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THE UNIVERSITY OF ALBERTA

METHANOGENIC METABOLISM OF m-CRESOL

by

DEBORAH J. ROBERTS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

Edmonton, Alberta

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YEAR THIS DEGREE GRANTED: 1990

(SIGNED) Deborah Roberts

2445 105 St. Edmonton, Alberta

DATE: October 27, 1989

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P. Fedorak

Supervisor

Susan Krasny

External Examiner

Date Oct 26, 1989

Dedicated to my family

Especially to my parents

Donald McLean Roberts

and

June Roberts

and most of all my daughter

Brie-anne Heather Roberts

for all their love and support.

ABSTRACT

The methanogenic metabolism of m-cresol was examined using the techniques of anaerobic microbiology, analytical chemistry and chemical synthesis. The use of different culture methods to obtain m-cresol-degrading enrichment cultures from anaerobic sewage sludge showed that m-cresol degradation but not methane production was inhibited by concentrations of soluble sulfide as low as 0.5 mM.

The m-cresol-metabolizing consortium contained three nutritional types of bacteria. Each type had a distinct cellular morphology. The hydrogen-utilizing methanogens were long, slender, rods that were capable of UV fluorescence. The acetate-utilizing methanogens were encased in very long, broad, flat-ended sheaths. These organisms did not possess strong UV fluorescence capabilities. The proposed m-cresol-metabolizing members of the consortium were short, fat, Gram-negative rods. The acetate-utilizing methanogens appeared to be the most prevalent organisms in the consortium, the m-cresol-metabolizing bacteria the second most prevalent and the hydrogen-utilizing methanogens the least prevalent in the consortium.

The presence of 220 mg/L m-cresol was seen to moderately inhibit the methanogenic members of the consortium whereas the m-cresol-metabolizing members of the consortium withstood 710 mg/L m-cresol but could not degrade all of the m-cresol present without an active methanogenic population. The consortium could not use sulfate, thiosulfate, nitrate or oxygen as alternate electron acceptors during m-cresol metabolism.

A comparison of the fates of the methyl carbons of m- and p-cresol in methanogenic consortia was performed using $^{14}CH_3$-m-cresol and $^{14}CH_3$-p-cresol. The majority of the methyl carbon of m-cresol was converted to methane whereas the majority of the methyl carbon of p-cresol was oxidized to carbon dioxide.

The examination of the metabolic intermediates of m-cresol degradation showed that an initial step in m-cresol metabolism was the carboxylation of the ring to produce

4-hydroxy-2-methylbenzoic acid. This compound was presumed to be degraded by reductive ring fission and β-oxidation reactions. A total of 4.13 mol of methane were produced for each mol of m-cresol degraded. In bromoethanesulfonic acid-inhibited cultures, 4 mol of acetate were formed per mol of m-cresol metabolized.

When bromoethanesulfonic acid-inhibited cultures were incubated with $^{14}CH_3$-m-cresol, ^{14}C-methyl-labeled acetate accumulated. The ^{14}C-label from $H^{14}CO_3^-$ was incorporated into 4-hydroxy-2-methylbenzoic acid and was found as the carboxyl carbon of acetate. 2-Methylbenzoic acid was also detected in the cultures but was determined to be a dead-end product.

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LIST OF ABBREVIATIONS

ACS	Aqueous Counting Scintillant (Amersham)
AFS	amorphous ferrous sulfide
BESA	bromoethane sulfonic acid
F_{420}	5-diazaflavin (a cofactor involved in electron transport in methanogenesis).
GC	gas chromatography
GPC	gas proportional counter
HPLC	high performance liquid chromatography
LSC	liquid scintillation counting
MS	mass spectrometry
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
PCA	plate count agar
SRB	sulfate-reducing bacteria
TLC	thin layer chromatography
U	uniformly labeled
UV	ultraviolet

1. Introduction

The microbial degradation of xenobiotic compounds is one mechanism of removing these compounds from the environment. The last ten years have seen a steady increase in the number of literature reports of the microbial degradation or detoxification of many of the compounds introduced to the environment by man. These reports have shown many xenobiotic compounds are biodegradable but there is still much work to be done to ensure the preservation of our environment.

The compound m-cresol is a common constituent of industrial wastes. Reports have also indicated that m-cresol has been produced by the fungus *Valsa freisii* and has been detected as an intermediate in the patulin biosynthesis pathway of *Penicillium urticae*. There have been no reports of the accumulation of m-cresol in pristine environments, indicating the small amounts of m-cresol produced by fungi are not an environmental concern.

There are, however, reports of the accumulation of m-cresol in environments polluted by man. This accumulation occurs when pollution exceeds the environment's natural ability to deal with this compound. m-Cresol is suspected to be a toxic compound and causes taste and odor problems at the μg/L level in waters receiving industrial wastes.

The aerobic fate of m-cresol has been well documented but there is little information on its fate in anaerobic environments. These environments, such as aquifers, sediments and sewage digestors, are commonly found to accumulate compounds that are not metabolized anaerobically.

The reports found in the literature concerning the amenability of m-cresol to anaerobic degradation are often conflicting. The general consensus is that if m-cresol degradation occurs at all in anaerobic conditions, long periods of adaptation are required. Other related compounds such as p-cresol and phenol have not been observed to require

these long adaptation times, while o-cresol was even less amenable to degradation under anaerobic conditions. The position of the methyl group on the aromatic ring of phenolic compounds greatly affects the fate of the compound in the environment.

The conflicting reports on m-cresol degradation may be due to the use of different inocula and culture methods. An investigation of culture methods for the growth of methanogenic m-cresol-degrading consortia may provide information for a more efficient treatment method for waste-waters containing this compound. This information may also be applied to other culture methods and, perhaps, allow the enrichment of cultures capable of degrading other compounds that to date have not been seen to be amenable to methanogenic breakdown.

The objectives of the study were: (A) to determine reliable culture methods to obtain and maintain active methanogenic m-cresol-degrading consortia and (B) to use these cultures as inocula to study the metabolic intermediates of this degradation as well as define the microbial interactions involved in m-cresol degradation.

2. Literature Review

The literature review covered five topics relevant to the scope of the study. These topics were the occurrence of cresols in the environment, whether from industrial or biological sources; the fate of m-cresol in these environments; characteristics of methanogens and methanogenic degradations; the methods used to study anaerobic degradations; and the pathways of aerobic and anaerobic metabolism of selected aromatic compounds. The main emphasis of the discussion of the literature will be placed on information about m-cresol but some discussion will deal with cresols or aromatic compounds in general in order to provide a background for analysis of the results to be presented.

2.1 Occurrence of Cresols in the Environment

2.1.1 Cresols in Industrial Wastes

The last twenty years have seen an increase in the technology used to analyze and understand the problems and hazards associated with the waste-waters generated by organic chemical industries. The advances in analytical technology for the identification of organic compounds in waters and waste-waters have increased the awareness of the scientific and industrial communities to the many organic pollutants found in waste-waters. Numerous publications are available in the literature concerning the organic makeup of individual industrial effluents especially those of the newer industries such as the synthetic fuels industry. Review articles published by De Renzo (1980) and Neufeld (1984) summarize the constituents of many waste-waters. These articles present the results for classes of compounds (such as phenolic compounds) as they occur in waste-waters of different types of industries.

Phenolic-rich waste-waters can originate from five major industries. These are: petroleum refining, organic chemicals and synthetics production, pulp and paper production, coal conversion and steel milling, and the textiles industry (De Renzo 1980). The reports concerning concentrations of organic chemicals in individual waste-waters often list all phenolic compounds as a group rather than as individual phenolic compounds. The Environmental Sources and Emissions Handbook (Sittig 1975) listed several examples of concentrations of phenolic compounds in waste-waters from industrial sources. These included up to 3,900 mg/L in ammonia liquor from coke ovens; up to 185 mg/L in sour water from oil refineries; up to 600 mg/L in general petrochemical wastes; and, among others, up to 6,000 mg/L in a stocking factory waste. These values were reported as concentration of phenol or phenolic compounds.

Neufeld (1984) summarized the individual constituents of waste-waters produced in the synthetic fuels industry. The summary indicated that the cresols as a group were found in almost all waste-waters from the synthetic fuels industries and ranged in concentration from 20 mg/L (an oil shale waste-water) to as much as 3,600 mg/L (a Synthane waste-water). The cresols were often the main constituents of some of these waste-waters.

Cresols were also found in creosotes distilled from wood tar. Creosotes were used as wood preservatives and constituted a large industry in North America. Materials from creosotes have contaminated ground water at several places in the United States leaving large areas of contamination to be dealt with by the environment (Ehrlich et al. 1982).

2.1.2 Cresols from Biological Sources

Cresols are widely distributed in the natural environment as constituents of essential oils but are rarely produced as free metabolites (Child et al. 1969). There have been no reports of cresols accumulating in the environment in pristine areas but there

have been some reports of cresol production by laboratory cultures. Phenol and p-cresol have been reported as products or intermediates during tyrosine degradation (Updegraff 1949, Balba and Evans 1980a) and p-cresol has been found in the defensive secretions of millipedes (Eisner et al. 1963). Phenol has been found to be produced by *Aerobacter* strains when grown on casein digest (Williams and English 1950) or on a defined medium containing p-hydroxybenzoic acid (Patel and Grant 1969). Phenol was found to be a constituent in the needles and cones of *Pinus sylvestris* (Harborne and Simmons 1964).

The fungus *Valsa friesii* has been found to produce the compound m-cresol in significant amounts as a free metabolite. Child et al. (1969) found that *V. friesii* produced m-cresol up to 160 mg/L when grown on glucose phytone broth. The production of m-cresol was improved to 1 g/L by incubation of the fungal culture with shaking at 28°C in the dark in a defined phosphate buffered medium with glucose as the carbon source. *V. friesii* was found to be tolerant to higher concentrations of m-cresol than were other fungi. This indicated an unusual resistance to this antimicrobial agent. There was no discussion as to the mechanism of m-cresol synthesis or possible advantages to the organism from the production of this compound (Child et al. 1969, Child and Haskins 1970).

Another fungus, *Penicillium urticae,* produced m-cresol as an intermediate during the production of patulin. Patulin is a potent antibiotic and mycotoxin produced by a variety of fungi. The first committed pathway metabolite was determined to be 6-methylsalicylic acid (2-hydroxy-6-methylbenzoic acid) which was formed from acetyl-CoA and malonyl-CoA. 6-Methylsalicylic acid was converted to m-cresol by the enzyme 6-methylsalicylic acid decarboxylase. m-Cresol was then oxidized through a number of steps to gentisaldehyde which underwent further transformations to the final product patulin (Gaucher et al. 1981).

It appears that phenolic compounds can be found in the environment due to both

biological and industrial activities. The presence of phenolic compounds due to biological processes has not been observed to cause environmental problems while the presence of phenolic compounds due to pollution from industrial sources has been seen as a threat to the environment and has merited legislative action to control the content of phenolic compounds in industrial effluents.

2.1.3 Consequences of Cresols in the Environment

The literature concerning the toxicity of m-cresol or cresols in general has not been explicit about the actual affects these compounds have on biological systems. Krenkel (1974) stated phenols are objectionable in respect to taste and odor in amounts as low as 0.001 mg/L in a water supply. The United States Public Health Service has limited allowable phenolic compounds in water supplies to this level. The Occupational Health and Safety and the American Conference of Governmental Hygenists have set the threshold limit value for skin exposure of m-cresol to 5 mg/L. In the United States of America, m-cresol is monitored under the Toxic Substances Control Act; the Clean Water Act; the Clean Air Act; the Comprehensive Environmental Response Compensation and Liability Act; and the Superfund Amendments and Reauthorization Act. Guidelines for the transportation of m-cresol are set out by the Department of Transportation Hazardous Materials Act and m-cresol is on the list of the National Toxicology Testing Program as an agent to be tested (Clansky 1987).

Phenolic compounds are used as antiseptics in cleaning solutions and are antimicrobial agents. Child and Haskins (1970) found that the growth of some fungi was inhibited by 250 mg/L m-cresol. The inhibition of the natural microbial communities in the environment by m-cresol would effect the degradation of natural substrates present in the environment. The accumulation of other compounds would then occur and the natural turnover of organic matter would be upset.

Large quantities of organic compounds in waste-waters, no matter whether toxic or not, must be treated before the waste-water is released to the environment to avoid causing depletion of the dissolved oxygen in the receiving waters. Action of the aerobic heterotrophs on the organic compounds would remove oxygen from receiving waters, causing anaerobic conditions. The study of the degradation of m-cresol will help to determine its fate under environmental conditions and in waste-water treatment facilities.

2.2 Biodegradation of m-Cresol in Aerobic Environments

The ability of aerobic soil microorganisms to metabolize cresols has been demonstrated by many workers (Tabak et al. 1964). Of the aerobic bacteria, *Achromobacter*, *Micrococcus*, *Xanthomonas*, *Vibrio*, *Flavobacterium* and *Pseudomonas* species have been shown to be capable of oxidizing the aromatic ring of nitrophenols, chlorophenols, alkylphenols, arylphenols, hydroxyphenols and phenol (Tabak et al. 1964).

Dobbins and Pfaender (1988) used the respiration and uptake of labeled substrates by pristine soil samples from the subsurface environment as indicators of the ability of soil microorganisms to metabolize natural amino acids and m-cresol. The results for m-cresol metabolism indicated that, in unsaturated zones, the first order rate constant (K_1) was similar for both uptake and respiration. In saturated-soils uptake was comparable to the uptake in unsaturated zones but respiration was as much as 50 times less than uptake. The uptake and respiration of amino acids in both saturated and unsaturated zones were at least ten-fold greater than that for m-cresol. These results suggested a recalcitrance of m-cresol to aerobic degradation, especially in samples from saturated-soils, that was not observed with amino acid degradation. Dobbins and Pfaender (1988) did not suggest whether anaerobic mechanisms such as fermentation occurred in the saturated samples. The higher levels of uptake as compared to low levels of respiration could indicate that other metabolic mechanisms were taking place.

Palumbo et al. (1988) examined the response of coastal and estuarine environments to pollution with m-cresol and nitrilotriacetic acid (NTA). The work evaluated the effect of environmental conditions on the degradation of the two compounds by unadapted populations of heterotrophic aerobic bacteria. The uptake and respiration of radiolabelled compounds in samples taken from a freshwater site in the Mississippi river, an estuarine site and a high-salinity offshore site were examined over an 18 month period. The results showed that m-cresol uptake by the natural population was maximal from freshwater samples in the summer, although the bacterial numbers and activity were highest in the estuarine site. There was no significant correlation between the uptake of m-cresol and any of the indicators of biological activity, temperature, soluble reactive phosphorous, or total particulate concentration. Palumbo et al. (1988) reported that m-cresol uptake was greatly reduced at increasing salinity. In the estuarine areas where the bacterial numbers were greatest, the uptake of m-cresol was inhibited by the increasing salinity.

The turnover time for m-cresol at its maximum rate, as determined from the kinetic parameters, was 1-3 d. This was calculated to be fast enough to significantly effect the fate of low levels of m-cresol (1-10 µg/L) during its passage through the freshwater and estuarine environment which had a mean flushing time of 5 d. For higher concentrations of m-cresol (70 µg/L) an estimated turnover time of 100-1,000 d was expected. During colder periods and in the more saline environments, the degradation of even low concentrations of m-cresol was not expected to significantly alter its fate in the environment (Palumbo et al. 1988).

