Ontario); [methyl-^{14}C]m-cresol was custom synthesized by Pathfinder Laboratories (St. Louis, MO). CoA and benzoyl-CoA were obtained as lithium salts from Sigma.

Other chemicals used included: phenylacetic acid (Sigma); bromoethanesulfonic acid (BESA) (Aldrich); 4-hydroxybenzyl alcohol (Aldrich); copper (II) oxide and copper (II) nitrate hemipentahydrate (Aldrich); 5,5'-dithiobis-2-nitrobenzoic acid (Aldrich); and BSA (Pierce, Rockford IL).

Gases were obtained from Linde, a division of Union Carbide Canada Ltd. (Toronto). Other chemicals and reagents including ether, methanol, and $Na_2S \cdot 9H_2O$ were obtained from BDH Chemicals (Toronto).

3 RESULTS AND DISCUSSION

3.1 2-Methylbenzoic Acid Degradation

2-Methylbenzoic acid was identified as a metabolite in m-cresol-degrading cultures (Roberts et al. 1990). In these cultures, m-cresol was transformed to 4-hydroxy-2-methylbenzoic acid, and 2-methylbenzoic acid arose from the dehydroxylation of 4-hydroxy-2-methylbenzoic acid. Although abundant in culture supernatants, 2-methylbenzoic acid was not readily metabolized by the m-cresol-degrading culture. In fact, 2-methylbenzoic acid was considered a dead-end product in m-cresol-degrading cultures because it accumulated in 2-L enrichment cultures (Roberts et al. 1990). During this project, 2-methylbenzoic acid was consistently detected in m-cresol-degrading cultures, although it did not continue to accumulate in the enrichment cultures (refer to section 3.1.1.2).

There have been very few investigations of the metabolism of 2-methylbenzoic acid, even under aerobic conditions. Engelberts et al. (1989) and Higson and Focht (1992a) reported that the aerobic degradation of 2-methylbenzoic acid began with the formation of 3-methylcatechol, which was subsequently degraded by the *meta*-fission pathway. Similarly, the halogenated analogue 3-chloro-2-methylbenzoic acid was transformed to 4-chloro-3-methylcatechol, which was subsequently metabolized through the *meta*-fission pathway (Higson and Focht 1992b). There have been no reports of 2-methylbenzoic acid degradation under anaerobic conditions, although trends noted for other aromatic compounds suggest that one of three mechanisms are most likely involved: methyl group oxidation (as for p-cresol) to form phthalic acid, ring carboxylation (as for phenol) to yield a methyl-substituted dicarboxylic acid, or ring hydroxylation (as for toluene) to yield a hydroxy-methylbenzoic acid.

The goal of this investigation with 2-methylbenzoic acid was to determine whether this dead-end product from m-cresol-degrading methanogenic consortia could be degraded by an anaerobic population. Thus, methanogenic enrichment cultures were established with 2-methylbenzoic acid as the sole added carbon source. Factors affecting the degradation of 2-methylbenzoic acid were studied as well as the initial steps of its degradation.

3.1.1 Establishing 2-methylbenzoic acid-degrading cultures

Over the course of the study, a total of three sets of enrichment cultures were established with methanogenic sewage sludge as inoculum. These were analyzed for such factors as 2-methylbenzoic acid degradation, methane production, and intermediate formation. Several of these cultures were used as inocula for subcultures. The results of these investigations are presented below.

Enrichment cultures using rumen content as inoculum were also established, in an attempt to enrich methanogenic cultures capable of 2-methylbenzoic acid-degradation. An initial concentration of 200 mg/L 2-methylbenzoic acid was used, and the cultures were monitored for gas production and substrate concentration. No significant increase in methane production, or decrease in substrate concentration, was observed over the 28 weeks incubation. These cultures were discarded, and no other analyses were performed with these cultures.

3.1.1.1 Evidence of 2-methylbenzoic acid degradation

The concentration of substrate in the cultures was monitored by HPLC analysis of samples of culture supernatants. The depletion of 2-methylbenzoic acid in all cultures was typical of biodegradation; that is, there was a typical lag in which no degradation occurred, followed by a relatively fast and complete degradation of the available substrate (Figure 3.1-1). Subsequent feedings of 2-methylbenzoic acid were similarly degraded.

An increase in the methane concentration in the headspace of the cultures was observed concomitant with 2-methylbenzoic acid degradation. However, measuring this increase in cultures that already had large background levels of methane due to the metabolism of substrate carried over in the sludge, was difficult. For example, the background methane concentrations in cultures with 25% (v/v), 50% (v/v), or 94% (v/v) inoculum just prior to 2-methylbenzoic acid degradation were 31%, 41%, 51%, whereas the maximum value of methane determined by this GC method was 55%. Background levels of methane were reduced by flushing the headspaces of selected enrichment cultures from the second set. An

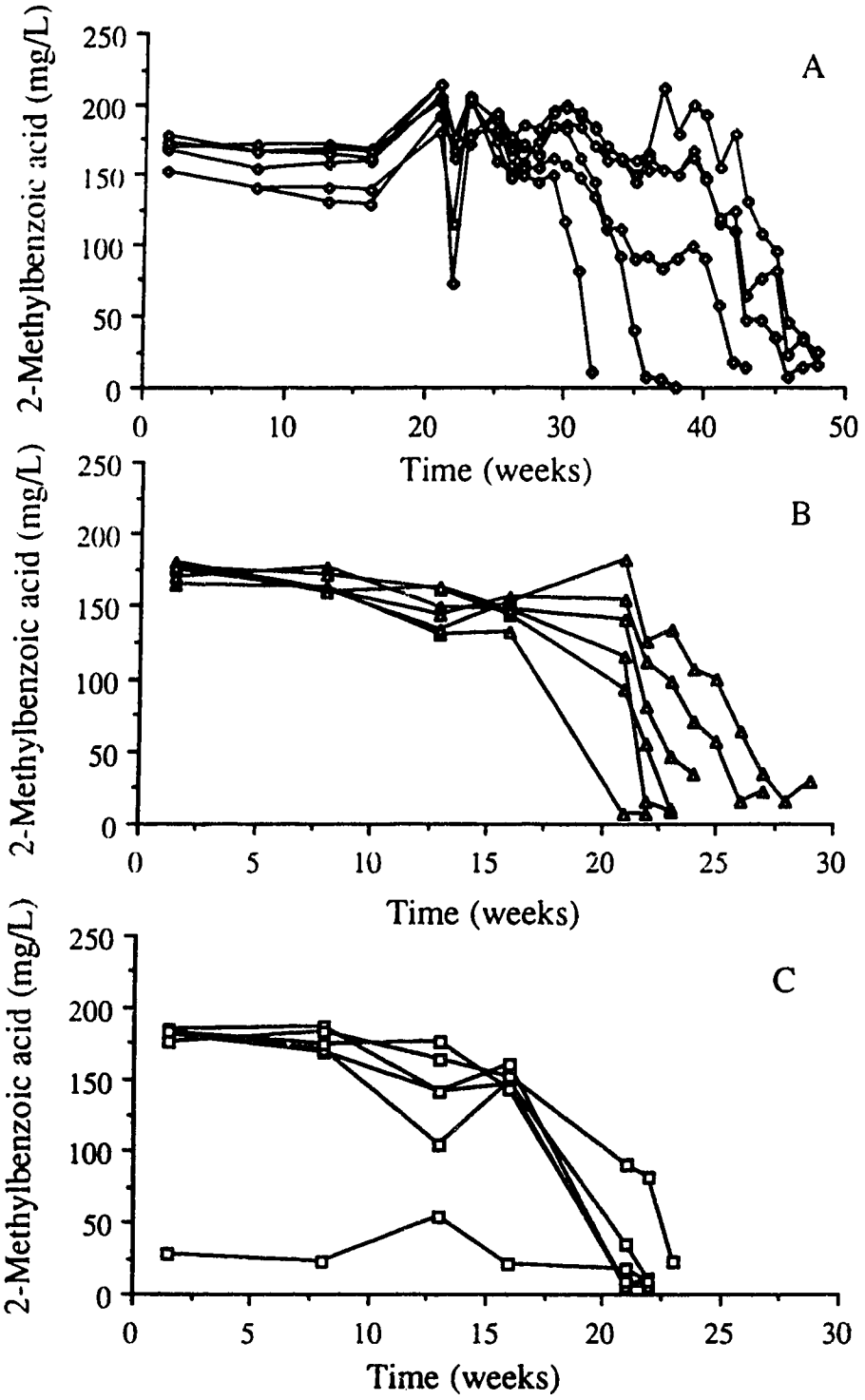


Figure 3.1-1: Degradation of 2-methylbenzoic acid in replicate 80-mL methanogenic enrichment cultures (second set) inoculated with 94% (v/v) (A), 50% (v/v) (B), or 25% (v/v) (C) sludge, demonstrating dependence of lag time on inoculum. One replicate (C) apparently did not receive 200 mg/L 2-methylbenzoic acid initially.

increase in methane above control levels was observed for cultures with a 94% (v/v) inoculum, but not for those with 25% (v/v) or 50% (v/v) inoculum (Figure 3.1-2A), that was consistent with the observed degradation of 2-methylbenzoic acid in only the 94% (v/v) cultures. Therefore a definitive increase in methane production due to 2-methylbenzoic acid degradation was observed in select cultures. Increases in the concentration of methane in the headspaces of control cultures, that were proportional to the amount of inoculum added, were also observed over the incubation period (Figure 3.1-2B).

3.1.1.2 Lag times prior to 2-methylbenzoic acid degradation

The degradation of 2-methylbenzoic acid proceeded only after long lag times. The acclimation period depended on the amount of inoculum and on the initial concentration of 2-methylbenzoic acid. The lag time was fairly consistent among replicate cultures with different inocula (Table 3.1-1). The degradation time, defined as the time prior to 90% reduction of 2-methylbenzoic acid, was also determined as additional evidence of the combined lag time and degradation rate within the cultures. The degradation times, which were more precisely determined, for the various inocula and among the three sets of enrichment cultures were statistically compared using Duncan's multiple range test, and all results were determined for $P<0.05$. Comparison of all nine parameters determined that the shortest average degradation time was 15 weeks for the 50% (v/v) inoculum from the third set. The longest average degradation time was 42 weeks for the 94% (v/v) inoculum from the second set, which was significantly larger than all other averages. Between the first and second sets of enrichment cultures, the degradation times for the 25% (v/v) inocula were not significantly different, nor were those of the 50% (v/v) or 94% (v/v) inocula, indicating that the two sets of enrichment cultures were indeed almost identical.

Comparison of the three sets of enrichment cultures revealed that the average time prior to complete degradation was significantly shorter for the third set of cultures. The difference in the degradation time was attributed to the reduced concentration of 2-methylbenzoic acid, although differences among the sewage sludge inocula could also be responsible. Reports of the inhibitory effect of high concentrations of otherwise

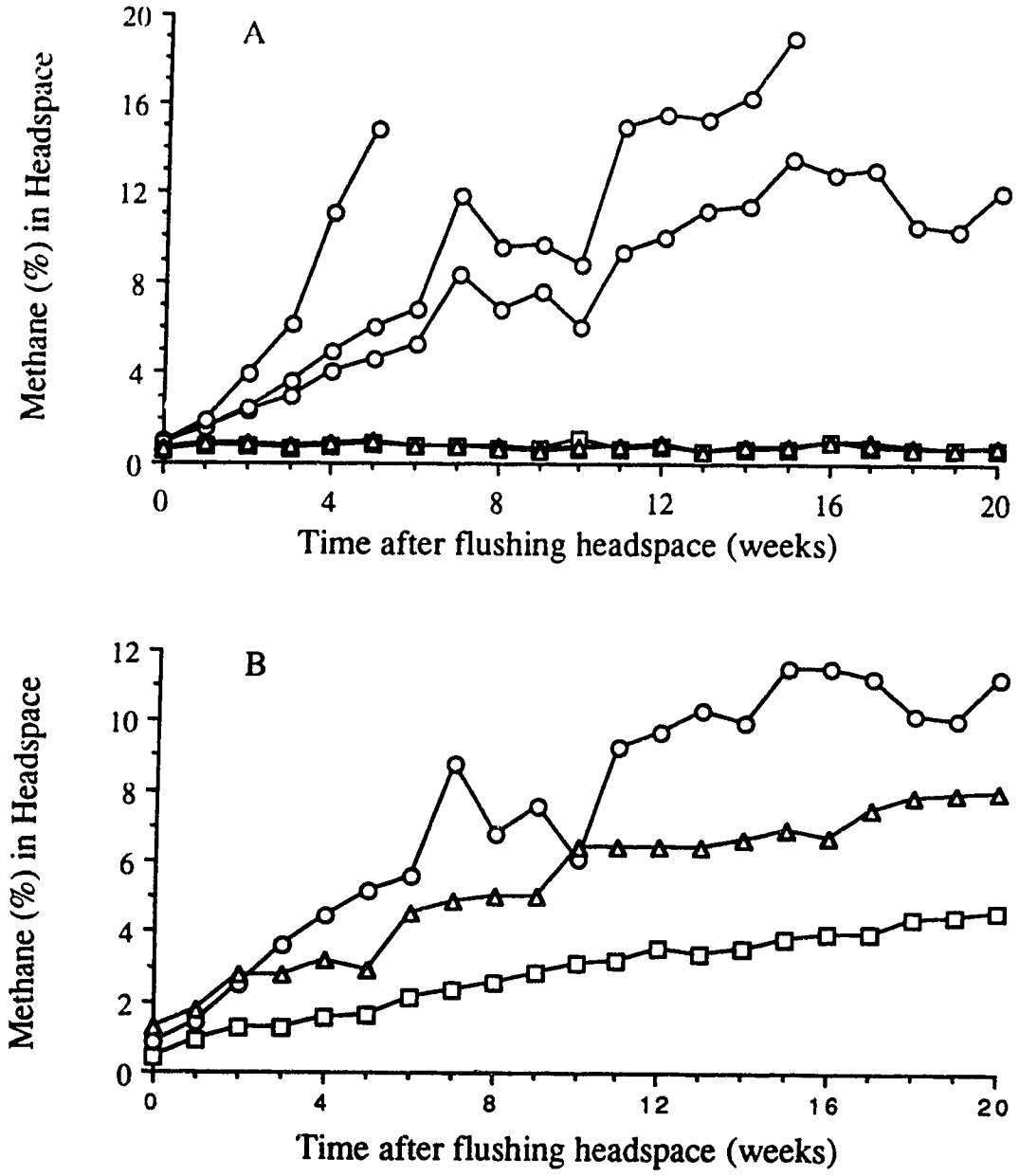


Figure 3.1-2: Enrichment cultures monitored for additional methane production due to 2-methylbenzoic acid degradation after the headspaces were flushed to remove large background levels of methane. Increases in methane concentration in headspaces are shown for 2-methylbenzoic acid-containing cultures (A) and controls without substrate (B), with 25% (v/v) (□), 50% (v/v) (Δ), or 94% (v/v) (○) inocula.

Table 3.1-1: Lag times prior to 2-methylbenzoic acid degradation in three sets of enrichment cultures with different proportions of inocula and two different initial concentrations of 2-methylbenzoic acid.

Set	Initial concentration (mg/L)	Inoculum (v/v)	Number of replicates	Average lag time ± S.D. (weeks)[a]	Average degradation time(weeks)[b]
1	200	25%	6	26 ± 2	32 ± 4
		50%	6	16 ± 1	23 ± 2
		94%	5[c]	25 ± 3	34 ± 7
				22 ± 5[d]	30 ± 7[d]
2	200	25%	5[e]	18 ± 2	21 ± 2
		50%	6	20 ± 2	24 ± 3
		94%	6	36 ± 5	42 ± 6
				24 ± 11[d]	29 ± 10[d]
3	100	25%	6	16 ± 2	18 ± 1
		50%	6	12 ± 2	15 ± 2
		94%	6	12 ± 1	16 ± 2
				13 ± 2[d]	16 ± 2[d]

[a] Lag time refers to incubation time prior to onset of 2-methylbenzoic acid degradation.