Pollutant chemicals that escape biodegradation by microbial populations from surface environments make their way into the subsurface environment through natural seepage resulting in polluted ground water and aquifers. Swindoll et al. (1988) examined the aerobic biodegradation of amino acids, as model natural compounds, and several pollutant compounds such as m-cresol by subsurface microbial communities.

The study used the conversion of radiolabelled substrate to $^{14}CO_2$ as well as the incorporation of label into biomass as indicators of biodegradation. Samples of pristine aquifer solids were used as inoculum.

Results indicated that subsurface communities were able to degrade both natural and xenobiotic compounds. The total metabolism of natural organic compounds (amino acids) was found to become saturated at low concentrations (10-20 µg/g) whereas that of xenobiotic compounds was not. In the case of ring-U-^{14}C-m-cresol there was no saturation observed for either uptake or respiration (up to 700 µg/g tested). The calculated turnover time for m-cresol at a concentration of 0.5 ng/g soil was 1,370.3 h and the calculated turnover time for amino acids at this concentration was 46.9 h. There were 100-fold differences between the first order rate constants observed for the uptake and respiration of m-cresol and those for amino acids. The first order rate constant for respiration of m-cresol was 51.1×10^{-5} h^{-1} and for uptake was 23.4×10^{-5} h^{-1}. The first order rate constant for respiration of amino acids was 11.3×10^{-3} h^{-1} and for uptake was 2.56×10^{-3} h^{-1}(Swindoll et al. 1988).

These results suggested that the subsurface microorganisms were adapted to relatively high concentrations of organic compounds. At very low environmental concentrations the organisms would be functioning at rates far below the maximum potential for the enzymes they possess (Swindoll et al. 1988). It was suggested that, because the input of chemicals into the environment is discontinuous, it could be an advantage for an organism to possess an enzyme system capable of responding to high concentrations when they occurred. This study gave no indication of the residence time of the ground water in the aquifer so an estimate of the effect of biodegradation on the fate of m-cresol in this environment cannot be made. The results do indicate that aerobic heterotrophic communities from pristine environments are capable of metabolizing m-cresol.

The studies described above have used very low concentrations of cresols relative to those found in industrial effluents. The higher levels of cresols found in some industrial effluents require treatment not only because cresols are toxic at these concentrations, but to ptorect the receiving waters from the taste and odor problems associated with phenolic compounds. It is also important to remove the biological oxygen demand that the oxidation of the high levels of these compounds would place on the receiving waters.

2.3 An Introduction to Anaerobic Environments

Anaerobic environments are created when oxygen consumption exceeds its supply. Examples of anaerobic environments are; soils with impeded drainage, stagnant water, municipal landfills, sewage treatment digestors, the alimentary tract of all animals, and sediments of oceans and other natural bodies of water (Evans and Fuchs 1988). The degradation of organic compounds in these environments depends on the availability of inorganic electron acceptors such as ferric iron, nitrate, sulfate or carbon dioxide and the ability of the microbial populations present to use these electron acceptors. Anaerobic degradation of organic compounds may also occur under photometabolic or fermentative conditions. There is usually a separation of dominant metabolic processes depending on the availability of electron acceptors, the presence of suitable microorganisms and the energetic benefit of each process to the microbial communities involved (Suflita et al. 1988). These factors must be considered when attempting to describe the capabilities of an anaerobic environment to metabolize pollutant chemicals.

The thermodynamic equations as presented by Young (1984) and Thauer et al. (1977) demonstrate the relative energetics of the use of different terminal electron acceptors.

$NO_3^- + 2\ H^+ + 4\ H_2 \rightarrow NH_4^+ + 3\ H_2O$ $\Delta G^{\circ\prime} = -599.6$ kJ/mol Eqn. 2.1

$2\ Fe^{3+} + H_2 \rightarrow 2\ Fe^{2+} + 2\ H^+$ $\Delta G^{\circ\prime} = -228.3$ kJ/mol Eqn. 2.2

$SO_4^{2-} + 2\ H^+ + 3\ H_2 \rightarrow H_2S + 3\ H_2O$ $\Delta G^{\circ\prime} = -151.9$ kJ/mol Eqn. 2.3

$HCO_3^- + H^+ + 4\ H_2 \rightarrow CH_4 + 3\ H_2O$ $\Delta G^{\circ\prime} = -135.6$ kJ/mol Eqn. 2.4

The large negative free energy of reaction for nitrate reduction (Eqn. 2.1) indicates the reaction would proceed more readily than ferric iron, sulfate or carbonate reduction (Eqns. 2.2-2.4 respectively). If coupled to an ATP-generating system the reduction of nitrate would yield more energy than the other systems. The methanogenic reduction of bicarbonate would yield the least amount of energy/mol of any of the anaerobic terminal reductions.

2.4 Anaerobic Degradation of Aromatic Compounds

Several classes of aromatic compounds have been shown to be degraded under anaerobic conditions by pure cultures or mixed cultures of anaerobic bacteria. These include hydrocarbons such as toluene, benzene, naphthalene; oxidized aromatic compounds such as phenolic compounds and benzoic acids; sulfur heterocycles such as benzothiophene and dibenzothiophene; and nitrogen heterocycles such as quinoline (Grbic´-Galic´ in press).

This review of the anaerobic degradation of aromatic compounds will be limited to a discussion of the degradation of benzoate, phenol and the cresols. The information will be considered according to the terminal electron acceptor used during the metabolism - nitrate, ferrous iron, sulfate or carbon dioxide.

2.4.1 Nitrate-Reducing Environments

Nitrate-reducing organisms are likely to be found in soil, freshwater and marine environments (Young 1984). The anaerobic nitrate reduction process can be

respiratory in which nitrogenous oxides, principally nitrate and nitrite, are reduced to dinitrogen gases, N_2O or N_2 (respiratory nitrate reduction); or are reduced to ammonium (dissimilitory nitrate reduction to ammonia). An excellent review on the biochemistry of these processes is given by Tiedje (1988).

Williams and Evans (1975) examined a nitrate-reducing species of *Moraxella* that was capable of metabolizing benzoate and several other aromatic compounds and found that it could not metabolize phenolic compounds. Bakker (1977) demonstrated that a mixed culture consisting of mainly *Pseudomonas* and *Spirillum* species could degrade the cresols under nitrate-reducing conditions. Bossert et al. (1986) isolated two bacterial species capable of utilizing p-cresol in coculture under nitrate-reducing conditions. This coculture could not utilize the other isomers of cresol.

Tschech and Fuchs (1987) isolated two pure cultures of facultatively anaerobic bacteria (designated K172 and S100) capable of the degradation of phenolic compounds under nitrate-reducing conditions. Of these two strains, only S100 was capable of utilizing m-cresol whereas both strains could utilize p-cresol. Neither strain could utilize o-cresol.

From these limited number of studies, it appears that the utilization of m-cresol by nitrate-reducing microorganisms is not a common phenomenon. The majority of the studies performed have examined the ability of organisms enriched from the environment on benzoate or phenol to degrade other compounds. Perhaps, inocula from environmental samples would provide a better test for the ability of nitrate-reducing organisms to degrade m-cresol or other compounds of interest.

2.4.2 Ferric Iron-Reducing Environments

In most freshwater environments, ferric iron is the most abundant potential electron acceptor for the oxidation of organic compounds (Lovley et al. 1989). Although some organic compounds, such as catechol, have been shown to be oxidized

by ferric iron in the absence of biological activity it has recently been shown that microbial activity was essential for the coupling of ferric iron reduction to the oxidation of most aromatic compounds in aquatic sediments.

This group examined a glacial outwash aquifer that had been contaminated with crude oil from a pipeline break. It was found that the concentrations of compounds leaching into anaerobic ground waters from the spill area decreased rapidly along the ground water flow path. The decrease in concentration of isomeric alkylbenzenes occurred at different rates indicating that more than simple physical-chemical processes were involved. The concentration of ferrous iron increased and the concentration of ferric iron decreased over time in the contaminated ground water, but not in uncontaminated areas, indicating that the reduction of ferric to ferrous iron was occurring. When the concentration of ferric iron was reduced sufficiently, methane production began, indicating a sequential use of terminal electron acceptors. There were insufficient concentrations of nitrate or sulfate for the use of these electron acceptors to be considered feasible.

Inocula from a site demonstrating active iron reduction was used to isolate an organism, GS-15, which could obtain energy for growth by oxidizing benzoate, toluene, phenol and p-cresol with ferric iron as the sole electron acceptor. There was no mention of the ability of this organism to degrade m-cresol although enrichment cultures were obtained from the same inoculum that could degrade a wide variety of aromatic and other organic compounds (Lovley et al. 1989).

2.4.3 Sulfate-Reducing Environments

The utilization of aromatic compounds by sulfate-reducing organisms would be of significance in marine anaerobic environments where sulfate is usually not limiting (Sleat and Robinson 1984). The microbial reduction of sulfate occurs in stepwise reactions through sulfite to sulfide. A comprehensive discussion of the biochemistry

and microbiology of sulfate reduction can be found in The Sulfate Reducing Bacteria (Postgate 1984).

Until recently, it was believed that sulfate-reducing bacteria (SRB) could only utilize very simple organic molecules such as pyruvate and lactate. In addition, the majority of SRB utilize hydrogen as a substrate (Postgate 1984). Studies using pure cultures or cocultures of SRB have shown that aromatic compounds can be utilized. Balba and Evans (1980c) found that a coculture of *Pseudomonas aeruginosa* and *Desulfovibrio vulgaris* could utilize benzoate. Mountfort and Bryant (1982) also characterized a sulfate-reducing coculture capable of utilizing benzoate. Bak and Widdel (1986) isolated *Desulfobacterium phenolicum*, the first obligate anaerobe that completely oxidizes phenol with sulfate as a terminal electron acceptor.

Studies concerning the degradation of phenolic compounds by inocula from sulfate-reducing environments have also been performed. Bak and Widdel (1986) cultured sulfate-reducing organisms capable of degrading p- and o-cresol from anaerobic marine sediments. There was no indication that the inoculum was tested against m-cresol. Smolenski and Suflita (1987), and Suflita et al. (1988) examined the ability of inocula from a shallow anaerobic alluvial sand aquifer to degrade the three cresol isomers. Inocula from two areas adjacent to a municipal landfill site were collected and used in the study. One site had sulfate concentrations ranging from 2-11 mM while the other had no detectable amounts of sulfate (Smolenski and Suflita 1987).

Inocula from each site were incubated with each of the three cresols to determine whether these substrates could be utilized. These incubations showed that the inocula from either site could degrade all three isomers of cresol but different lag times were observed before the substrates were metabolized. It was generally observed that lag times were shorter under sulfate-reducing conditions than for methanogenic conditions, possibly reflecting the thermodynamic favorability of the reduction of sulfate over the reduction of carbon dioxide. Lag times observed for the sulfate-reducing inoculum

were less than 10 d for p-cresol metabolism, m-cresol metabolism occurred after 43 d and o-cresol metabolism was not observed until after at least 90 d incubation (Smolenski and Suflita 1987).

It appears from these studies that nitrate and sulfate were both capable of serving as terminal electron acceptors for the degradation of phenolic compounds. Perhaps the addition of nitrate or sulfate to polluted anaerobic environments could increase the capability of these environments to degrade phenolic compounds (Suflita et al. 1988).

2.4.4 Methanogenic Environments

The methanogenic process involves the production of methane from carbon dioxide and hydrogen or small organic compounds such as acetate, methanol and methylamines. Methanogens do not utilize complicated organic molecules, but instead, form the last link in a chain of organisms that ferment more complex substrates to simple organic acids, which are mineralized to methane and carbon dioxide by the methanogens. Methanogenic consortia degrading complex substrates are, of necessity, mixed cultures of fermentative microorganisms and methanogens.

There have been reports on the ability of methanogenic consortia to produce methane from aromatic compounds since 1906 (Soehngen) cited by Evans (1969). Recent reviews on the subject include Young (1984), Sleat and Robinson (1984) and Berry et al. (1987). This section of the literature review will be restricted to studies concerning the ability of inocula from methanogenic environments to degrade phenol and the cresol isomers.

Chmielowski et al. (1965) examined the ability of anaerobic sewage sludge enrichment cultures to metabolize phenol and the cresols by gradual introduction of the substrate into methanogenic fermentation chambers. The results showed that phenol and p-cresol underwent total mineralization to CH_4 and CO_2, whereas m- and o-cresol were resistant to degradation under these conditions.

Ehrlich et al. (1982) examined the concentrations of phenolic contaminants in ground water samples from a coal-tar distillation (creosote production) and wood treatment plant in St. Louis Park, Minnesota. The phenolic compounds were present in concentrations of 30 mg/L at the source of contamination but were reduced to less than 0.2 mg/L at a distance of 430 m immediately downgradient from the source. There was very little sorption of phenolic compounds to the alluvial sand and dilution alone could not account for the disappearance of the phenolic compounds. Methane was found in soil zones contaminated with 2-20 mg/L phenolic compounds but not in uncontaminated soils. Methanogenic bacteria were also found in samples from contaminated areas and not in samples from uncontaminated areas. Methane was produced by laboratory cultures of contaminated water inoculated with bacteria from the contaminated zone. These results were interpreted as an indication that the removal of phenolic compounds from the contaminated ground waters was carried out by methanogenic degradation (Ehrlich et al. 1982).

Horowitz et al. (1982) tested the ability of inocula from freshwater eutrophic lake sediments and two anaerobic municipal sewage sludges to metabolize several aromatic compounds. The freshwater sediments did not mineralize the three cresol isomers over a 29-week incubation period. The studies using municipal sludges showed that the compounds were degraded after various acclimation times. p-Cresol generally had the shortest lag times (3-4 weeks). m-Cresol lag times were intermediate (4-5 weeks). o-Cresol was indicated to be a persistent compound in all three habitats whereas m- and p-cresol were considered to be metabolized.

Boyd et al. (1983) tested the ability of an anaerobic sewage sludge to degrade phenolic compounds and found that 50 mg/L m-cresol was slowly metabolized to methane and carbon dioxide after a 4-week acclimation period. Fedorak and Hrudey (1984) also tested the ability of an anaerobic sewage sludge inoculum to metabolize m-cresol and found that it was not metabolized during a six week incubation period.

Shelton and Tiedje (1984a) tested the ability of nine anaerobic sewage sludges to metabolize several aromatic compounds. The results showed that p-cresol was metabolized to methane by all of the sludges whereas m-cresol was only metabolized to methane by half of the sludges tested in the 8 week incubation period. Lag times of at least 1 week were observed for p-cresol degradation. The lag time before m-cresol degradation began was not presented.

Young and Rivera (1985) examined the ability of inocula from a municipal sewage treatment plant digestor to metabolize several aromatic compounds. The initial degradation of the substrates required different lengths of incubation for each substrate. Phenol was metabolized completely by 12 d whereas the degradation of p-cresol required a 25 d incubation period. It was seen that complete p-cresol mineralization did not occur until the microbial community had been acclimated to the compound by repeated feedings over a period of about 6 months. Results also showed that m-cresol was metabolized by the sludge inoculum but no details were presented (Young and Rivera 1985).

Smolenski and Suflita (1987) examined the biodegradation of o-, m- and p-cresol in samples from a sulfate-reducing environment (see section 2.4.3) and a methanogenic environment. The results obtained were in agreement with those of Horowitz et al. (1982). Under methanogenic conditions p-cresol was the first isomer to be degraded (46 d lag), m-cresol the second (46-90 d lag) and the o-cresol was not degraded during a 90 d incubation period.

The reports in the literature concerning the degradation of cresols indicate that while p-cresol was considered to be generally amenable to metabolism to methane by methanogenic consortia the metabolism of m-cresol to methane was not universally observed and occurred at different rates with different lag times for different inocula.

2.4.5 Proposed Use of Methanogenic Processes for Treating Phenolic Waste-waters

The methanogenic process is considered as a desirable process for treating industrial waste because it does not require energy input for aeration, it does not produce large amounts of biomass for disposal and, most importantly, methane is produced. Methane can be used as a fuel source to offset the demands of the process and could be a source of revenue in times when non-renewable fuel reserves have been depleted.

Although no full scale anaerobic processes have been used to treat phenolic waste-waters, there have been studies concerning the methanogenic treatment of these waste-waters with laboratory-scale methanogenic bioreactors. These have been reviewed by Fedorak and Hrudey (1988). Many of the studies concerning anaerobic degradation of phenolic waste-waters have incorporated activated carbon in the test reactors (Cross et al. 1982, Harper et al. 1983, Suidan et al. 1983a, 1983b). The actual toxicity of the individual organic compounds in the waste-waters is difficult to determine under these conditions because the relationship between adsorption onto the carbon and biological activity has not been fully defined. Fox et al. (1988) used activated carbon in reactors fed a synthetically prepared coal conversion waste-water. They determined that o- and m-cresol resisted biodegradation and were toxic to the anaerobic culture even though activated carbon was present in the reactor. Their results indicated that phenol and p-cresol were degraded by the culture.

In studies of the ability of cultures that were not amended with activated carbon the toxicity of phenolic compounds is more clear. Fedorak and Hrudey (1986a) tested the ability of methanogenic consortia to degrade phenolic compounds in a waste-water from the H-coal liquefaction process. Their results showed that although phenol, p- and m-cresol could be removed from this waste-water, m-cresol would eventually begin to accumulate indicating a loss in the ability of the culture to degrade this

compound.

Blum et al. (1986) tested the ability of batch cultures in serum bottles and anaerobic filters, not amended with activated carbon, to degrade constituents of a coal conversion waste-water. The results showed that serum bottle cultures degraded 91% of 1000 mg/L phenol over the 35 week incubation whereas anaerobic filters with 18-h hydraulic retention times degraded 99% of 1890 mg/L phenol. Concentrations of 1500-3000 mg/L phenol caused 50% inhibition of the conversion of acetate or propionate to methane in cultures enriched on acetate or propionate. Serum bottle cultures degraded 80% of 500 mg/L p-cresol over the 35 week incubation whereas the anaerobic filter cultures with 18-h hydraulic retention times degraded 93% of 180 mg/L p-cresol. Concentrations of 750-2500 mg/L p-Cresol caused 50% inhibition of the conversion of acetate to methane by cultures enriched on acetate. Concentrations of 750-1250 mg/L p-cresol caused 50% inhibition of the conversion of propionate to methane by cultures enriched on propionate. o-Cresol was not degraded to methane by batch cultures or anaerobic filter cultures. The degradation of m-cresol was not examined.

The development of a treatment process for waste-waters containing m-cresol would rely on an understanding of why some inocula were observed to degrade m-cresol to methane while others were not and why the degradation of m-cresol was unstable in cultures shown to be able to degrade this compound.

2.5 Characteristics of Methanogens and Methanogenic Degradations

The methanogenic degradation of complex organic compounds relies on the cooperation of a consortium of microorganisms. These consortia consist of at least two metabolic types of bacteria: those that ferment complex organic substrates to acetate, carbon dioxide and hydrogen; and the methanogens, which utilize the products of the above fermentations. The first group of organisms may consist of a number of

different microbial species capable of degrading the original substrates or intermediates produced by the initial degradations. The second group of microorganisms, the methanogens, consists of two subgroups, methanogens that can utilize small organic molecules such as acetate and those that utilize the hydrogen/carbon dioxide couple for energy and growth. Some methanogens can utilize both acetate and the hydrogen/carbon dioxide couple.

The energetics of the utilization of different substrates for methanogenesis have been reviewed by Daniels et al. (1984) and the free energies for the utilization of carbon dioxide, formate and acetate are shown below.

$HCO_3^- + H^+ + 4\ H_2 \rightarrow CH_4 + 3\ H_2O$	$\Delta G^{\circ\prime} = -135.6$ kJ/mol	Eqn. 2.4
$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$	$\Delta G^{\circ\prime} = -138.8$ kJ/mol	Eqn. 2.5
$4\ HCOOH \rightarrow 3\ CO_2 + CH_4 + 2\ H_2O$	$\Delta G^{\circ\prime} = -119.5$ kJ/mol	Eqn. 2.6
$CH_3COOH \rightarrow CH_4 + CO_2$	$\Delta G^{\circ\prime} = -27.6$ kJ/mol	Eqn. 2.7

The production of 1 mol of ATP has been reported to require approximately -37 kJ/mol of energy (Thauer et al. 1977). An examination of Eqns. 2.4-2.7 has led to the assumption that a maximum of two-three mol of ATP could be synthesized per mol of methane formed from carbon dioxide (Eqns. 2.4 and 2.5) or formate (Eqn. 2.6) but less than 1 mol of ATP could be synthesized per mol of methane produced from acetate (Eqn. 2.7). Daniels et al. (1984) pointed out that almost all methanogens can reduce carbon dioxide with hydrogen, half the genera can utilize formate and very few will utilize acetate.

Methanosarcina barkeri has been reported to grow with acetate as a carbon source although it has been seen to use the carbon dioxide/hydrogen couple or methanol as substrates for methanogenesis in preference over acetate (Krzycki et al. 1982, Smith

and Mah 1978, Weimer and Zeikus 1978). *Methanothrix soehngenii* and *Methanothrix concilii* were reported to be capable of an acetoclastic splitting of the acetate molecule to methane and carbon dioxide. The *Methanothrix* sp. were not capable of utilizing the carbon dioxide/hydrogen couple and were reported to be the most numerous methanogens in sewage treatment plants. *M. concilii* has been cultured in mineral medium with acetate as the only source of carbon and energy. (Huser et al. 1982, Patel 1984, and Zehnder et al. 1980)

An excellent review of the biochemical process of methanogenesis is given by Daniels et al. (1984). The production of ATP by methanogens occurs via electron transport phosphorylation systems and as such has been termed respiration (Daniels et al. 1984). A variety of novel and traditional coenzymes which function both in electron transport and as methyl transfer agents have been seen to be involved in methanogenesis.

The most important compound involved in electron transport in methanogens is 5-deazaflavin (F_{420}). F_{420} is a two electron transfer agent and is found in all methanogens in levels about 1.1-4.7 nmol/mg protein (Daniels et al. 1984). F_{420} accepts 2 electrons from hydrogen and 2 H^+ from water through the action of a hydrogenase. The electrons are transferred to nicotinamide adenine dinucleotide phosphate (NADP), but not nicotinamide adenine dinucleotide (NAD), by NADP-F_{420} oxidoreductase. Formate, CO and possibly, methanol and acetate also donate electrons to F_{420}. F_{420} is responsible for the green UV fluorescence observed in methanogens. Methanogens also possess nicotinamides, flavins and folates in concentrations comparable to those found in eubacteria. Cytochromes have been identified only in methanogens capable of the reduction of methyl containing substrates (Daniels et al. 1984).

The first novel cofactor found in the methanogens was Coenzyme M (2-mercaptoethanesulfonic acid). It has been found in cells in levels of 3-50 nmol/mg protein and was seen to be essential for the last two electron reduction in methanogenesis. The thiol portion of the cofactor carries the methyl group during the final reduction catalyzed by methyl-coenzyme M reductase. Bromoethanesulfonic acid (BESA), an analog of coenzyme M, has been reported to be a potent inhibitor of methanogenesis and has been used as an aid to study methanogenic fermentations. Other carbon carrying compounds identified in methanogens are methanopterin, formylmethanopterin, formaldehyde-activating factor and methanofuran, as well as conventional carbon transferring coenzymes such as biotin, thiamine and pantothenic acid (Daniels et al. 1984).

The reduction of carbon dioxide to methane has been reported to begin with the binding of carbon dioxide to methanofuran. The carbon from the bound carbon dioxide undergoes a two electron reduction before transfer to formaldehyde-activating factor. This factor was reported to carry the carbon through three reduction states to a methyl group. The methyl group was transferred to coenzyme M for the final two electron reduction and release of methane (Daniels et al. 1984). The biochemical processes involved in the methanogenic metabolism of acetate have not been elucidated.

Although methanogens have been isolated and grown in pure cultures, attempts to isolate pure cultures of aromatic-degrading organisms from methanogenic enrichment cultures have not been successful to date. This has indicated a dependency on microbial interactions during the degradation of aromatic compounds under these conditions. Young (1984) has illustrated the need for microbial interactions in the anaerobic degradation of benzoic acid through the use of chemical equations.

$$4\ C_6H_5COOH + 24\ H_2O \rightarrow 12\ CH_3COOH + 4\ HCOOH + 8\ H_2 \quad \text{Eqn. 2.8}$$

$$12\ CH_3COOH \rightarrow 12\ CH_4 + 12\ CO_2 \quad \text{Eqn. 2.9}$$

$$4\ HCOOH \rightarrow 4\ CO_2 + 4\ H_2 \quad \text{Eqn. 2.10}$$

$$12\ H_2 + 3\ CO_2 \rightarrow 3\ CH_4 + 6\ H_2O \quad \text{Eqn. 2.11}$$

$$4\ C_6H_5COOH + 18\ H_2O \rightarrow 15\ CH_4 + 13\ CO_2 \quad \text{Eqn. 2.12}$$

Eqn. 2.8 illustrates the degradation of benzoic acid to the products formate, acetate and hydrogen (Ferry and Wolfe 1976). The free energy for this reaction was calculated to be 45.2 kJ/reaction (11.8 kJ/mol). This positive free energy of formation can be interpreted to suggest that the reaction is thermodynamically unfavorable and equilibrium would be reached with the benzoate only partially removed from the system. It has been postulated that a removal of the intermediates (Eqns. 2.9-2.11) would allow the reaction to proceed to the right and the benzoic acid could be removed from the system (Young 1984).

Complete utilization of benzoic acid would require a benzoate-catabolizing microorganism, a formate-utilizing methanogen, an acetate-utilizing methanogen and a carbon dioxide/hydrogen-utilizing methanogen. Without the complete consortium the benzoic acid would not be completely mineralized to methane and carbon dioxide (Eqn. 2.12).

The thermodynamic equilibria calculated for chemical equations for the non-methanogenic degradation of other aromatic compounds such as the cresols are also believed to yield positive free energies. Kaiser and Hanselman (1982) have calculated the free energy for the conversion of 1 mol of phenol to 3 mol of acetate to be + 6.55 kJ/mole. This again indicated a need for product removal to allow the reaction to go to completion. The free energies of the non-methanogenic degradations of other

phenolic compounds can only be calculated once the products of the non-methanogenic degradations are known.

It is apparent that the degradation of aromatic compounds under anaerobic conditions requires an interspecies cooperation and that pure culture techniques used to study the metabolism of compounds under aerobic circumstances may not be applicable to anaerobic circumstances. The study of methanogenesis has led to suitable methods of cultivating these extremely oxygen sensitive anaerobes and has opened the doors to the study of the other members of the methanogenic consortia which to date are poorly understood.

2.6 Metabolic Pathways Described for the Microbial Metabolism of Selected Aromatic Compounds

There is very little information in the literature on the anaerobic pathway of m-cresol degradation. Information on the aerobic degradation of the cresol isomers and the anaerobic degradation of benzoate, phenol and p-cresol is available and will be presented to use as a comparison when the findings of the investigation of the intermediates of m-cresol degradation are presented.

2.6.1 Aerobic Metabolism of the Cresol Isomers

The aerobic pathways for the metabolism of the cresol isomers are well documented (Bayly et al. 1966, Tabak et al. 1964). A review article by Bayly and Barbour (1984) summarized some of the pathways and control mechanisms for the metabolism of these compounds. Studies using different strains and species of microorganisms have shown that there is no single pathway for the degradation of these compounds but the number of mechanisms is limited. The main variations in the observed metabolic pathways are the fate of the methyl group and the position at which the aromatic ring is cleaved.

The methyl groups of m- or p-cresol have been found to be metabolized in two manners. The methyl group may be left intact on the aromatic nucleus prior to ring cleavage (Figure 2.1) or it may be oxidized to give a benzoic acid derivative (Figures 2.2 and 2.3). The methyl hydroxylase enzyme (enzyme A, Figures 2.2 and 2.3) was found to use water as the source of oxygen and pass electrons directly to the electron transport chain through flavin and c-type cytochrome subunits (Hopper 1978). Further oxidations were performed by dehydrogenase enzymes (enzymes B and C in Figures 2.2 and 2.3) producing m-hydroxybenzoic acid (from m-cresol, Figure 2.2) or p-hydroxybenzoic acid (from p-cresol, Figure 2.3). There was no reference in the literature to an oxidation of the methyl group of o-cresol (Bayly and Barbour 1984).

The benzoic acid derivatives produced when the methyl groups were oxidized were then oxidized by monooxygenase enzymes to gentisic acid from m-cresol (Figure 2.2) or to protocatechuic acid from p-cresol (Figure 2.3) which were the substrates for ring fission. When the methyl groups were not oxidized, the ring fission substrates were the corresponding catechols: o- and m-cresol were oxidized to form 3-methylcatechol (compound 4, Figure 2.1) and p-cresol was oxidized to form 4-methylcatechol (compound 5, Figure 2.1).