[b] Degradation time refers to incubation time prior to 90% reduction of initial 2-methylbenzoic acid concentration; standard deviation is also given.

[c] One replicate never degraded the initial 2-methylbenzoic acid.

[d] Average and standard deviation of all cultures of each set.

[e] One replicate apparently did not receive 200 mg/L 2-methylbenzoic acid initially.

biodegradable aromatic compounds are common, such as for m-cresol (Roberts et al. 1988) and phenol (Fedorak and Hrudey 1984).

The degradation time was also dependent on the proportion of sewage sludge used to establish the 80-mL cultures. Within the first set of enrichment cultures, the 50% (v/v) average was significantly lower than the 25% (v/v) and 94% (v/v) averages. Within the second set, the 50% (v/v) and 25% (v/v) averages were significantly different than the 94% (v/v) average. Within the third set, the 50% (v/v) average was significantly lower than the 25% (v/v) average, but not significantly lower than the 94% (v/v) average. Combining the values from all three sets for each inoculum level, the use of 50% (v/v) inoculum had the shortest time at 20 weeks, which was not significantly different than the 24 weeks of the 25% (v/v) inoculum. However the degradation times for these inocula were significantly different than the 31 weeks of the 94% (v/v) inocula. In summary, the trend was that the shortest degradation time was observed for cultures that had 50% (v/v) inoculum, although it was not consistently lower, and overall not significantly different from the 25% (v/v) inoculum. The significantly longer degradation time associated with cultures that received the 94% (v/v) inoculum could be attributed to an increased sulfide concentration in the cultures as a result of the increased inoculum, as observed by Roberts et al. (1988). The slightly longer degradation time associated with 25% (v/v) cultures could be associated with dilution of the organisms required to carry out the initial transformations, thereby necessitating a longer time to generate the critical population size.

3.1.1.3 Maintenance of 2-methylbenzoic acid-degrading activity

After the degradation of the initial allotment of 2-methylbenzoic acid, the substrate was replenished by the draw-and-feed method and the analyses continued. Overall, the cultures did not readily maintain their 2-methylbenzoic acid-degrading activity. Therefore, a variety of conditions were compared to attempt to elucidate the best procedures or culture conditions for maintaining the 2-methylbenzoic acid-degrading ability in the enrichment cultures.

For the first set of enrichment cultures, subsequent degradation of the first feeding of 200 mg/L 2-methylbenzoic acid added was noted for only 4 of 15 possible cultures, with an average of 6 weeks required to

degrade the substrate. Two of the cultures were used as inocula for subculture experiments, and one never degraded the initial allotment, so these were omitted from the comparison. None of the cultures degraded a second feeding of 2-methylbenzoic acid within the 55 weeks total incubation.

For the second set, enrichment cultures were maintained for up to 75 weeks incubation, although three of the 25% (v/v), and three of the 95% (v/v) cultures were used as inocula for subcultures once they had degraded the initial allotment of 2-methylbenzoic acid. Of the remaining 12 cultures, 10 degraded the first feeding of 100 mg/L 2-methylbenzoic acid, and one degraded a second feeding. The average length of time between the addition of 2-methylbenzoic acid by the draw-and-feed procedure, and its subsequent degradation, was 10 weeks. After about 45 weeks incubation, the concentration of 2-methylbenzoic acid in 10 of the 12 remaining cultures levelled off and remained constant. After 75 weeks incubation, the cultures were discarded. The concentration at which each culture levelled off varied from 30 mg/L to 120 mg/L, averaging 65 mg/L. The reason for the cessation of 2-methylbenzoic acid-degrading activity was unknown.

The third set of enrichment cultures could only be maintained for a total of 46 weeks because of time constraints. During this time, half of the cultures were used either as inocula or for extractions. Nevertheless, all the cultures degraded the first feeding of 100 mg/L of 2-methylbenzoic acid within the subsequent 17 weeks. Generally, subsequent feedings of 2-methylbenzoic acid were degraded only after 18 or 20 weeks incubation. Occasionally, cultures could become very active, degrading repeated feedings of 2-methylbenzoic acid without a lag. The average lag time for degradation of 2-methylbenzoic acid added by the draw-and-feed procedure was 11 weeks. At the time the cultures were discarded, a maximum of four feedings of 2-methylbenzoic acid were degraded by one of the cultures.

The time before subsequent feedings of 2-methylbenzoic acid were degraded was dependent on the length of delay between substrate depletion and the next feeding. For the third set of cultures, the interval between the time when the substrate was 25 mg/L or lower, and the time when more substrate was added, was compared to the time it took to degrade this added

substrate. For an interval of 0-1 weeks, there were 25 occurrences, with 3 incidences where the degradation was ≥14 weeks but could not be precisely determined because the cultures had to be discarded. Of the remaining 22, the average degradation time was 9.2 ± 5.2 weeks. For intervals of ≥2 weeks, only once was the limit ≥17 weeks, and for the other 9 the average was 14.1 ± 1.5 weeks. If the times for the cultures in which no degradation occurred were omitted, a statistical comparison of the two means (student's t-test) revealed that they were significantly different at $P<0.05$. Therefore, a delay of 2 or more weeks prior to replenishing the substrate of a culture that had degraded the 2-methylbenzoic acid, prolonged the time prior to subsequent degradation.

There was no apparent correlation between the concentration of 2-methylbenzoic acid and the degradation time, within the range of 100 - 130 mg/L (data not shown). The rate at which the 2-methylbenzoic acid was degraded did not change with the number of feedings. Therefore the rate of degradation was not significantly different for subsequent feedings. Likewise, the rate of degradation of 2-methylbenzoic acid was not affected by the age of the culture.

In summary, the factors affecting the maintenance of the 2-methylbenzoic acid-degrading activity were not elucidated but some general trends were noted. For example, addition of 100 mg/L 2-methylbenzoic acid resulted in a much greater probability of continued activity compared to the addition of 200 mg/L. After the initial allotment was degraded, the degradation of subsequent feedings of 2-methylbenzoic acid generally required ≥10 weeks. Occasionally, cultures degraded feedings of substrate without a lag, but the conditions that led to this desirable activity were unknown. In general, a delay of ≥ 2 weeks between replenishing the substrate once substrate was depleted, increased the time required to degrade the subsequent feeding of 2-methylbenzoic acid. However, the rate of degradation after the lag period was not affected either by the amount of 2-methylbenzoic acid previously degraded, nor by the age of the culture.

3.1.1.4 Gas production due to 2-methylbenzoic acid degradation

With the first set of enrichment cultures, increases in methane production due to 2-methylbenzoic acid degradation could not be reliably

measured because the headspaces of the cultures had such large percentages of methane from the metabolism of fermentable organic compounds associated with the inoculum. Attempts to measure an increase in methane due to 2-methylbenzoic acid degradation were successful after the headspaces of selected enrichment cultures (second set) were flushed to remove existing methane at the time of feeding (Figure 3.1-2A).

Gas production measurement was improved for the third set of enrichment cultures by flushing the headspaces of all cultures after 10 weeks incubation and adjusting the headspace volume. Enhanced methane production (Figure 3.1-3) was observed for cultures with all three inoculum levels, concurrent with the degradation of 2-methylbenzoic acid, the lag times of which are shown in Table 3.1-1. The cultures were fed 100 mg/L 2-methylbenzoic acid once the initial allotment was depleted, resulting in further methane concentration increases. After 30 weeks incubation, the total volume in each culture was measured using the 2-syringe device (Fedorak and Hrudey 1983) and compared to the amount of gas volume predicted by Equation 3.1-1 based on the amount of 2-methylbenzoic acid degraded in each culture.

$$C_8H_8O_2 + 5\ H_2O \rightarrow 3.5\ CO_2 + 4.5\ CH_4 \quad \text{(Equation 3.1-1)}$$

The results for the 25% (v/v) and 50% (v/v) cultures above control levels are shown in Table 3.1-2. The average recovery of methane in the 25% (v/v) cultures and 50% (v/v) cultures was 100 ± 8% and 64 ± 17% respectively, whereas the recovery of total gases were 81 ± 22% and 102 ± 7% respectively. The results for the 94% (v/v) cultures (data not shown) were especially low because the volumes measured were at the limit of the capacity of the methods used, and therefore the true increases above control levels could not be measured. A gradual increase in the concentration of methane in the headspaces of controls was once again observed.