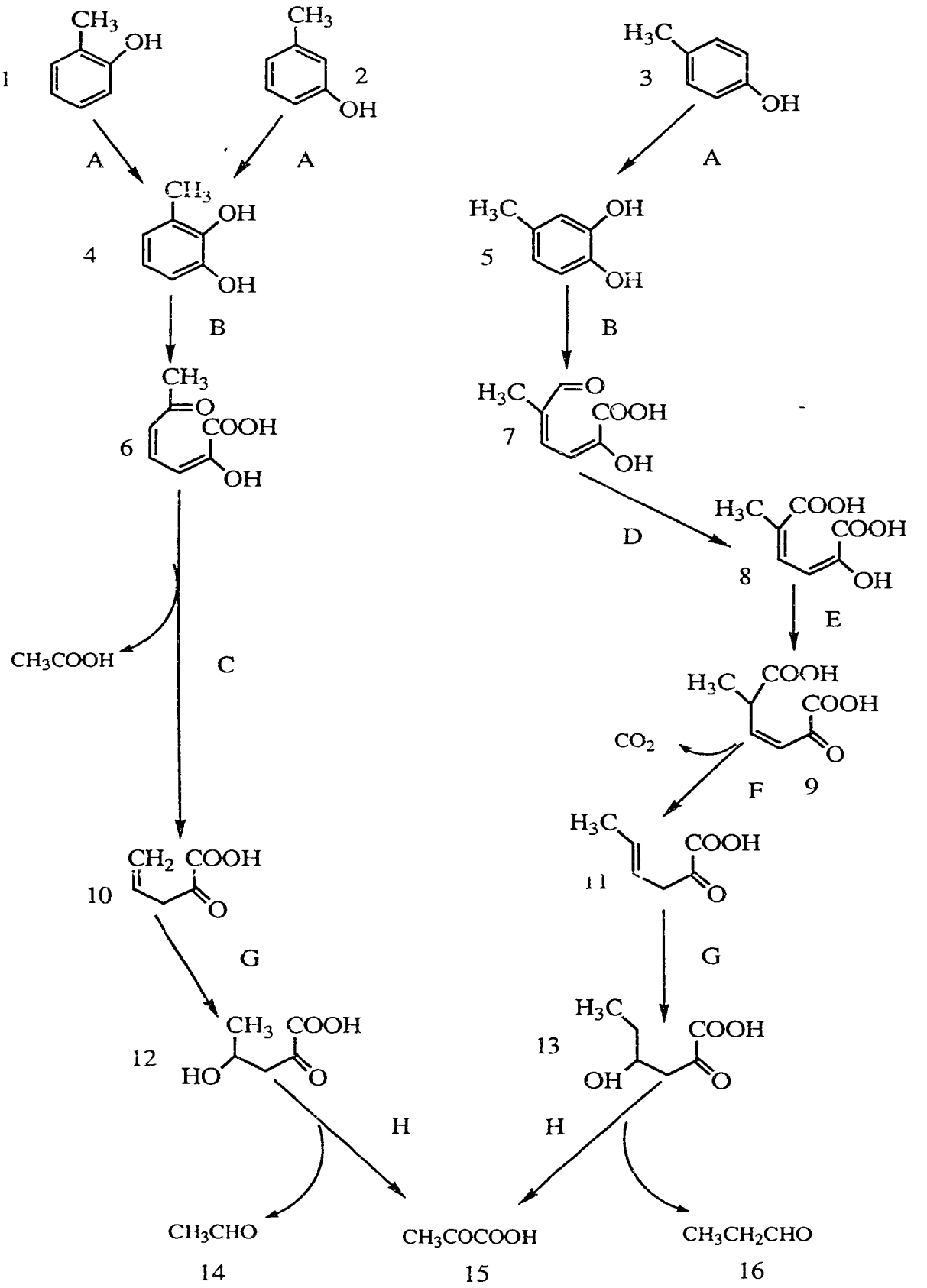
Ring cleavage of the diol substrates was reported to be accomplished by one of three pathways depending on the original substrate and the organism involved. The cleavage of the aromatic nucleus between adjacent hydroxyl groups is termed the ortho (or β-keto-adipate) pathway; cleavage between two adjacent carbons only one of which contains a hydroxyl group is termed the meta pathway; and when the hydroxyl groups are para to each other, as in gentisic acid, the pathway is termed the gentisate pathway (Bayly and Barbour 1984).

Figure 2.1 illustrates the use of the meta pathway to degrade the three cresol isomers by *Pseudomonas putida*. The cleavage of 2-methylcatechol produced from m- and o-cresol was between the carbon bearing the methyl group (C-3) and the adjacent

Figure 2.1. The meta cleavage pathway for the aerobic degradation of the three cresol isomers by *P. putida*. After Bayly and Barbour (1984).

Key to compounds: 1. o-cresol, 2. m-cresol, 3. p-cresol, 4. 3-methylcatechol, 5. 4-methyl-catechol, 6. 2-hydroxy-6-ketohepta-2,4-dienoate, 7. 2-hydroxy-5-methyl-muconic semialdehyde, 8. 2-methyl-4-oxalocrotonate (enol), 9. 2-methyl-4-oxalo-crotonate (keto), 10. 2-ketopent-4-enoate, 11. 2-ketohex-4-enoate, 12. 2-keto-4-hydroxyvalerate, 13. 2-keto-4-hydroxy-hexanoate, 14. acetaldehyde, 15, pyruvate, 16. propionaldehyde.

Key to enzymes: A. phenol hydroxylase, B. catechol 2,3-dioxygenase, C. hydrolase, D. aldehyde dehydrogenase, E. isomerase, F. decarboxylase, G. hydratase, H. aldolase.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A
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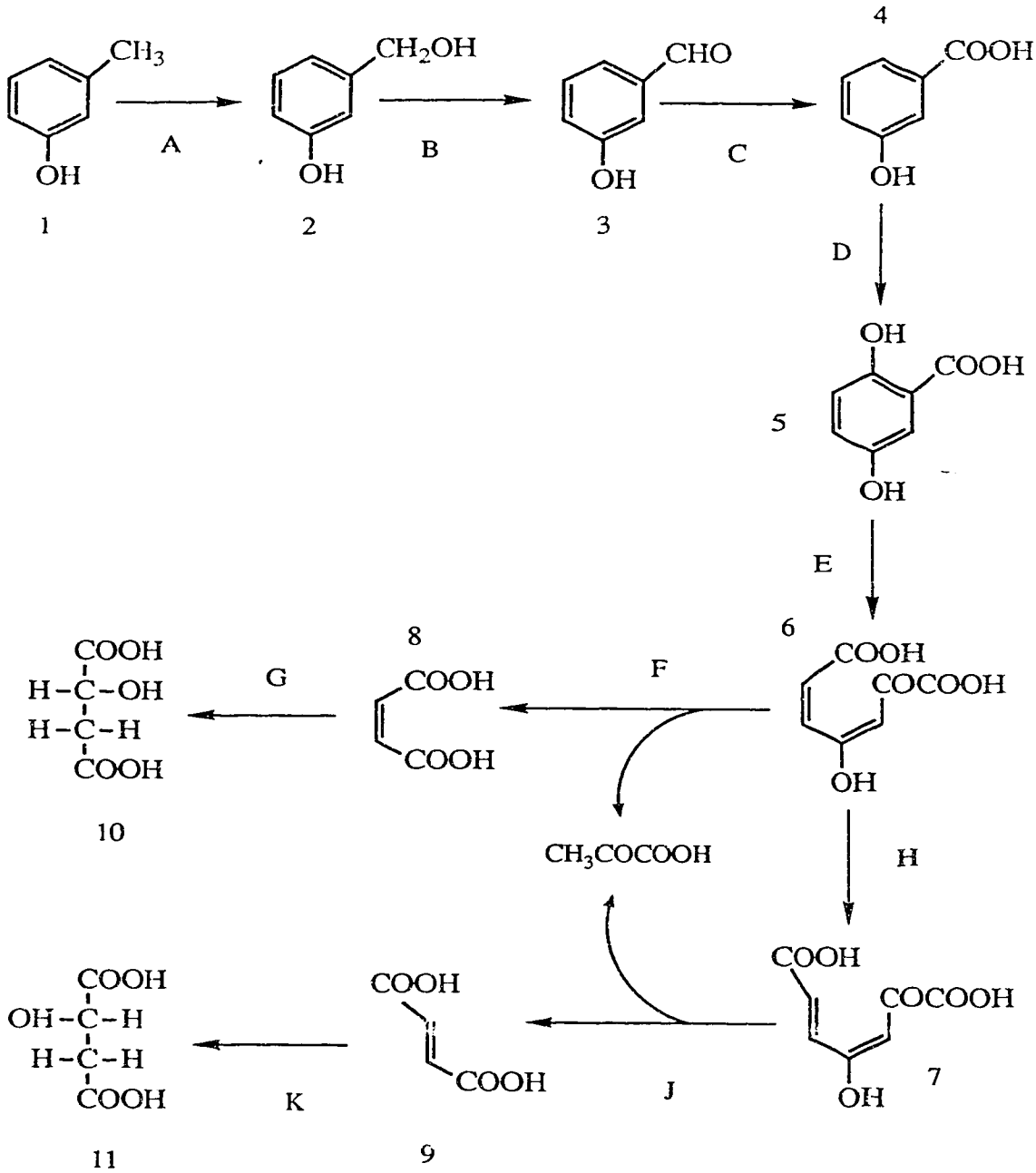
A
B
B
C
D
E
F
G
G
H
H
CH3COOH
CO2
CH3CHO
CH3COCOOH
CH3CH2CHO

Figure 2.2. The gentisate pathway for the aerobic degradation of m-cresol by *Pseudomonas* sp. From Bayly and Barbour (1984). Compound 6 can be metabolized through one of two routes.

Key to compounds: 1. m-cresol, 2. m-hydroxybenzylalcohol, 3. m-hydroxybenzaldehyde, 4. m-hydroxybenzoic acid, 5. gentisic acid, 6. maleylpyruvic acid, 7. fumarylpyruvic acid, 8. maleic acid, 9. fumaric acid, 10. D-malic acid, 11. L-malic acid.

Key to enzymes: A. methylhydrolase, B. alcohol dehydrogenase, C. aldehyde dehydrogenase, D. 6-mono-oxygenase, E. gentisate 1,2-dioxygenase, F. maleylpyruvate hydrolase, G. maleate hydratase, H. isomerase, J. fumarylpyruvate hydrolase, K. fumarase.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OH
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CHO
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COOH
COCOOH
OH
F
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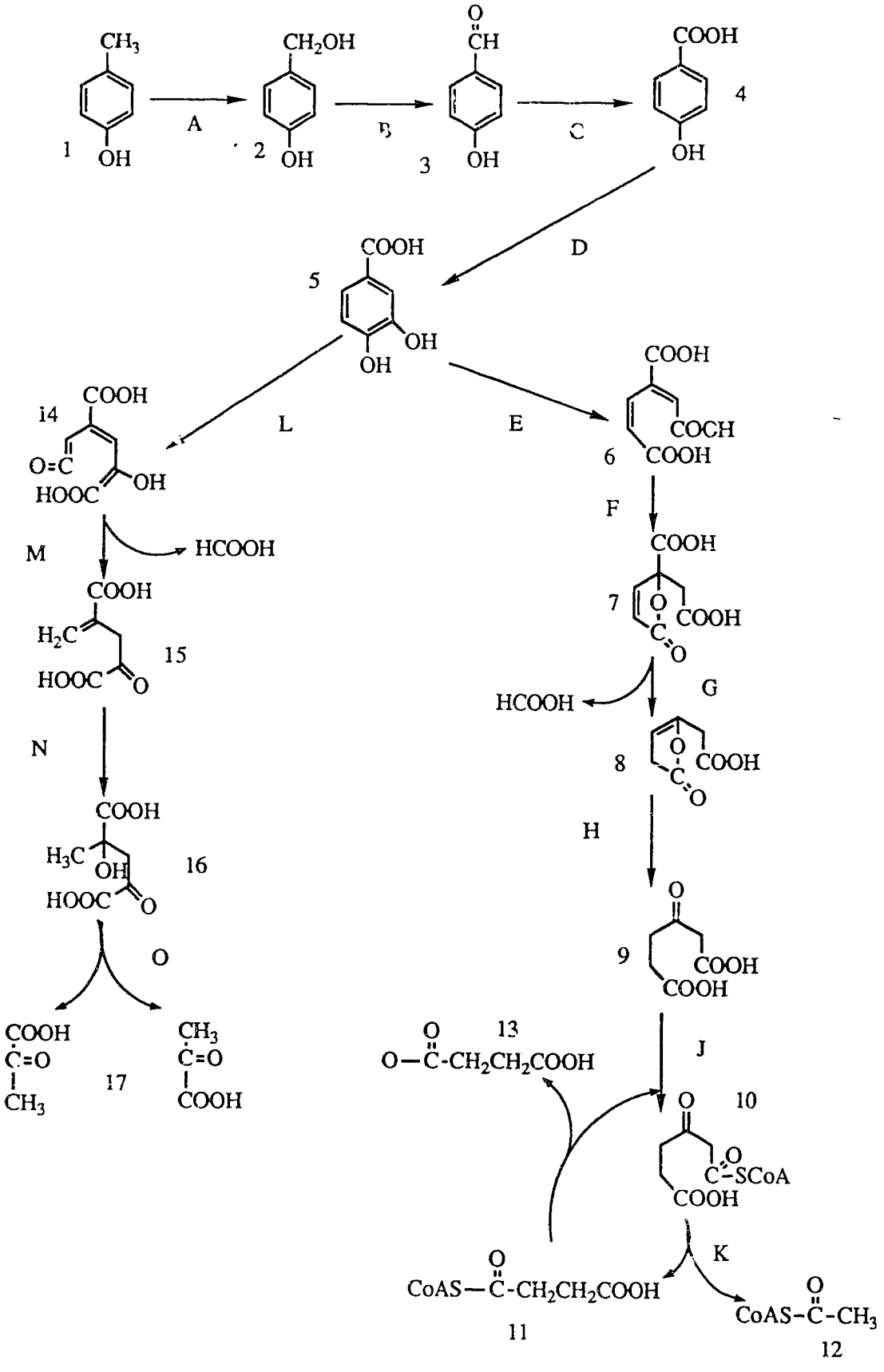
COOH
COOH
G
COOH
H-C-OH
H-C-H
COOH
10
CH3COCOOH
H
COOH
COCOOH
OH
7
J
COOH
COOH
K
COOH
OH-C-H
H-C-H
COOH
9
11

Figure 2.3. The meta and ortho (β-ketoadipate) pathways for the degradation of p-cresol when the methyl group is oxidized. After O'Reilly and Crawford (1989) and Stanier and Ornston (1973).

Key to compounds: 1. p-cresol, 2. p-hydroxybenzylalcohol, 3. p-hydroxybenzaldehyde, 4. p-hydroxybenzoic acid, 5. protochatechuic acid, 6. β-carboxy-cis-cis-muconic acid, 7. γ-carboxymuconolactone, 8. β-ketoadipic enol-lactone, 9. β-ketoadipate, 10. β-ketoadipyl-CoA, 11. succinyl-CoA, 12. acetyl-CoA, 13. succinic acid, 14. 4-carboxy-2-hydroxymuconic semialdehyde, 15. 4-carboxy-2-ketopent-4-enoic acid, 16. 4-carboxy-2-keto-4-hydroxyvaleric acid, 17. pyruvate.

Key to enzymes: A. p-cresol methylhydroxylase, B. p-hydroxybenzylalcohol dehydrogenase C. p-hydroxybenzaldehyde dehydrogenase, D. p-hydroxybenzoic acid monooxygenase, E. protocatechuate 3,4-dioxygenase, F. β-carboxy-cis-cis-muconate lactonizing enzyme, G. γ-carboxymuconolactone decarboxylase, H. β-ketoadipate enol-lactone hydrolase, J. β-ketoadipate succinyl-CoA transferase, K. β-oxidation enzymes, L. protocatechuate 4,5-dioxygenase, M. 4-carboxy-2-hydroxymuconic semialdehyde hydrolase, N. 4-carboxy-2-ketopent-4-enoic acid hydratase, O. 4-carboxy-2-keto-4-hydroxyvaleric acid aldolase.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COOH
COOH
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13
O—C-CH2CH2COOH
J
O
10
O
C-SCoA
COOH
K
O
CoAS—C-CH2CH2COOH
11
O
CoAS-C-CH3
12

carbon bearing a hydroxyl group (C-2). The cleavage of 4-methylcatechol produced during p-cresol degradation occurred between the C-2 and C-3 carbons as in 3-methylcatechol degradation but the carbon bearing the methyl group was not involved.

The final products found in the pathway for o- and m-cresol degradation were acetate, acetaldehyde, and pyruvic acid whereas the degradation of p-cresol through the meta pathway produced carbon dioxide, propionaldehyde, and pyruvate. The main difference in the products was due to the removal of the methyl group by a deacetylation reaction during m-cresol degradation (enzyme C, Figure 2.1) whereas the methyl group was not removed in the decarboxylation reaction during p-cresol degradation (enzyme F, Figure 2.1).

Figure 2.2 illustrates the aerobic degradation of m-cresol through the gentisate pathway. In this case the methyl group was oxidized before ring cleavage. The cleavage occurred between the carboxyl carbon (C-1) (produced by oxidizing the methyl carbon) and the carbon bearing the added hydroxyl group (C-2). The final products of the pathway were: D and/or L malic acid (some strains were capable of producing both while some strains produced one or the other) and pyruvate (Bayly and Barbour 1984).