The observed increases in methane and total gas in enrichment cultures degrading 2-methylbenzoic acid were reasonably consistent with predicted values, especially considering the 30 week total incubation time, the size of the cultures, the background methane concentrations, and the small total amounts of 2-methylbenzoic acid degraded. Therefore,

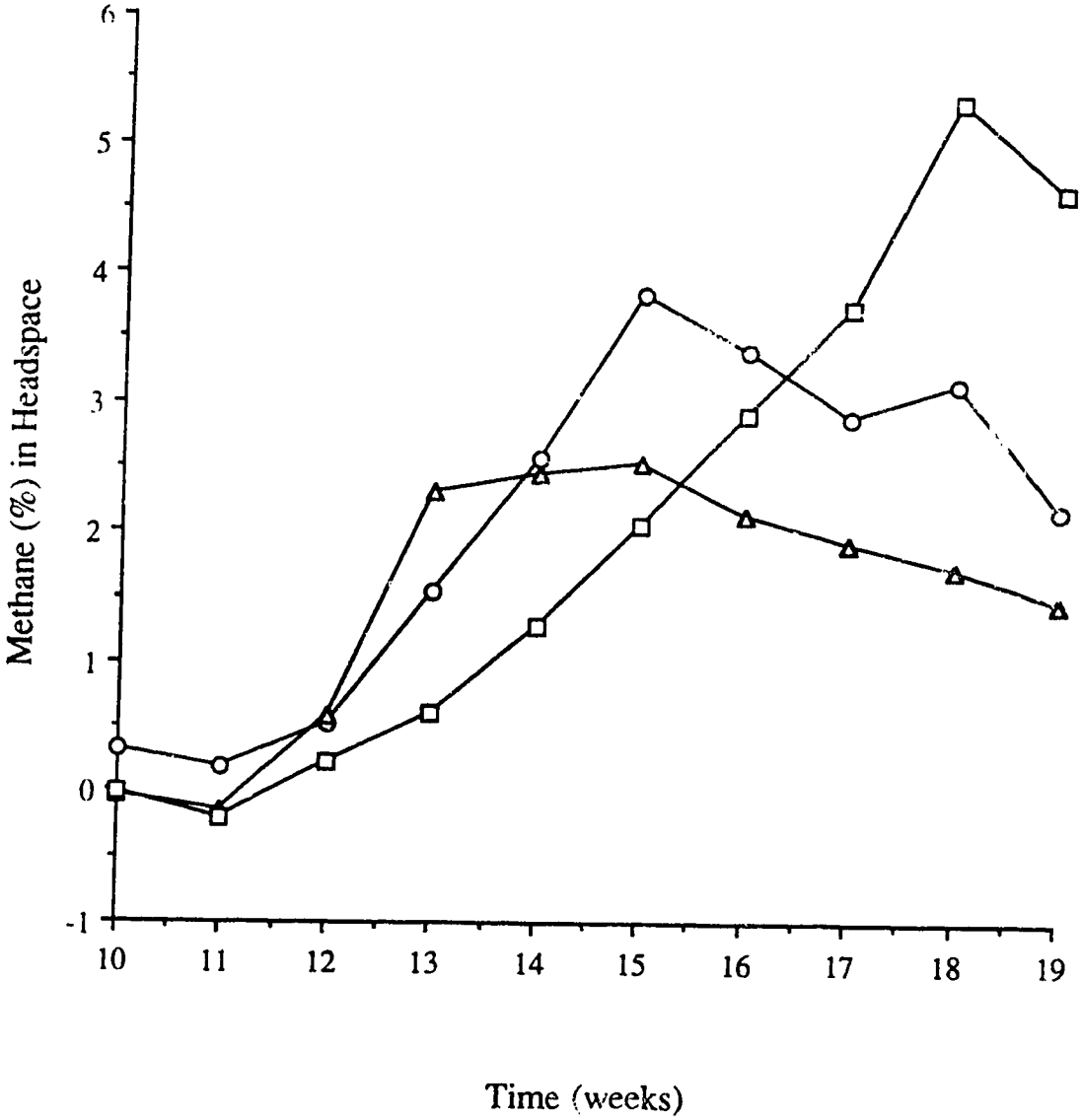


Figure 3.1-3: Increases in methane production due to 2-methylbenzoic acid degradation in the third set of enrichment cultures, after the headspaces had been flushed after 10 weeks incubation. Values are the averages of six replicates above control levels for 25% (v/v) (□), 50% (v/v) (Δ), and 94% (v/v) (O) inocula.

Table 3.1-2. Increases in methane and total gas volume after 30 weeks incubation in enrichment cultures degrading 2-methylbenzoic acid.

Cultures[a] (replicate)	2-Methylbenzoic acid consumed (mg)	Expected methane[b] (mL)	Observed methane[c] (mL)	Observed / Expected (%)	Expected total gas[b] (mL)	Observed total gas[c] (mL)	Observed / Expected (%)
25%(v/v) (1)	15.5	11.4	10.2	89	20.4	8.6	42
(2)	28.9	21.3	19.7	92	37.9	26.9	71
(3)	24.3	17.9	19.2	107	31.9	29.2	92
(4)	11.1	8.2	8.2	100	14.6	14.7	101
(5)	14.6	10.8	11.8	105	19.2	16.5	86
(6)	15.0	11.0	11.8	107	19.6	18.8	96
Avg. ± S.D.[c]	18.2 ± 6.8	13.4 ± 5.0	13.4 ± 4.8	100 ± 8	23.9 ± 8.9	19.1 ± 7.7	81 ± 22
50%(v/v) (1)	13.0	9.6	6.9	72	17.1	17.5	102
(2)	13.4	9.9	5.7	58	17.5	16.0	91
(3)	11.5	8.5	6.1	72	15.1	16.2	107
(5)	13.4	9.9	8.1	82	17.6	19.3	110
(6)	9.8	7.2	2.7	38	12.8	13.0	102
Avg. ± S.D.	12.2 ± 1.6	9.0 ± 1.2	5.9 ± 2.0	64 ± 17	16.0 ± 21	16.4 ± 2.3	102 ± 7

a Replicates of cultures from the third set of enrichment cultures inoculated with 25% (v/v) or 50% (v/v) sludge; fourth replicate was sacrificed before 30 weeks incubation were complete.

b Volume of methane predicted based on amount of 2-methylbenzoic acid consumed.

c Corrected for control levels of methane at standard temperature and pressure.

d Average and standard deviation of the replicates.

combining the increases in methane concentration in the headspace volumes (section 3.1.1.1), and the gas volume determinations from the third set of enrichment cultures, it was concluded that the 2-methylbenzoic acid degraded in these cultures was indeed mineralized to methane and carbon dioxide.

3.1.2 Studies with subcultures containing 2-methylbenzoic acid

A total of fourteen sets of fifteen subcultures per set were established with 2-methylbenzoic acid-degrading enrichment cultures as inocula. These were used to determine the effects of various conditions on substrate degradation. The subcultures were monitored for enhanced methane production, but because of the small 10-mL total volume, the substrates were not routinely analyzed by HPLC except when noted.

3.1.2.1 Effects of 2-methylbenzoic acid concentration

The inhibitory effects of high concentrations of 2-methylbenzoic acid were evidenced by the difference in lag times between the two sets of enrichment cultures. The cultures that received 100 mg/L initially had lag times about half of those that received 200 mg/L initially. Five sets of subcultures were established using cultures from all three sets of enrichment cultures as inocula, to try to determine whether the concentration of 2-methylbenzoic acid had any effect on its rate of degradation. The conditions used are summarized in Table 3.1-3.

Only in the first of these sets of subcultures was 2-methylbenzoic acid degradation observed. For this set of cultures, substrate concentration did affect methane production (Figure 3.1-4). As predicted, cultures that received 50 mg/L or 100 mg/L 2-methylbenzoic acid had proportionally enhanced methane production relative to the controls. The lag time and the initial rate of methane production were not inhibited by 200 mg/L 2-methylbenzoic acid. However, the methane yield did not exceed that of those cultures that received 100 mg/L 2-methylbenzoic aicd. Thus, the high concentration of 2-methylbenzoic acid caused some form of inhibition.

Several variations were applied in subsequent attempts to obtain active subcultures to verify the concentration-dependence observed in the

Table 3.1-3: Specifications for five sets of subcultures established to determine the effects of 2-methylbenzoic acid concentration on degradation.

Subculture series (date)	Source of inoculum set #	inocul. (v/v)	history (weeks)	Na_2S added[a]	Conc. tested (mg/L)	Time analyzed (weeks)	Enhanced methane (> controls)
1 (Apr. 18 1991)	1	94%	39[b]	2 mM	50	16	yes
					100	16	yes
					200	16	yes
2 (May 30 1991)	1	94%	45[b]	1 mM	100	10	no
					200	10	no
					300	10	no
3 (Sept. 17 1991)	2	25%	22[b]	1 mM	50	9	no
					100	9	no
					200	9	no
					300	9	no
4 (Jul. 8 1992)	3	94%	30[c]	0.5 mM	50	16	no[d]
					100	16	no[d]
					200	16	no[d]
					300	16	no[d]
5 (Aug. 25 1992)	3	25%	37[e]	0.5 mM	50	9	no[d]
					100	9	no[d]
					200	9	no[d]
					300	9	no[d]

[a] Final concentration of sodium sulfide in reduced feed solution.
[b] The enrichment culture had degraded 200 mg/L 2-methylbenzoic acid.
[c] The enrichment culture had degraded 100 mg/L 2-methylbenzoic acid.
[d] Total gas volume measured using pressure transducer.
[e] The enrichment culture had degraded three portions of 100 mg/L.

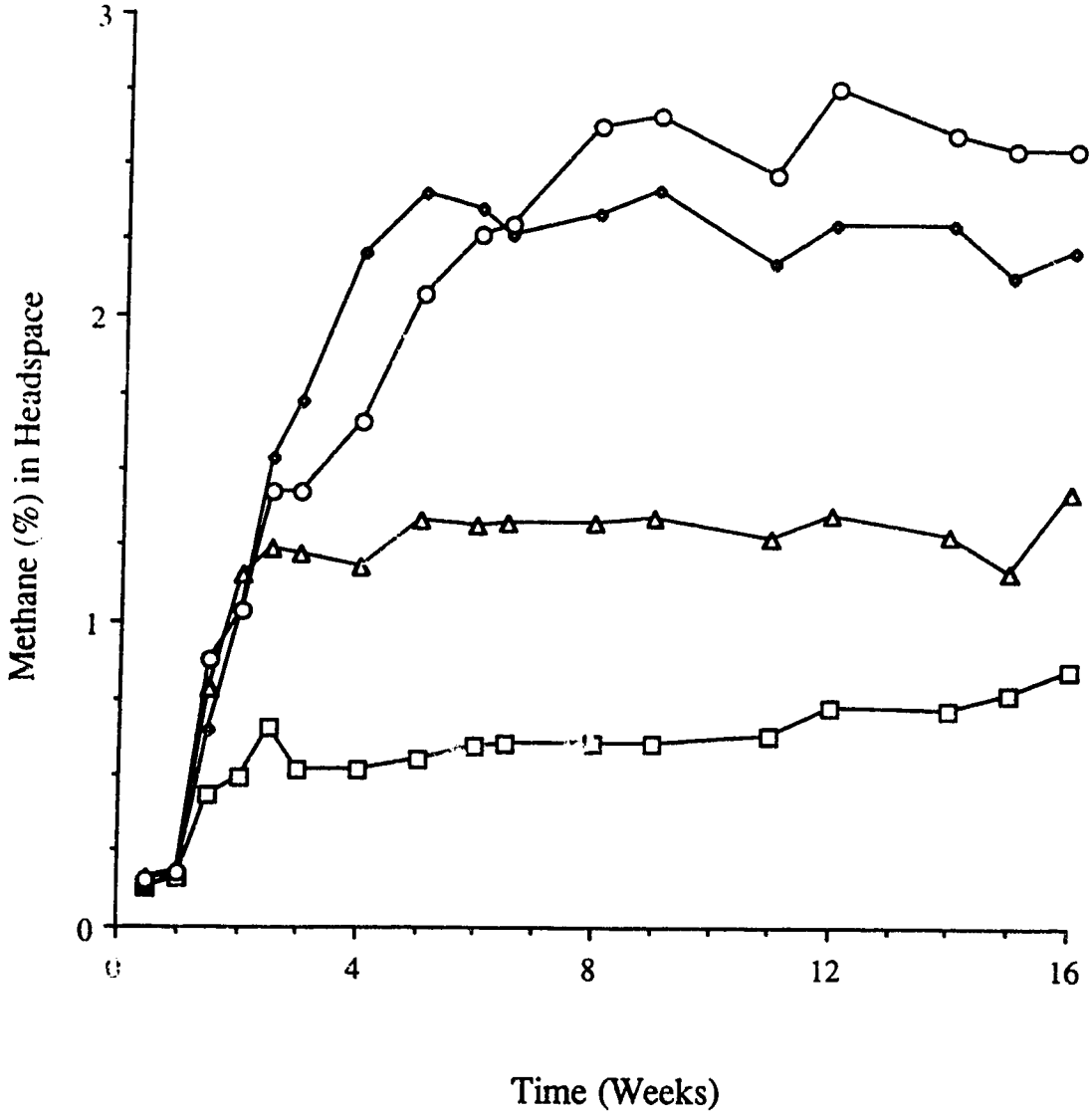


Figure 3.1-4: Effects of 2-methylbenzoic acid concentration on methane production in subcultures containing 0 mg/L (□), 50 mg/L (Δ), 100 mg/L (◊), or 200 mg/L (O) 2-methylbenzoic acid.

first set. However, regardless of the inocula or culture conditions used (Table 3.1-3), no 2-methylbenzoic acid degradation was observed. Neither 25% (v/v) nor 94% (v/v) cultures from any of the three sets of enrichment cultures were effective inocula, regardless of the length of incubation (22-45 weeks), or amount of 2-methylbenzoic acid previously degraded (100 mg/L, 200 mg/L, or 3 x 100 mg/L). None of the concentrations of 2-methylbenzoic acid tested (50-300 mg/L) were degraded within the 9-16 weeks that cultures were analyzed, even when gas volume was also determined. While it may appear that the higher Na_2S concentration used initially (2 mM in the medium) may have been a requirement, other subcultures (to be discussed later) had exhibited 2-methylbenzoic acid-degrading activity in media reduced with 0.5 mM or 1 mM Na_2S. In spite of repeated attempts, it was impossible to confirm the results of the first experiment, that higher concentrations of 2-methylbenzoic acid were inhibitory to the cultures.

3.1.2.2 Effects of various reducing agents on 2-methylbenzoic acid degradation

Experiments were designed to test the effect of different reducing agents on 2-methylbenzoic acid degradation, based on the observation that increased amounts of inoculum (94% (v/v)) had increased lag times prior to degradation. Roberts et al. (1988) had previously reported similar results in m-cresol-degrading cultures and proceeded to show that it was due to sulfide inhibition. Sulfide can originate from the sludge inoculum, but it is also routinely added at a concentration of 1 mM to reduce media prior to use. To test whether 2-methylbenzoic acid-degrading cultures were inhibited by sulfide, two concentrations of sulfide (1 mM and 0.5 mM) were compared with alternate reducing agents, iron nails, and titanium citrate, to determine whether sulfide affected methane production.