Figure 2.3 illustrates the aerobic degradation of p-cresol through the meta and ortho cleavage pathways when the methyl carbon was oxidized prior to ring cleavage. The hydroxyl groups are adjacent in protocatechuate so meta or ortho cleavage can occur. The use of one pathway or the other was dictated by the particular species of *Pseudomonas* studied. Meta cleavage occurred between the carbon bearing the hydroxyl group (C-4) and the C-5 carbon. This was not in the same position as the meta cleavage of p-cresol when the methyl carbon was left intact (Figure 2.1). The final products were different for each method of cleavage; meta cleavage produced 1 mol of formate and 2 mol of pyruvate while ortho cleavage produced 1 mol of carbon

dioxide, 1 mol of acetyl-CoA, and 1 mol of succinate.

Although studies with $^{14}CH_3$-m-cresol or $^{14}CH_3$-p-cresol were not performed, the fate of the methyl group of these two compounds can be traced through the pathway intermediates. When the methyl group of m-cresol was not oxidized prior to ring cleavage it could be traced to the methyl group of acetate. When the methyl group of m-cresol was oxidized prior to ring cleavage it could be traced to the carboxyl group of pyruvate. When the methyl group of p-cresol was not oxidized it could be traced to the methyl group of propionaldehyde. When the methyl group of p-cresol was oxidized prior to ring cleavage it could be traced to the carboxyl group of pyruvate (meta cleavage) or to carbon dioxide (ortho cleavage). In these cases, the methyl groups remained intact unless they were oxidized on the aromatic ring. No oxidation reactions affected the methyl groups during the metabolism of ring cleavage products.

The aerobic metabolism of the cresol isomers is reliant upon molecular oxygen during the oxidation of the ring by monooxygenase enzymes and also during the subsequent ring fission reactions by dioxygenase enzymes. These requirements designate the reactions as aerobic reactions. For the degradation of these compounds under anaerobic conditions, another mechanism for ring cleavage must have evolved. If the model of aerobic metabolism of aromatic compounds can be applied to anaerobic conditions, it would be reasonable to assume that there could be more than one way to metabolize substituents on the ring while there would probably be one common mechanism for ring fission reactions. Although the rings were opened at different carbon atoms the fission was always an oxidative process and always involved at least one carbon bearing a hydroxyl substituent.

2.6.2 Anaerobic Metabolism of Aromatic Compounds

The discussion in section 2.4 of the degradation of aromatic compounds in anaerobic environments has provided evidence that these compounds can be

metabolized in anaerobic environments with a variety of terminal electron acceptors. However, there is very little evidence concerning the actual metabolic intermediates involved in these degradations.

One report has been published concerning metabolic intermediates of m-cresol degradation. Godsy and Goerlitz (1984) found both acetate and formate as intermediates in the methanogenesis of m-cresol. When cultures were inhibited with BESA and incubated for two weeks they found formate and acetate accumulated to concentrations of 194 mg/L and 186 mg/L respectively. This corresponds to a formate/acetate molar ratio of 1.4/1. Godsy and Goerlitz (1984) proposed the following steps in the conversion of m-cresol to methane and carbon dioxide.

$$4\ C_7H_8O + 32\ H_2O \rightarrow 10\ CH_3COOH + 8\ HCOOH + 20\ H_2 \qquad \text{Eqn. 2.13}$$

$$10\ CH_3COOH \rightarrow 10\ CH_4 + 10\ CO_2 \qquad \text{Eqn. 2.14}$$

$$8\ HCOOH \rightarrow 8\ CO_2 + 8\ H_2 \qquad \text{Eqn. 2.15}$$

$$28\ H_2 + 7\ CO_2 \rightarrow 7\ CH_4 + 14\ H_2O \qquad \text{Eqn. 2.16}$$

$$4\ C_7H_8O + 18\ H_2O \rightarrow 17\ CH_4 + 11\ CO_2 \qquad \text{Eqn. 2.17}$$

Eqn. 2.13 predicts a molar ratio of 0.8/1 formate to acetate whereas their experimental data indicated a 1.4/1 ratio of these compounds. No other intermediates were detected in this study. The accumulation of monocarboxylic acids with chain lengths of 3-6 carbons or dicarboxylic acids with chain lengths of 4-6 carbons did not occur in the presence of BESA. The study also suggests that "a single but unique organism is responsible for the non-methanogenic step in the conversion of phenol and methylphenol to their respective intermediates" (Godsy and Goerlitz 1984) but no supporting data were presented.

There have been reports concerning the pathways of the anaerobic metabolism of benzoic acid, phenol and p-cresol as well as other aromatic compounds. These results have been summarized in review articles (Berry et al. 1987, Evans and Fuchs 1988, Schink 1988 and Young 1988). There appears to be one major method of ring fission and at least two mechanisms have evolved to metabolize ring substituents.

The results indicate that the aromatic ring is reduced to a cyclohexyl compound by the addition of 6 hydrogen equivalents; this is then the substrate for ring fission. There have been no reports of the reduction of an unsubstituted benzene ring, instead, as in aerobic metabolism, the ring must bear an oxygen containing substituent such as an alcohol (phenol) or carboxyl group (benzoic acid). The ring fission is an oxidative process producing a carboxylic acid as the ring fission product.

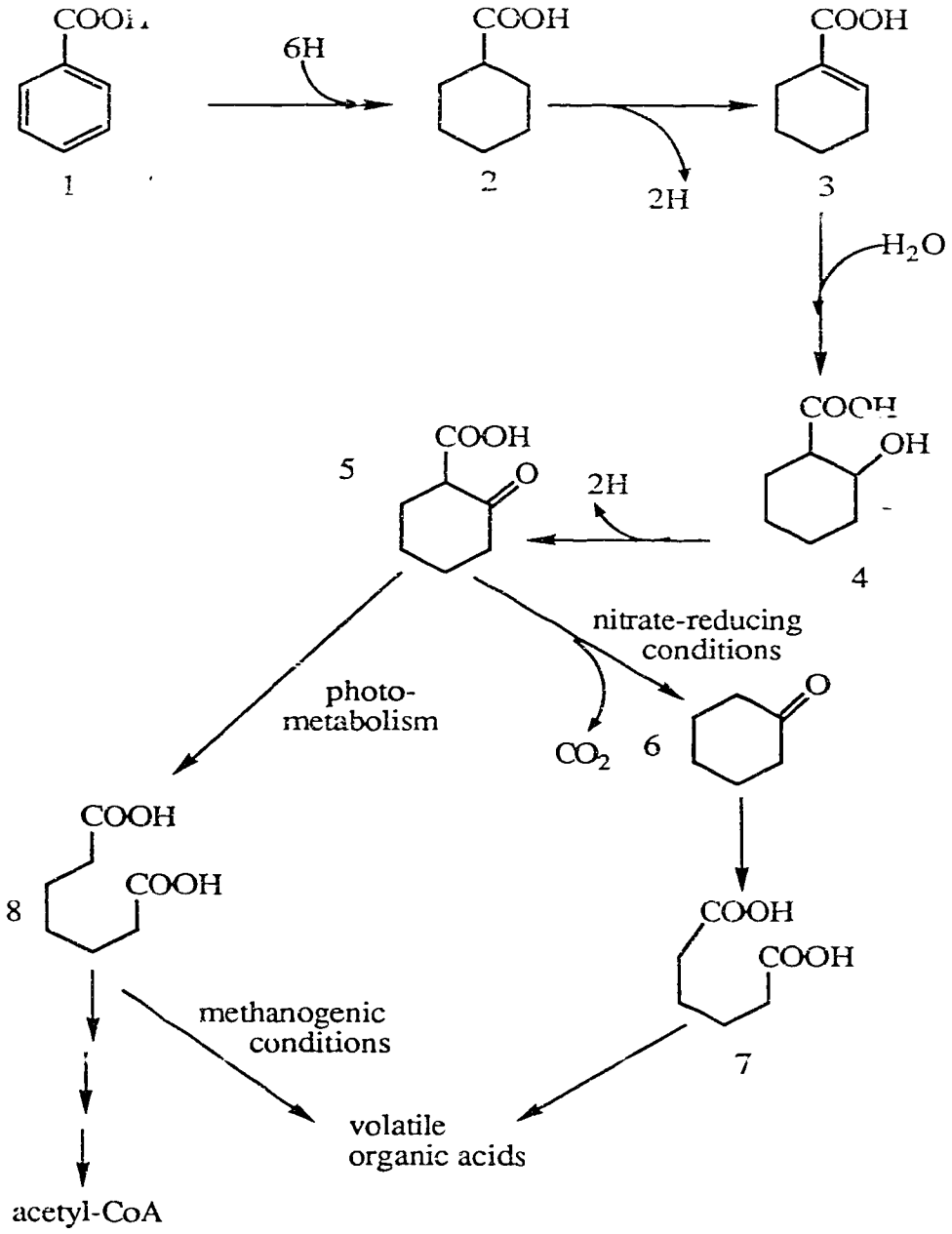
The most exhaustive studies of the anaerobic degradation of any one compound have been carried out for the degradation of benzoate. The anaerobic degradation of benzoate has been studied using pure cultures of the phototrophic organism *Rhodopseudomonas palustris*, (Dutton and Evans 1968, 1969, 1970; Guyer and Hegeman 1969, Hutber and Ribbons 1983); pure cultures of a nitrate-reducing *Moraxella* sp. (Williams and Evans 1973, 1975) actually believed to be *Paracoccus denitrificans* (Evans and Fuchs 1988); pure cultures of *Pseudomonas* strain PN-1 (Taylor et al. 1970, Taylor and Heeb 1972) [This organism has been reclassified as *Alcaligenes xylosoxidans* subsp *denitrificans* (Blake and Hegeman 1987)]; and in methanogenic enrichment cultures (Fina and Fiskin 1960, Fina et al. 1978, Keith 1972, Keith et al. 1978). The pathways are summarized in Figure 2.4.

Whittle et al. (1976) demonstrated that cell free extracts, from cells of *R. palustris* grown photosynthetically, were able to catalyze the thioesterification of benzoate to benzoyl-CoA. They proposed that all of the acidic intermediates of benzoate degradation (compounds 2-6, and 9, Figure 2.4) were actually CoA thioesters in cells of *R. palustris*. Harwood and Gibson (1986) also detected benzoyl-CoA and some

Figure 2.4. The anaerobic metabolism of benzoic acid. After Evans and Fuchs (1988).

Key to compounds: 1. benzoic acid, 2. cyclohexanecarboxylic acid, 3. cyclohex-1-enecarboxylic acid, 4. 2-hydroxycyclohexanecarboxylic acid, 5. 2-oxocyclohexanecarboxylic acid, 6. cyclohexanone, 7. adipic acid, 8. pimelic acid.

Cultures of *R. palustris* grown anaerobically in the light with ^{14}C-labeled benzoate yielded compounds 2, 3, 4, 5, and 8 containing the radioactive label. Cultures of *P. denitrificans* incubated with ^{14}C-U-ring-benzoate incorporated the label into compounds 2, 3, 4, 5 and 8 whereas *P. denitrificans* incorporated the label of ^{14}C-carboxyl-benzoate into compounds 2, 3, 4 and 5. Methanogenic consortia incorporated the label from ^{14}C-benzoate into compounds 2, 3, 4, and 5 as well as volatile organic acids.


COOH
6H
COOH
COOH
1
2
2H
3
H_2O
COOH
OH
COOH
5
O
2H
4
nitrate-reducing
conditions
photo-
metabolism
CO_2
6
O
COOH
8
COOH
COOH
COOH
methanogenic
conditions
7
volatile
organic acids
acetyl-CoA

reduction products of benzoyl-CoA in cells of *R. palustris* grown phototrophically. Benzoyl-CoA has also been identified in the degradation of benzoic acid under nitrate-reducing conditions (Evans and Fuchs 1988). The enzyme benzoyl-CoA synthetase has been identified in cell free extracts of *R. palustris* as the enzyme responsible for the thio-esterification of benzoic acid under phototrophic conditions (Hutber and Ribbons 1983). The enzyme required CoASH and ATP for this reaction. There has been no indication of the involvement of CoA in the methanogenic degradation of benzoic acid.

Under nitrate-reducing conditions, it has been reported that 2-oxocyclohexane-carboxylic acid (compound 6 Figure 2.4) was decarboxylated to produce cyclohexanone (compound 7) rather than a direct fission to produce pimelic acid (compound 9) found in methanogenic and *R. palustris* cultures. Acetate (or acetyl-CoA) has been detected as the major product of the fermentation in all of the environments although heptanoate, n-caproate, valerate, butyrate and propionate were detected in methanogenic consortia.

The metabolism of phenol by anaerobic microorganisms has been examined under nitrate-reducing conditions (Bakker 1977, Tschech and Fuchs 1987), and under methanogenic conditions (Balba and Evans 1980b, Knoll and Winter 1987, 1989). Phenol was degraded under ferric iron-reducing conditions (Lovley et al. 1989) and sulfate-reducing conditions (Bak and Widdel 1986) but no pathways have been proposed for these degradations.

Figure 2.5 summarizes the two separate pathways believed to be used for phenol degradation under anaerobic conditions. Bakker (1977) suggested that, in a nitrate-reducing enrichment culture, phenol was reduced to cyclohexanol, which was dehydrogenated to cyclohexanone. The cyclohexanone underwent a hydrolytic ring fission producing n-caproate (compound 4, Figure 2.5). When ^{14}C-labeled phenol was included in the growth medium ^{14}C-labeled n-caproate and acetate were found in

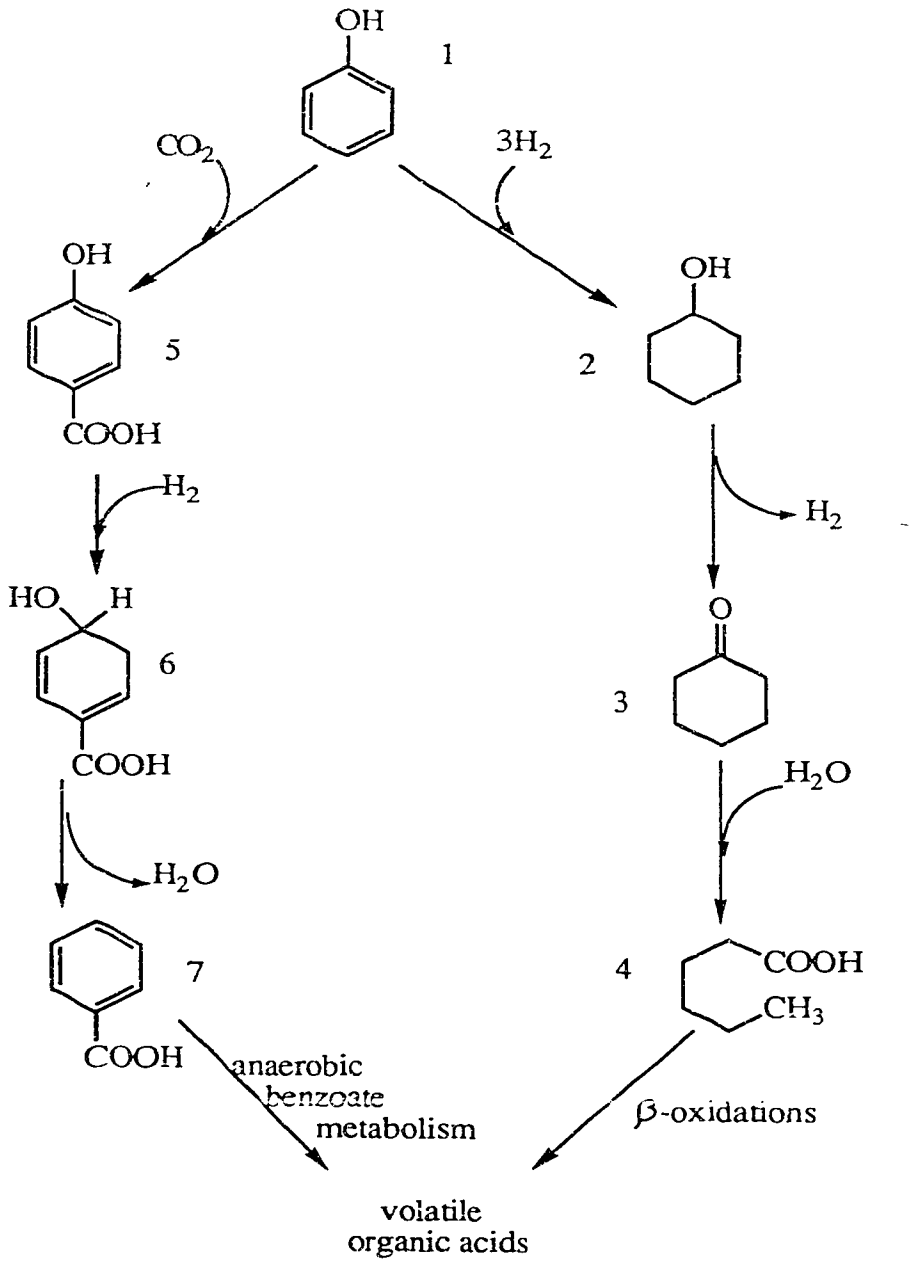


Figure 2.5. The anaerobic metabolism of phenol. After Evans and Fuchs (1988). Key to compounds: 1. phenol, 2. cyclohexanol, 3. cyclohexanone, 4. caproic acid, 5. p-hydroxybenzoic acid, 6. p-hydroxycyclohex-1,5-enecarboxylate, 7. benzoic acid.

the culture fluids.