Several experiments comparing methane production in subcultures containing 2-methylbenzoic acid reduced with iron nails, titanium citrate, or Na_2S, to methane production in controls reduced with 0.5 mM Na_2S with no added substrate, clearly demonstrated enhanced methane production in the cultures reduced with nails or titanium citrate (Figure 3.1-5). However, when the 2-methylbenzoic acid in these cultures was measured by HPLC analysis, it was discovered that the increased methane

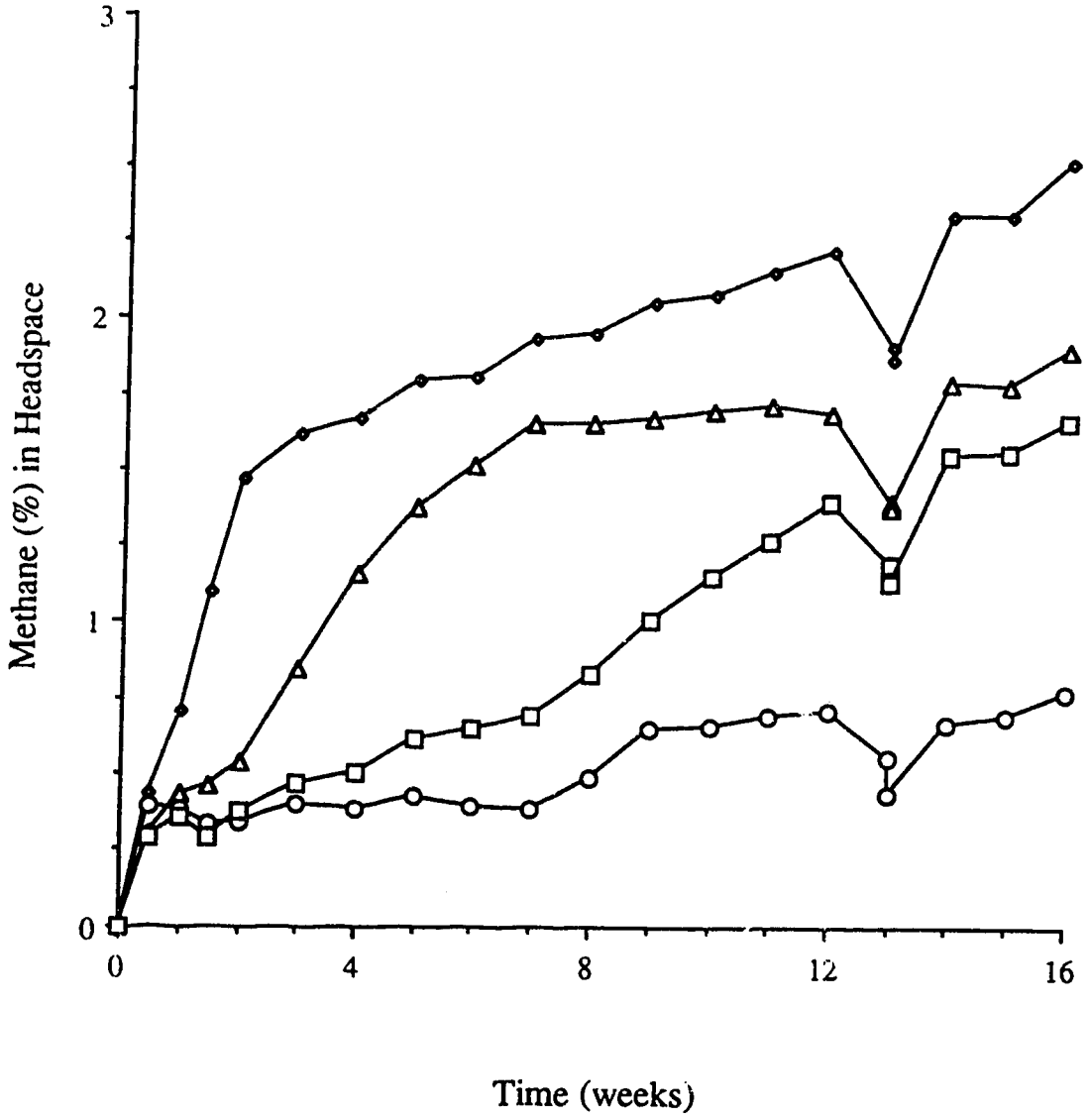


Figure 3.1-5: Effects of various reducing agents on methane production in 2-methylbenzoic acid-containing subcultures. Cultures contained 100 mg 2-methylbenzoic acid, with media reduced with iron nails (◊), titani. citrate (Δ), 0.5 mM Na_2S (□), or 1 mM (O) Na_2S.

in the iron- or titanium citrate-reduced cultures was not due to 2-methylbenzoic acid degradation.

Further experiments showed that the increase in methane production observed in cultures reduced with nails or titanium citrate were identical regardless of whether 2-methylbenzoic acid was added. Therefore, it was concluded that reducing the medium with these agents did not reduce the lag time prior to 2-methylbenzoic acid degradation. The reason for the increases in methane in the cultures reduced with iron nails could be the effect reported by Daniels et al. (1987), that methanogenic bacteria can use either pure elemental iron or iron in mild steel as a source of electrons in the reduction of CO_2 to CH_4, by a process called cathodic depolarization, in which electrons from iron and H^+ from water produce H_2, which is then released for use by the methanogens. As well, it was hypothesized that the methanogenic consortium could have metabolized the citrate present from the titanium citrate reducing agent.

To test whether these explanations were probable, hydrogen gas was added to cultures reduced with nails, and a solution of sodium citrate was added to cultures reduced with titanium citrate, after 2 weeks incubation when the initial gas production had plateaued. These triplicate cultures were then compared to triplicate unamended cultures. Figure 3.1-6 shows that there was a subsequent increase in the volume of methane in these cultures due to the addition of either hydrogen or citrate. Therefore, it was quite probable that these were the sources of the initial burst of methane production. Because this initial methane production would interfere with the detection of methane from the degradation of 2-methylbenzoic acid or other substrates, iron nails and titanium citrate are consequently not recommended for use as alternate reducing agents for subcultures derived from this sewage sludge.

Comparison of cultures reduced with either 1 mM or 0.5 mM Na_2S demonstrated that Na_2S was indeed inhibitory to 2-methylbenzoic acid degradation at the higher concentration. The recovery of methane from the degradation of 100 mg/L 2-methybenzoic acid was complete after 10 days when the medium was reduced with 0.5 mM Na_2S, and 16 days when 1 mM was used. In a subsequent experiment, two of the three replicates with media reduced with 0.5 mM Na_2S degraded 100 mg/L 2-methylbenzoic acid in 16 weeks, whereas no degradation was observed in