Alternatively, Tschech and Fuchs (1987) found that Pseudomonads grown with phenol, under nitrate-reducing conditions, catalyzed the exchange of ^{14}C-labeled carbon dioxide with the carboxyl carbon of p-hydroxybenzoic acid. Cells grown on p-hydroxybenzoic acid did not catalyze this reaction. They proposed that phenol was converted to p-hydroxybenzoic acid by phenol carboxylase. The p-hydroxybenzoic acid was then converted to benzoic acid by a dehydroxylation reaction. The benzoic acid produced was assumed to be degraded through one of the reductive pathways of benzoate metabolism discussed earlier (Figure 2.4).

The results of studies with methanogenic consortia indicate that a reductive ring fission was involved and the same two mechanisms for this fission were proposed as were proposed for the metabolism of phenol under nitrate-reducing conditions. Phenol was either carboxylated to p-hydroxybenzoic acid prior to ring reduction or ring reduction occurred immediately, forming cyclohexanol. Balba and Evans (1980b) reported the reduction of phenol to cyclohexanol, a dehydroxylation to cyclohexanone, then ring fission yielding caproate. Alternatively, Knoll and Winter (1987, 1989) proposed the degradation of phenol via a carboxylation to benzoic acid. Knoll and Winter (1987) found that ^{14}C-labeled benzoate was formed from U-^{14}C-phenol or from non-labeled phenol and $^{14}CO_2$ in sewage sludge cultures in the presence H_2/CO_2 gas phase. Hydrogen gas was included in the cultures to provide [illegible] inhibition of benzoate degradation, allowing the accumulation of benzoate. It may be assumed that phenol degradation could occur via either pathway shown in Figure 2.5, although the evidence supporting each of the pathways could be much stronger. Many of the proposed intermediates have not been detected in the culture fluids and the complete sequence of events leading to mineralization of phenol has not been elucidated in any of the studies.

The metabolism of organic acids produced as ring fission products (dipimelic acid

from benzoate, caproic acid from phenol) (Figures 2.4 and 2.5 respectively) has been reported to involve a series of β-oxidation reactions. Cell free systems of benzoate-degrading organisms have been demonstrated to have the enzymes responsible for β-oxidation (Blakely 1978, Rho and Evans 1975).

The fate of methyl substituents on aromatic rings has been studied in detail for very few compounds. Studies of the degradation of p-cresol under nitrate-reducing conditions (Bossert and Young 1986, Bossert et al. 1986, and Tschech and Fuchs 1987) have shown that the methyl group was oxidized to a carboxylic acid. The intermediates observed were the same as those found for the aerobic oxidation of the methyl group of p-cresol, forming p-hydroxybenzoic acid (Figure 2.3). The further metabolism of p-hydroxybenzoic acid in these cultures has not been documented.

Young and Rivera (1985) found that methanogenic enrichment cultures removed the methyl group of p-cresol from the ring producing phenol which was then metabolized to methane and carbon dioxide. There was no mention of further metabolites. Alternatively, Senior and Balba (1984) found that methanogenic enrichment cultures fed p-cresol accumulated p-hydroxybenzoic acid, indicating that the methyl carbon of p-cresol was oxidized.

Suflita et al. (1989) studied the degradation of o-, m- and p-cresol under sulfate-reducing conditions and found that the methyl group of p-cresol was oxidized to produce p-hydroxybenzoic acid. There was evidence that the methyl group of o-cresol was also oxidized to yield a hydroxybenzoic acid. However, there was no indication of the oxidation of the methyl carbon of m-cresol and no intermediates were detected during m-cresol degradation.

Kuhn et al. (1988) studied the degradation of toluene under nitrate-reducing conditions and found that the ring underwent a hydroxylation reaction producing p-cresol. The methyl carbon was then oxidized as in the aerobic sequence of oxidations producing p-hydroxybenzoic acid. Vogel and Grbić-Galić (1986) found that

methanogenic consortia incorporated an oxygen atom from ^{18}O-labeled water onto the aromatic ring of toluene producing p-cresol. These same cultures produced ^{14}C-labeled carbon dioxide from ^{14}C-methyl-labeled toluene. These observations indicated that the methanogenic cultures oxidized the methyl group of toluene (through p-cresol) to a carboxyl group.

From the limited studies performed it can be seen that two mechanisms for the metabolism of aryl methyl substituents have evolved under anaerobic conditions. Methyl constituents were oxidized to form benzoic acids or were removed before reductive ring fission took place under anaerobic conditions. Unlike the aerobic mechanisms evolved for the metabolism of aryl methyl groups, there were no reports of methyl substituents left intact on the ring or during subsequent cleavage reactions.

2.7 Methods for the Study of Biodegradations

The objectives of this research were to identify the intermediates of methanogenic m-cresol metabolism and to describe the microbiological interactions involved in this degradation. To achieve these goals a multidisciplinary approach was required. The methods used in this study were from the fields of anaerobic microbiology, analytical chemistry and organic chemical synthesis. These methods are explained in detail in the Methods and Materials section of this thesis. However, a brief overview and a general background of the approaches used to carry out similar studies with other aromatic compounds are given below.

2.7.1 Microbiological Techniques

The complete mineralization of aromatic compounds by methanogenic cultures requires the cooperation of fermentative organisms and methanogens. The culture media and methods used to study the mineralization of aromatic compounds have been developed to cater to the methanogenic members of the consortia. These archaebacteria

are the strictest anaerobes known and are not only extremely sensitive to very short exposure to oxygen (Bryant 1974) but rely on enzymes that require very low redox potentials for activity ($E^{\circ\prime}$ of coenzyme F_{420} is -370 mV, (Daniels et al. 1984)). The culture methods developed by Hungate (1950) have been used extensively to obtain pure cultures of methanogenic organisms. Miller and Wolin (1974) developed a serum bottle modification of the Hungate technique which has been commomly used to study methanogenic enrichment cultures. Owen et al. (1979) modified the procedure of Miller and Wolin (1974) to allow determinations of biochemical methane potentials and anaerobic toxicities of organic compounds. Media used to culture methanogenic enrichment cultures are usually simple mineral salts media buffered with carbon dioxide and containing ammonia-nitrogen and phosphate.

Several different methods may be employed to reduce the media and poise the E_h to suitable values for the growth of methanogenic bacteria. These methods involve the anaerobic techniques of boiling media to expell dissolved O_2 and the use of O_2-free gases in the headspace of vessels during media preparation and dispensing (Hungate 1950). In most cases, additional methods of media reduction, such as the addition of chemical reducing agents are necessary. The most common reducing agents used in the culture of methanogens and methanogenic consortia are the addition of 1 mM sodium sulfide (Owen et al. 1979) or addition of a mixture of cysteine/sulfide Patel (1984). The use of amorphous ferrous sulfide (AFS) as a reducing agent has also been reported (Brock and O'Dea 1977). Another method that has been used to reduce media for the cultivation of anaerobes is the use of iron nails (Butlin et al. 1949). This method is more commonly used for the cultivation of SRB.

Inocula capable of the anaerobic degradation of aromatic compounds have been obtained from anaerobic muds and sewage digestors. Aromatic substrates are added to the inoculum to concentrations in the range of 50-300 mg/L and the production of methane by the cultures is monitored. The concentration of substrate in the culture

fluids may also be monitored. After the first allotment of substrate has been depleted the cultures are usually fed more substrate until a stable rate of degradation is achieved (Balba et al. 1981, Boyd et al. 1983, Fedorak and Hrudey 1984, Horowitz et al. 1982, Shelton and Tiedje 1984a).

Young and Rivera (1985) examined the degradation of four phenolic compounds and found that the acclimation procedure greatly enhanced the ability of the consortia to degrade the substrate presented to them and that acclimated consortia were able to withstand concentrations of substrate that would have been toxic to an unacclimated consortium. Fedorak and Hrudey (1984) observed that the acclimation process was dependant upon concentration of the substrate. In tests of anaerobic sewage sludge incubated in the presence of various concentrations of phenol, the lag times required before the methane concentrations exceeded those of unamended controls was 15 d for concentrations in a range of 100-300 mg/L but at higher concentrations of 400 and 500 mg/L 18 and 26 d lag times were observed, respectively. Lag times observed before methane production exceeded that of unamended controls for various concentrations of p-cresol were observed to be 15 d for 100 mg/L and 39 d for concentrations in the range of 200-400 mg/L.

The consortia involved in the degradation of aromatic compounds can also be studied by the coculture method. This method employs the dilution of an enrichment culture or the isolation of individual members of the consortia in agar roll tubes and the reconstitution of an active defined consortium by the incubation of the fermentative organisms with various hydrogen- or acetate-utilizing organisms. Mountfort and Bryant (1982) and Mountfort et al. (1984) isolated a syntrophic benzoate-degrading organism, *Syntrophus buswelii*, by inoculating dilutions of an enrichment culture into roll tubes containing agar with benzoate as the sole carbon source and *Methanosarcina hungateii* or *Desulfovibrio* sp. as hydrogen scavenger. Colonies growing on benzoate in the agar were picked and grown in liquid medium with either *M. hungateii* or

Desulfovibrio sp. This method allowed the workers to establish a defined coculture degrading benzoate. The benzoate-catabolizing organism was identified on the basis of cellular morphology in comparison to the morphology of *M. hungateii* or *Desulfovibrio* sp.

In some instances, the fermentative organism does not require a syntrophic association with a hydrogen scavenging organism for growth on a particular substrate. These organisms usually only perform an initial part of the fermentation. Strain DCB-1 was isolated in pure culture and dechlorinated 3-chlorobenzoate without the need for coculture with hydrogen scavenging organisms (Shelton and Tiedje 1984b). However, the organism could not metabolize the aromatic ring so it only served as the initial member of the consortium required for the complete mineralization of 3-chlorobenzoate. The metabolism of the aromatic ring required a benzoate-catabolizing organism (strain BZ-1) which could only be grown in coculture with *Desulfovibrio* strain PS-1. A hydrogen-utilizing methanogen (*Methanospirillum* strain PM-1) was also isolated from the same enrichment culture. These organisms were used to reconstitute a defined consortium that metabolized benzoic acid to acetate and the further study of this degradation was carried out with the reconstituted consortium (Dolfing and Tiedje 1986, 1987, Shelton and Tiedje 1984b, Stevens et al. 1988 and Stevens and Tiedje 1988).

Barik et al. (1985) used coculture techniques to isolate anaerobes designated strains PA-1 and P2 which catabolized many aromatic compounds in syntrophy with the hydrogen-utilizing organisms *M. hungateii,* or *Wollinella succinogenes*. It was found that strain PA-1 grew much better in coculture with *W. succinogenes* than *M. hungateii*. The reduction of fumarate to succinate by hydrogen as catabolized by *W. succinogenes* was proposed to be much more energetically favorable than the reduction of carbon dioxide or sulfate.

Microbiological techniques have also incorporated the use of specific inhibitors as

an aid in the study of the metabolism of aromatic substrates. The inhibition of methanogens by BESA has been used to study the fermentative population and cause the accumulation of intermediates that would otherwise be removed by methanogenic populations. Young and Rivera (1985) found that phenol accumulated in p-cresol-degrading methanogenic enrichment cultures inhibited by BESA. Grbic´-Galic´ (1986) used BESA to inhibit ferulic acid-degrading methanogenic consortia and found that several intermediates accumulated in the culture supernatants. Healy et al. (1980) enhanced the accumulation of several intermediates in cultures of methanogenic ferulic acid-degrading bacteria by incubation in the presence of BESA. Kaiser and Hanselman (1982) found acetate accumulated in syringic acid-degrading cultures fed syringic acid, 2,6-dimethoxyphenol, gallic acid and pyrogallol. Frazer and Young (1985) used BESA to obtain pure cultures of O-demethylating organisms from ferulic acid-degrading enrichment cultures.

2.7.2 Analytical Techniques

A broad spectrum of analytical techniques have been used to study the biodegradation of aromatic compounds. Common methods employ gas chromatography (GC) and high performance liquid chromatography (HPLC) as well as thin layer chromatography (TLC) and mass spectrophotometric (MS) methods to separate, quantitate and identify aromatic substrates and intermediates of their degradation.

Radiotracer techniques are used to help identify which of the large number of unidentified and often unrelated compounds found in enrichment cultures from sewage sludge are actual intermediates in the degradations of the specific aromatic compounds under investigation. ^{14}C-Labeled compounds are not always commercially available so must be prepared by custom synthesis. The production of ^{14}C-labeled carbon dioxide from ^{14}C-labeled substrates has been quantitated by flushing the headspace gases from

acidified cultures and trapping the carbon dioxide in basic solutions (Fedorak et al. 1982, Frazer and Young 1986). The production of ^{14}C-labeled methane has been quantitated by a gas proportional counter (GPC) attached to the outflow of a GC column (Fina and Fiskin 1960).

Phenolic compounds used as substrates for the growth of cultures have been analyzed by GC (Fedorak and Hrudey 1984); reverse phase HPLC analysis (Young and Rivera 1985, Kaiser and Hanselman 1982); ultra violet (UV) absorbance (for studies using pure substrates) (Young and Rivera 1985) or the 4-aminoantipyridine method (APHA 1980). GC and HPLC methods are advantageous over the UV absorbance and 4-aminoantipyridine methods because they allow separation of the compounds in the supernatant before quantitation. The GC method of Bartle et al. (1977) used by Fedorak and Hrudey (1984) employs a direct aqueous injection of culture fluids. This is an advantage over most GC methods that require extensive sample preparation, in the form of extractions, before analysis can be performed. The use of Tenax GC coated with 5% polymetaphenyl ether gives symmetrical peaks and improved sensitivity over the use of uncoated Tenax GC (Bartle et al. 1977). Retention times of phenolic compounds are quite short (< 5 min) so many samples can be processed in a short time. The areas of the sample peaks are compared to the peak areas produced by the analysis of standards to allow quantitative analysis of specific aromatic compounds in the GC and HPLC methods.

A method to quantitate the production of methane by methanogenic cultures has been developed by Fedorak and Hrudey (1983). The method involves the quantitation of gas volumes using a double syringe apparatus and quantitation of the amount of methane in the gas by GC analysis. Young and Rivera (1985) also used a syringe method to measure gas production, and a gas partitioner was used to determine gas composition. Shelton and Tiedje (1984a) reported the use of a pressure transducer to measure the volume of gas in serum bottle cultures.

Volatile organic acids produced during anaerobic fermentations have been analyzed by GC (Boone 1982, Healy et al. 1980, Kaiser and Hanselman 1982, Young and Rivera 1985, Knoll and Winter 1987, 1989) and by HPLC (Tholozan et al. 1988). These methods allow the separation and quantitation of each specific volatile organic acid and can be coupled to a GPC or fraction collector, respectively, for the detection of labeled volatile organic acids. The GC method of Boone (1982) employs a direct aqueous injection, eliminating the need for extensive sample preparation such as extraction from the culture fluids and the preparation of the methyl esters of the acids.

The position of the radioactive label in volatile organic acids can be determined by the Schmidt degradation (Phares 1951, Fuchs et al. 1980). This method employs a decarboxylation reaction which releases the carboxyl moiety from the molecule and leaves the rest of the molecule in solution as an amine. Further oxidations can lead to a release of subsequent carbon atoms as carbon dioxide. The carbon dioxide released is trapped in basic solutions such as in the method of Fedoral et al. (1982).

The identification of intermediates such as aromatic acids has been accomplished with the use of TLC methods after extraction from the culture fluids (Balba and Evans 1980a, Dutton and Evans 1969, Fina and Fiskin 1960, Harwood and Gibson 1986, Williams and Evans 1975), by HPLC methods (Knoll and Winter 1987, 1989 and Young and Rivera 1985) or by GC/MS methods (Grbić-Galić 1986, Suflita et al. 1989). TLC or HPLC methods allow the use of radiotracer techniques to determine which spots or peaks (respectively) contain the radioactive label. Quantitation of radioactivity has been accomplished by liquid scintillation counting (LSC) methods. GC/MS procedures are used to determine the molecular weight and fractionation patterns of intermediates. This information can be used to make preliminary identifications of compounds or to confirm identifications made using other methods.

Authentic standards of intermediate compounds are not always commercially available and often must be prepared in the laboratory. For example Ehman and

Gaucher (1977) synthesized 6-methylsalicylic acid (2-hydroxy-6-methylbenzoic acid) for use in the identification of this compound as an intermediate in the pathway of patulin synthesis by *P. urticae*. Similarly, Laborde and Gibson (1977) synthesized 2-hydroxydibenzothiophene and dibenzothiophene-5-oxide for use in the identification of the intermediates of the metabolism of dibenzothiophene by *Beijerinckia* species.

The research described in the following pages was performed using adaptations of the methods listed above as well as the basic concepts of microbiology and analytical chemistry as they applied to this research.

3. Materials and Methods

3.1 General Culture Techniques

Anaerobic technique was used for all inoculations and incubations of enrichment, experimental cultures and cocultures unless described otherwise. The vessels used to store solutions or cultures (serum bottles, Hungate tubes or roll tubes) were made anaerobic by purging the vessel for 1-2 min with a steady flow of 30% CO_2/N_2 that had been scrubbed free of O_2 by passage through a heated copper column (Hungate 1950). This gas will be referred to as anaerobic gas.

Medium WR86 (Fedorak and Hrudey 1986b) (Appendix 1.1) was boiled to remove O_2 before equilibration to pH 6.9-7.1 by bubbling anaerobic gas through it with a gas dispersion tube. The medium was maintained in an anaerobic state by continued flushing of the headspace with anaerobic gas. The anaerobic medium was transferred to the anaerobic receiving vessel using a pipette that had been rinsed with anaerobic gas by withdrawing and expelling anaerobic gas from the headspace of the medium flask several times. The vessel was flushed an additional 1 min with a butyl rubber stopper partially in place. The canula was removed and the stopper quickly seated. Stoppers were held in place with crimped aluminum caps or screw cap lids. The medium was sterilized by autoclaving (Hungate 1950).

Solutions added after sterilization were prepared anaerobically by the addition of distilled water, that had been boiled under a steady flow of anaerobic gas to remove O_2 (anaerobic distilled water), to quantities of the chosen compounds in Hungate tubes that had been purged of O_2 with anaerobic gas. The Hungate tubes were sealed as described above. The solutions were sterilized by autoclaving or passage through a sterile 0.45-μm filter into another anaerobic tube. The solutions were added with sterile syringes that had been rinsed with anaerobic gas by withdrawal and expulsion of

anaerobic gas from a serum bottle adapted to allow a steady flow of anaerobic gas into and out of the bottle. The medium or medium and added solutions were reduced with 1.0 or 0.5 mM sodium sulfide just prior to inoculation, unless otherwise indicated. The inoculum was added with glass syringes that had been rinsed with anaerobic gas as described above (Miller and Wolin 1974). All cultures were incubated in the dark at 35°C. Unless otherwise indicated 10-mL batch culture experiments were set up in triplicate using a 5 mL inoculum from an m- or p- cresol enrichment culture. The remaining volume was made up with 4 or 4.5 mL of Medium WR86 and various volumes of added test agents. The detailed protocols for each experiment presented in Chapters 4, 5 and 6 are presented in Appendix 2.

3.2 Enrichment Cultures

m-Cresol enrichment cultures were established in 158-mL serum bottles occasionally over the first three years of study. Each of the 8-10 replicates consisted of 75 or 85 mL domestic anaerobic sewage sludge taken from the Edmonton Goldbar Anaerobic Wastewater Treatment Plant, 1 mL of 0.01% resazurin and 4 mL of 6,000 mg/L m-cresol in distilled water.

These were analyzed weekly for m-cresol depletion and, when required, fed 1/10 of their volume of a 3,000 mg/L m-cresol in Medium WR86 using the draw and feed procedure of Fedorak and Hrudey (1986a). To do this, the cultures were allowed to settle in an inverted position before 8 or 9 mL of the supernate was removed and replaced with 8 or 9 mL of feed solution for 80- or 90-mL enrichment cultures, respectively. Medium WR86 contains minerals, trace elements and a vitamin B solution in bicarbonate buffer (Fedorak and Hrudey 1986a). m-Cresol was present as the major organic carbon source. A more complete description of Medium WR86 can be found in Appendix 1.1. Cultures were maintained on this program for at least 3 months before being used as inocula for further experiments.

Two 2-L m-cresol-degrading enrichment cultures were established during the period of study. The first 2-L enrichment culture was originally an 80-mL enrichment culture as described above. The 80-mL culture was fed a 1/10 volume of m-cresol in Medium WR86 solution (final m-cresol concentration of 300 mg/L), with no culture supernatant removal. Subsequent feed volumes were 1/10 of the accumulated total culture volume. This continued as necessary until the volume was 2 L.

The second 2-L enrichment culture was originally a 90-mL enrichment culture. This culture underwent a 50% transfer to fresh medium when m-cresol degradation was at a steady state. The resulting culture was then fed volumes of 1/10 the accumulated total culture volume with no culture supernatant removal as required until the volume was 2 L. Both 2-L enrichment cultures were maintained by removing 50 mL of the supernatant and replacing it with 50 mL of 12,000 mg/L m-cresol in Medium WR86 as necessary.

One aspect of this project considered the fates of the methyl groups of m-cresol and p-cresol. Inocula for p-cresol batch experiments were obtained from a 2-L enrichment culture that had been maintained with p-cresol as the major carbon and energy source for 9 months. This culture was obtained by incubating 2 L of fresh sewage sludge with 200 mg/L p-cresol and then feeding it 220 mg/L p-cresol every 2 days by drawing and feeding 50 mL of 8,800 mg/L p-cresol (Aldrich Chemicals Milwaukee, Wi.) in Medium WR86.

3.3 Pure Culture Methods

Liquid Medium WR86 required for attempts to obtain pure cultures of members of the m-cresol-metabolizing consortia was prepared as described in section 3.1. When hydrogen was required as a substrate, it was added to the vessels containing the medium before they were autoclaved. For manipulations requiring sterile technique the flushing gas was filter sterilized through a 1-mL syringe packed with glass wool. This

filter was sterilized by autoclaving before each use. Sterile syringes were rinsed with sterile anaerobic gas contained in a sterile serum bottle adapted to allow a steady flow of sterile gas to be maintained.

The roll tube technique was performed using the method of Hungate (1950). The anaerobic medium containing agar, roll tubes (not sealed), stoppers, flushing canula, safety pipette filler (Fisher) and pipettes were all sterilized by autoclaving. The roll tubes were removed from the autoclave immediately and stoppered with the sterile stoppers. The medium was removed from the autoclave and allowed to cool to 45°C in a water bath. When the medium was ready to be dispersed the bottle (158 mL serum bottle for 100 mL medium) was opened and a sterile canula with a steady slow stream of anaerobic gas flowing through it was inserted simultaneously. The medium was reduced with the appropriate reducing agent and gently mixed.

The pipette used to transfer the medium into the roll tubes was purged with sterile anaerobic gas. This was accomplished by attaching a line from the gas source, through a syringe filled with sterile cotton, to a sterilized safety pipette filler (Fisher) through the side-arm marked with an E. The sterile pipette was attached to the bottom outlet of the safety pipette filler. Depressing the E valve allowed a steady flow of gas through the pipette. The roll tube to which the medium was about to be transferred was opened while simultaneously inserting a sterile canula with a steady flow of anaerobic gas passing through it. The roll tube was purged in this way for 1 min before 9 mL of Medium WR86 plus 2% agar was added. The inoculum was added with a sterile syringe and the headspace of the tube was purged for an additional minute with the stopper partially in place. The roll tubes were kept in the waterbath during the whole procedure to avoid premature setting of the agar. The canula was removed and the stopper seated simultaneously. The tube was rolled by hand under a slow stream of cold water. An even film of agar was deposited on the side of the roll tube by twirling the tube with the fingers of both hands, one hand on each end of the tube. To ensure

good results a thin layer of agar was allowed to deposit on the inside surface of the rubber stopper.

Pure cultures of *Wollinella succinogenes* (ATCC #29543) were maintained on Chopped Meat Medium (Difco) or Medium WR86 plus 2% beef extract plus 5 mM formate and 7.5 mM fumarate. Pure cultures of *Methanosarcina barkeri* (ATCC #43241) were maintained on *Methanosarcina* medium (ATCC medium #1043).

3.4 Analytical Methods

3.4.1 Cresols by Gas Chomatography

Cresols were analyzed by GC on one of the following instruments (depending upon availability): Varian model 1700, Hewlett Packard (HP) model 5890 or 5790. Each GC was equipped with a flame ionization detector and a 2 m x 3 mm stainless steel column packed with 5% polyphenylether 6 ring (Chromatographic Specialties) coated Tenax GC 60/80 mesh (Alltech Assoc.). Helium (18 mL/min HP 5890, 30 mL/min HP 5790) and nitrogen (30 mL/min Varian) were used as the carrier gases. Air (400 mL/min HP 5890, 5790; 300 mL/min Varian) and hydrogen (30 mL/min) were supplied for the flame. The GC ovens, injectors and detectors were set at 200, 225 and 250°C respectively (Fedorak and Hrudey 1985). Peak areas were obtained using a HP model 3390A integrator.

The culture to be analyzed was inverted and the solids allowed to settle. A 10 µL Hamilton syringe was used to remove 2 µL of the supernatant through the butyl rubber stopper of the serum bottle. This sample was then injected directly onto the GC column. A calibration curve of m-cresol concentrations spanning the range of the sample data was run each day samples were analyzed. The equation of the line obtained from a simple regression analysis of the peak areas was used to calculate the concentrations of the samples. Standards were prepared in volumetric glassware with individually weighed amounts of m-cresol for each standard.

3.4.2 Capillary GC and Gas Chromatograph / Mass Spectrometry

Capillary GC was used to analyze the culture extracts and the methyl esters prepared from culture extracts. The analyses were performed on either a HP model 5730A or HP model 5890 GC depending on availability. Flame ionization detectors were supplied with 300 mL/min air and 30 mL/min hydrogen. Each instrument was equipped with 30-m, 0.25 μm film, DB-5 fused silica capillary column (J & W Scientific). The retention times of the sample compounds and the esters of these compounds were compared to the retention times of authentic standards or methyl esters prepared from authentic standards.

The extracts of sample compounds were analyzed by GC/MS performed by L. Harrower in Chemistry Spectral Services, University of Alberta. A 30-m DB-5 fused silica capillary column was used in a Varian Vista 6000 GC coupled to a VG7070E mass spectrometer and a VG11/250 data system. The effluent end of the GC column was inserted directly into the ion source of the MS. Scans were acquired at a rate of 1/s from mass 600 to 50 at a resolution of 1,000 at 70 eV. The mass spectra obtained were compared to standard mass spectra from a computer library or to mass spectra obtained from the authentic standards.

3.4.3 Volatile Organic Acids

Volatile organic acids with 2-5 carbon atoms in chain length were determined by the method of Boone (1982). An HP 5790 GC was equipped with a 2 m x 2 mm glass column packed with GP 10% SP1000/1%H_3PO_4 on 100/200 chromosorb WAW (Supelco). Helium was the carrier gas at 20 mL/min. The flame ionization detector was supplied with 300 mL/min air and 30 mL/min hydrogen. Injector, oven and detector temperatures were 225, 130 and 250° C respectively. For mono-acids with chain lengths 6-8 carbon atoms, the oven temperature was 170°C. Peak areas were obtained using a HP model 3390A integrator.

Samples of 100 µL of culture supernatant were prepared for analysis by acidification with 10 µL of 4 M phosphoric acid. One microlitre of this was injected onto the GC column. Standard concentrations of the individual acids prepared quantitatively were treated in the same manner as the samples. A calibration curve of concentrations spanning the sample concentrations was determined for each acid each day samples were analyzed. The equation of the line obtained from a simple regression analysis of the peak areas was used to calculate the concentrations of the samples.

3.4.4 Methane Analyses

Methane analyses of the headspace gases were routinely done using the GC method of Fedorak and Hrudey (1983). A Microtek GC equipped with a 2 m x 2 mm glass column packed with GP 10% SP1000/1% H_3PO4 on 100/120 chromosorb WAW (Supelco) was used to quantitate methane. Nitrogen was the carrier gas at 20 mL/min. The flame ionization detector was supplied with 300 mL/min air and 30 mL/min hydrogen.

Lo-dose gas-tight syringes (Becton Dickinson) were used to withdraw 0.1 mL of headspace gases from the culture bottles and inject this onto the GC column. The syringe was rinsed with carbon dioxide between samples to prevent O_2 contamination and carry-over of methane to the next sample. Quantitative standards were prepared by the addition of known volumes of methane to sealed serum bottles of known volume. Peak areas recorded by a HP model 3390A integrator were used to determine the percent methane in the headspace gas.