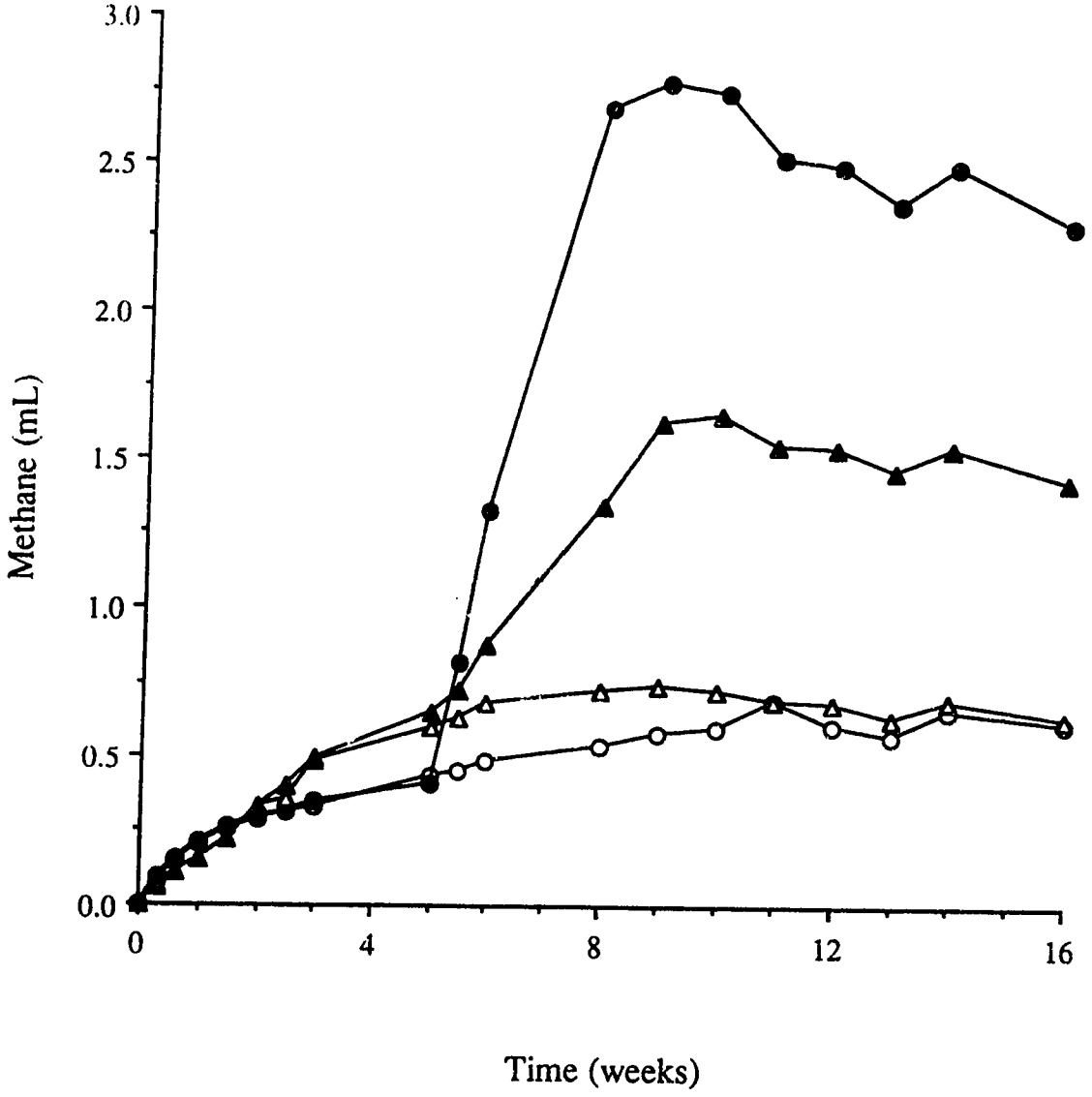


Figure 3.1-6: Methane production in 2-methylbenzoic acid-containing subcultures with feed solutions reduced with iron nails (○) or titanium citrate (△), compared to cultures to which hydrogen (●) or sodium citrate (▲) had been added after 5 weeks incubation.

cultures reduced with 1 mM Na_2S. It was concluded that reducing the medium with 1 mM Na_2S was inhibitory to 2-methylbenzoic acid degradation.

3.1.2.3 Ability of 2-methylbenzoic acid-degrading enrichment cultures to degrade other substrates

2-Methylbenzoic acid was detected as a transformation product from m-cresol (Roberts et al. 1990), so experiments were performed to determine whether 2-methylbenzoic acid-degrading cultures could also degrade m-cresol. Of the 12 subcultures that were amended with m-cresol, only one degraded the m-cresol. This degradation occurred after a 6 week lag, a typical lag period prior to m-cresol degradation in enrichment cultures (refer to section 3.4.1). Therefore, it was probable that this degradation was not related to the ability of the culture to degrade 2-methylbenzoic acid. Thus, it was concluded that 2-methylbenzoic acid degradation was not related to m-cresol degradation in these cultures.

2-Methylbenzoic acid was formed in the m-cresol-degrading cultures via a dehydroxylation of 4-hydroxy-2-methylbenzoic acid; the primary route of degradation for m-cresol was through this intermediate. Therefore, it was conceivable that the degradation of 2-methylbenzoic acid could also proceed via a ring hydroxylation yielding 4-hydroxy-2-methylbenzoic acid as the first metabolite. Several subcultures were amended with this compound to determine whether it could be degraded. In the first experiment, 100 mg/L 4-hydroxy-2-methylbenzoic acid was not degraded within 16 weeks, as evidenced by control levels of methane in the headspace, and by no decrease in substrate determined by HPLC analysis. Parallel cultures degraded 100 mg/L 2-methylbenzoic acid. In a subsequent experiment, 100 mg/L 4-hydroxy-2-methylbenzoic acid was not degraded within 11 weeks even though parallel cultures degraded benzoic acid. In one final experiment, the degradation of 100 mg/L 4-hydroxy-2-methylbenzoic acid, 20 mg/L 4-hydroxy-2-methylbenzoic acid, or 50 mg/L 4-hydroxy-2-methylbenzoic acid with 50 mg/L 2-methylbenzoic acid, were compared. It was postulated that at the lower concentrations, or in the presence of 2-methylbenzoic acid, 4-hydroxy-2-methylbenzoic acid could be more amenable to degradation. None of the cultures degraded 4-hydroxy-2-methylbenzoic acid, nor did they degrade 2-methylbenzoic acid,

so no definite conclusions could be drawn. In summary, 4-hydroxy-2-methylbenzoic acid was not degraded in 2-methylbenzoic acid-degrading cultures, and therefore is less likely to be an intermediate in its degradation.

Other substrates added to subcultures with 2-methylbenzoic acid-degrading cultures as inocula included benzoic acid, 3-methylbenzoic acid, and 4-methylbenzoic acid. Benzoic acid was degraded within 3 weeks, as evidenced by depletion of substrate and recovery of methane. No degradation was noted for either 3-methylbenzoic acid or 4-methylbenzoic acid, although parallel cultures did not degrade 2-methylbenzoic acid either.

3.1.2.4 Methane production in subcultures degrading 2-methylbenzoic acid

A total of sixty individual subcultures containing 2 methylbenzoic acid were analyzed for methane concentration in the headspace and total gas volume, in order to determine the actual volume of methane produced from 2-methylbenzoic acid degradation. A wide variety of 2-methylbenzoic acid concentrations, reducing agents, and types of inocula cultures were employed for these tests. Not one of these cultures actually degraded 2-methylbenzoic acid.

The transformation of other aromatic compounds such as phenol can be slowed or stopped if the methanogenic population is inhibited (Fedorak et al. 1986). Therefore, the effect of 2-methylbenzoic acid on the conversion of acetate to methane was studied by adding 500 mg/L of acetate to 2-methylbenzoic acid-containing subcultures which had not exhibited activity within 9 weeks incubation. The recovery of methane from the acetate was stoichiometric (Figure 3.1-7) in all subcultures to which the acetate was added. This indicated that the methanogenic component of the mixed culture was still active, and therefore it was the ability to degrade 2-methylbenzoic acid that was absent in these cultures.

However, the methane concentration in the headspaces of cultures that did degrade 2-methylbenzoic acid did exhibit an increase in the methane concentration in the headspace, which is a valid measure of methane production. The increase in methane in the headspace of 15 subcultures that degraded 2-methylbenzoic acid averaged 1.58% ± 0.30,

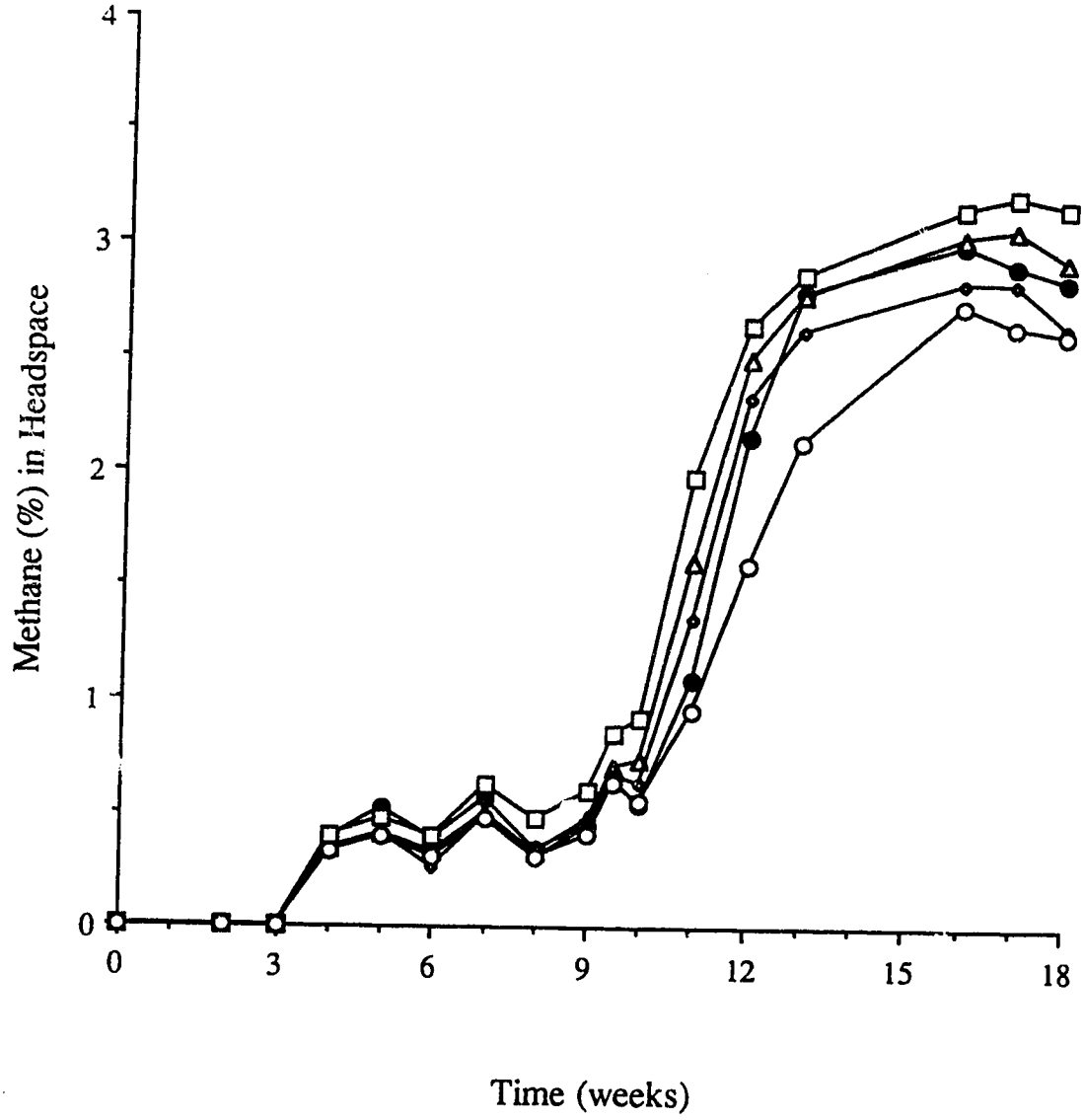


Figure 3.1-7: Methane production in subcultures containing 2-methylbenzoic acid. After 9 weeks incubation, 500 mg/L acetate was added to cultures. 2-Methylbenzoic acid concentration: 0 mg/L (●); 50 mg/L (Δ); 100 mg/L (□); 200 mg/L (◊); 300 mg/L (○).

which was very close to the 1.52% predicte[illegible] amount of 2-methylbenzoic acid added, based on Equation [illegible] his information provided further evidence that 2-methylbenzo[illegible]acid degradation was complete and conformed to the stoichiometric mineralization observed for other aromatic compounds.

3.1.3 Attempts to detect intermediates of 2-methylbenzoic acid degradation

Several approaches were used to search for metabolites in the pathway of 2-methylbenzoic acid degradation. The successful use of fluorinated analogues in phenol- and m-cresol-degrading cultures (sections 3.3 and 3.4), prompted the use of a fluorinated analogue in these cultures as well. Su[illegible]tants withdrawn from 2-methylbenzoic acid-degrading cultures at [illegible]f the draw-and-feed were analyzed for metabolites by two HPLC m[illegible] Supernatants and cultures were extracted, derivatives were made, and s[illegible]ples were ana[illegible]ed by GC-MS.

3.1.3.1 Use of a fluorinated analogue of 2-methylbenzoic acid

Fluorinated aromatic compounds have recently been used to elucidate the metabolic pathway of phenol degradation under methanogenic conditions (Sharak Genthner et al. 1989c, 1990). Replacement of a hydrogen with a fluorine has been widely used in other fields as a means of creating an analogue that can be analytically distinguished by the fluorine label, yet that is so structurally similar that biological systems will act upon the analogue without recognizing the difference. Sharak Genthner et al. (1990) discovered that their methanogenic consortia would transform 2-fluorophenol to 3-fluorobenzoic acid, and c[illegible]led that the pathway for phenol degradation involved a *para*-carboxylation followed by a dehydroxylation.

A fluorinated analogue of 2-methylbenzoic acid, 3-fluoro-2-methylbenzoic acid, was added to one of the enrichment cultures (from the second set) when it had degraded its substrate. This culture was monitored weekly by HPLC for substrate degradation and intermediate formation. However, there was no change in the 3-fluoro-2-methylbenzoic acid concentration and no transformation products or intermediates were observed over 48 weeks incubation.

The first attempt to test fluorinated 2-methylbenzoic acid in subcultures was inconclusive because 2-methylbenzoic acid was not degraded in the positive controls. For the second set of subcultures, the cultures that received 100 mg/L 2-methylbenzoic acid had enhanced methane production compared to controls without substrate. Cultures that received 100 mg/L 3-fluoro-2-methylbenzoic acid only had control levels of methane. The cultures that received 50 mg/L of 3-fluoro-2-methylbenzoic acid and 2-methylbenzoic acid had enhanced methane production similar to, but slightly slower than, cultures that received just 2-methylbenzoic acid. HPLC analyses of samples of these cultures revealed no metabolites, fluorinated or otherwise. In summary, 3-fluoro-2-methylbenzoic acid was not degraded, did not prevent 2-methylbenzoic acid degradation, and did not lead to the formation of fluorinated intermediates. 3-Fluoro-2-methylbenzoic acid did not act as either an analogue or as a potent inhibitor of 2-methylbenzoic acid degradation.

3.1.3.2 Analysis of supernatants of 2-methylbenzoic acid-degrading cultures

The supernatants withdrawn from cultures that had degraded 2-methylbenzoic acid were analyzed by HPLC. The analyses revealed small peaks that eluted at 2.4 min that are common to all sewage sludge cultures. No other compounds were detected in cultures from the first and second sets of enrichment cultures.

Routine HPLC analysis of weekly samples from the third set of 2-methylbenzoic acid-degrading cultures with a 70:30:0.5 CH_3OH:H_2O:H_3PO_4 mobile phase revealed the formation of a transient intermediate with a shorter retention time by HPLC than 2-methylbenzoic acid. A sample of culture supernatant from one of the cultures that had degraded most of its substrate was analyzed with a 30:70:0.5 CH_3OH:H_2O:H_3PO_4 mobile phase. Apart from a 2-methylbenzoic acid peak and a small resazurin peak, there was a small but definite peak at 3.15 min, the retention time of 4-hydroxy-2-methylbenzoic acid. Analysis of samples of the supernatants of all three controls showed that the controls had only very small peaks at 2.72 min, the common unknown peak observed from all sludge-based cultures. Therefore, the new compound was related to the 2-methylbenzoic acid degradation.

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