Gas volume measurements were determined using a pressure transducer (Micro switch 142 PC 30G; Honeywell, Freeport Il.) assembled as described by Shelton and Tiedje (1984a). Calibration standards were prepared by injecting known amounts of methane into serum bottles containing the same amount of water as the culture volume. Transducer readings of the standards were performed when sample analyses were

performed and were analyzed by linear regression to obtain the calibration line for determination of the volume in each experimental culture. The resulting volumes were used in conjunction with the determined percent methane to allow the calculation of the actual volume of methane in the culture bottles.

3.4.5 GC/GPC Analyses

3.4.5.1 $^{14}CH_4$ and $^{14}CO_2$ Analyses

Radioactive $^{14}CH_4$ and $^{14}CO_2$ in culture headspace gases were separated and measured using a Varian Aerograph model 700 GC with a 3 m x 0.5 cm column packed with Poropak R. The GC was fitted with a thermal conductivity detector operated at 24°C and 150 mV. Helium was the carrier gas at 107 mL/min with propane as the quench gas at 10.7 mL/min. GC oven and injector temperatures were 60°C and 24°C, respectively. The effluent from the GC flowed into a GPC (Packard Model 894) operating at 1750 mV with windows set from 0 to infinity.

Headspace samples from cultures were removed with Lo-dose gas tight syringes and injected onto the GC column. The output from the GPC was recorded on a HP model 7128A strip chart recorder. Peak area measurements were determined manually using the width at half height method. The disintegrations per minute (dpm) were calculated using the following equation obtained from the operating manual for the GPC (Packard Industries)

$$\text{dpm} = \frac{\text{A (counts)} \times 100\% \times \text{total flow rate (mL/min)}}{\%\ \text{efficiency} \times \text{tube vol (mL)}}$$

Where:

total flow rate = the flow of gas through the system as measured at the outlet port.

$$\%\ \text{efficiency} = \frac{\text{A(counts)} \times 100\% \times \text{total flow rate (mL/min)}}{\text{dpm (determined by LSC)} \times \text{tube vol (mL)}}$$

tube vol= the volume of the proportional tube = 20 mL.

$$\text{A (counts)} = \frac{\text{peak height (mm)} \times \text{range (cpm)} \times \text{peak width (mm)}}{\text{chart height (mm)} \times \text{chart speed (mm/min)}}$$

range = the counting range set on the GPC (e.g. 100, 200, 500, 1000 cpm etc.)

The counting efficiency of the GPC was determined each day samples were analyzed. To do this equal volumes of the headspace gas from sealed serum bottles containing acidified (pH 1) $H^{14}CO_3^-$ were injected into the GC/GPC and into a second sealed serum bottle. The total amount of radioactivity in that volume of headspace gas was determined by flushing the second serum bottle as described by Fedorak et al. (1982). Carbosorb (Packard Industries) (1 mL in 10 mL aqueous counting scintillant (ACS, Amersham) was used as the trapping agent in two scintillation vials connected in series to the serum bottle containing the sample. The $^{14}CO_2$ was flushed through into the trapping vials with nitrogen gas at a flow rate of 100 mL/min. Radioactivity in the trapping vials was counted in a Mark III-6881-C Analytical Liquid Scintillation Counter (LSC) (Searle Analytic Inc.) on program 2 or a Beckman LSC model LS 3801 with windows set to count ^{14}C. The counting efficiency of the LSC and thus dpm were determined by the external standard (Mark III operations manual, Searle Analytic Inc) or the H# (LS 3801 Operations manual, Beckman Instruments) methods.

The average counting efficiency of the GPC was 81.3 ± 11.5% (n=99). The lower detection limit for the GPC was taken to be 60 dpm per peak per sample. This value was double the usual background dpm. Injections of up to 1 mL were used.

Total gas volume measurements were determined as described for methane analysis above. These volumes were used to calculate the total dpm of labeled compound in the headspace gases. Total $^{14}CO_2$ in cultures was determined by acidifying the cultures with 2 mL 4 M sulfuric acid before flushing, trapping and counting the $^{14}CO_2$ as described above.

3.4.5.2 ^{14}C-Labeled Volatile Organic Acid Analyses

The radioactivity in volatile organic acids was analyzed using the GC system described in section 3.4.3. A GPC was coupled to the outlet of the GC detector

through an adapted flame ionization flame tip. The GPC was set up as described above. Counting efficiencies were determined each day samples were analyzed. Equal volumes of authentic 1-^{14}C-acetate were injected onto the column and counted by the GPC or into a vial of ACS and counted by LSC. The average counting efficiency was 77 ± 19% (n=28). The lower detection limit of the GPC was taken to be 60 dpm per peak per injection. Sample volumes were limited to 1 µL by the column requirements so the sample had to contain approximately 100 dpm/µL of the compound of interest in order for a peak to be seen.

3.4.6 Analysis of Water Soluble Intermediates by HPLC

Water-soluble radioactive intermediates were separated using a Waters HPLC system with a Lambda Max model 480 LC spectrophotometer set at 210 nm unless otherwise indicated. Separation was performed with an HP Lichrospher 100 RP-18, 5 µm 125 x 4 mm reverse phase HPLC column. Two mobile phases were used. Mobile phase 1 contained 20% acetonitrile in 6.66 g/L potassium phosphate buffer adjusted to pH 2.3 with 86% phosphoric acid. Whereas mobile phase 2 contained 8% acetonitrile in the buffer. A Gilson model 201 fraction collector was used to collect 1.0-mL fractions. The fractions were combined with 9 mL ACS prior to LSC.

The analyses of culture supernatants and organic extracts of acidic culture supernatants, not containing radioactive compounds, were done using an HP Series 1050 HPLC equipped with a variable wavelength detector that allowed UV-visible scans of eluting peaks. UV-visible spectra of standard compounds were performed on the HPLC or, alternatively, were obtained using a Phillips Pye Unicam PU/8740 spectrophotometer.

The HPLC method was most reliable when the column was washed with 20 mL methanol after each days use. If the column was not washed, the retention times of compounds would decrease each day. The optimum sample size was 50 µL for

samples from experimental cultures although larger sample sizes could be used when more dilute cultures were sampled. Solids in the samples were removed by centrifugation, thus prolonging the life of the column.

The optimum fraction size was found to be 1 mL, smaller fractions tended to spread the sample counts over too great an area. The detection limit of the LSC was considered to be 60 dpm/fraction. Peaks of at least 60 dpm were double the usual background.

3.4.7 Determination of the Position of the ^{14}C-Label in Radioactive Acetate

The acetate produced by cultures fed either $^{14}CH_3$-labeled-m-cresol or $H^{14}CO_3^-$ was purified by ion exchange chromatography as described by Thauer et al. (1970). Anion exchange columns were packed with 2 g AG1-X8, 100-200 mesh (Bio-Rad) in its formate form. Samples were removed from the cultures (1 mL), made basic with 1 drop 0.5 M potassium hydroxide, incubated 1 h at room temperature and then centrifuged to pellet solids. The supernatants were applied to the resin and washed with 10 mL water. Acetate was eluted with 20 mL of 0.05 M formic acid. Formate was eluted with 1.0 M formic acid. m-Cresol did not elute from the column under these conditions. The acetate-containing fractions were collected, made basic with 0.5 M KOH and dried under vacuum using a cold finger to speed the process.

The purified potassium acetate was redissolved in 1 mL distilled water, transferred to a 16 x 150 mL roll tube and dried in an 80°C oven overnight. The dry tube was then cooled to -20°C to prevent violent reactions when the acid was added. Cold, 100% sulfuric acid (1 mL) (prepared by the addition of 4 mL fuming sulfuric acid to 5 mL concentrated sulfuric acid) was added before the sample container was sealed and then refrozen to prevent explosion when the azide was added. The Schmidt degradation (Phares 1951) was initialized by the addition of 30 mg sodium azide (Fisher). A stir

bar was added before the tube was sealed with an adapted butyl rubber stopper as shown in Figure 3.1. The stopper was adapted by the addition of two glass tubes spanning the stopper and attached on the outside to rubber tubing. Leakage of gases was prevented by clamping the rubber tubing with two screw clamps on each tube. The remainder of the Schmidt degradation was carried out in a fume hood with the safety glass closed for maximum safety. Protective clothing was worn at all times. The reaction mixture was then warmed to room temperature before placing in an oil bath which was then heated to 80°C over about 30 min. The reaction mixture was kept at this temperature for 1 h with stirring.

The $^{14}CO_2$ produced from the carboxyl carbon of acetate by the Schmidt degradation was then trapped in ACS and Carbosorb as described above in section 3.4.5.1 using a modified flushing apparatus as shown in Figure 3.1. Glass tubes replaced metal parts in the trapping vials. The exit line from the reaction vessel was connected to the flushing train and the entry line was connected to a nitrogen source. The clamps on the exit tube were opened slowly to release the pressure in the reaction vessel then closed again. The reaction vessel was filled with nitrogen through the input line and the clamps on the exit line were opened again. The reaction vessel was flushed for 5 min with nitrogen bubbling through the reaction mixture at a flow rate of 100 mL/min. The radioactivity collected was counted by LSC.

In the reaction, the ^{14}C found in the methyl carbon of acetate was converted to methylamine that remained in the acidic reaction mixture. After flushing, the reaction mixture was diluted with 9 mL distilled water. Triplicate 1 mL samples of this were removed and added to 9-mL portions of ACS in separate scintillation vials and counted by LSC.

Standard 1-^{14}C-acetate and 2-^{14}C-acetate were used to test each step in the Schmidt degradation. The first step was the anion exchange purification of the acetate. Standard 1-^{14}C-acetate was used to determine the recovery of acetate from the column.

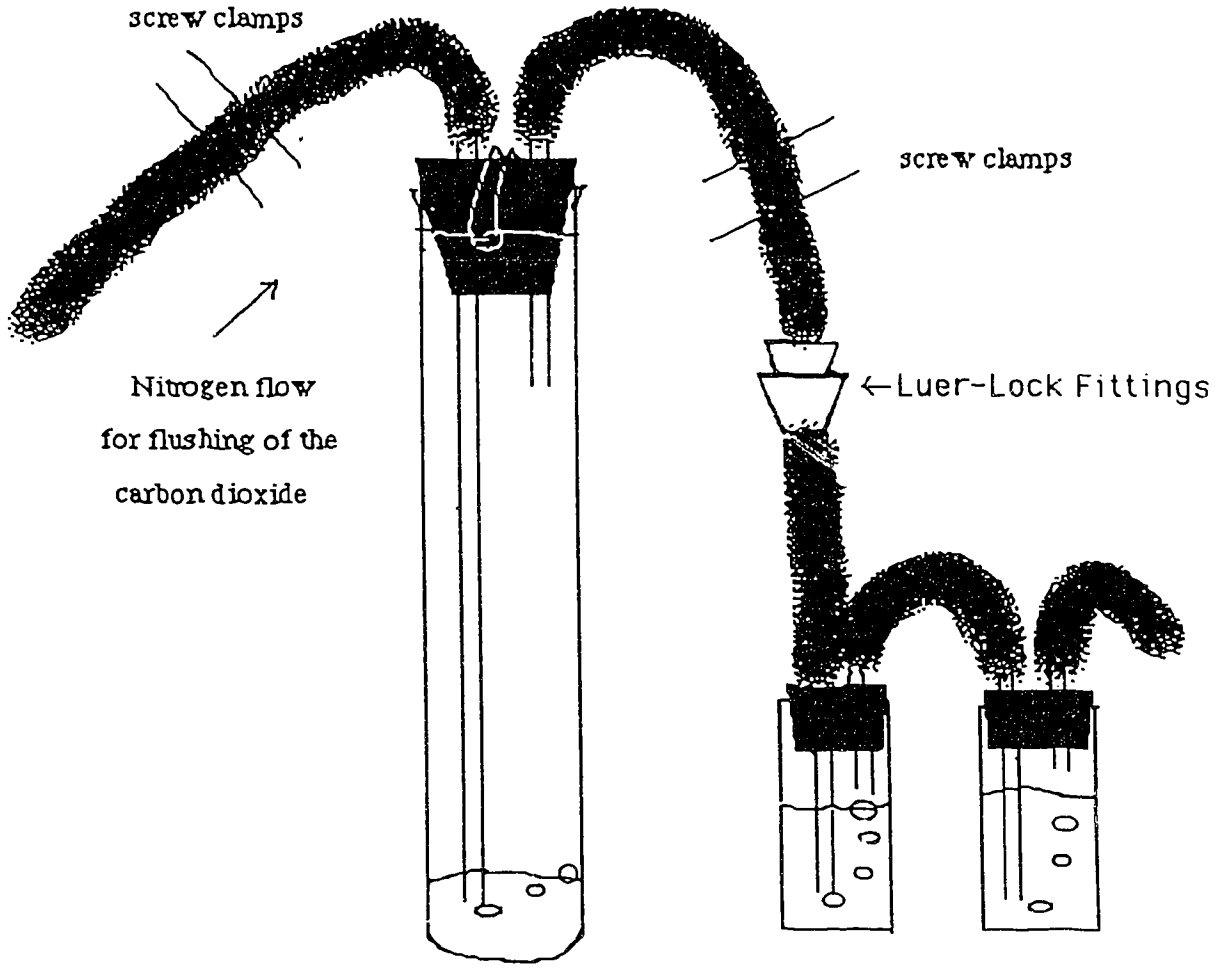


Figure 3.1. The modified reaction vessel and carbon dioxide trapping vials used for the Schmidt degradation.

The 1-^{14}C-acetate was recovered in four fractions containing 95 ± 5.4% (n=6) of the total ^{14}C applied to the column. The recovery from flash drying the pool of these four fractions was 97 ± 8% (n=19). The recovery from the transfer to the reaction vessel and the second drying was 97 ± 7% (n=12) of the ^{14}C used in each step.

The recoveries from the standard 1-^{14}C-acetate and 2-^{14}C-acetate subjected to the Schmidt degradation were as follows. The 1-^{14}C-acetate gave a mean recovery of 86 ± 3% carbon dioxide and 2 ± 0.7% was recovered in the liquid and assumed to be methylamine. This resulted in a total of 88% recovery of the radioactive label used (n=5). The 2-^{14}C-acetate gave a mean recovery of 1% carbon dioxide and 96% in the liquid (n=2).

The method itself was difficult to master as there were many separate steps each with its own hazards. The 100% sulfuric acid caused corrosion of any metal with which it made contact and extracted colored compounds from butyl rubber stoppers. Another problem encountered was the backflushing of trapping fluid into the lines. The method was finally perfected by using glass fittings on the flushing apparatus and extreme care in handling. Determinate errors occurred in approximately 7% of the analyses of standards (during the initial implementation of the method) but less often with the actual samples.

3.4.8 Determination of the Amount of ^{14}C Incorporated Into Macromolecules from Labeled Substrates

The amount of ^{14}C in macromolecules was determined by the method of Frazer and Young (1986). Samples of 1 mL of the culture that had been acidified and flushed to determine $^{14}CO_2$ content (section 3.4.5.1) were filtered through 0.22-µm filters (Millipore, Bedford, Ma.) then rinsed with 1 mL distilled water, 1 mL 10% trichloroacetic acid, 1 mL 5% trichloroacetic acid then 2 mL 70% methanol in water. Each rinse was collected separately. The filters and 1 mL of each rinse were counted

separately in 10 mL ACS by LSC.

3.4.9 Sulfide Analyses

The concentration of sulfide in sewage sludge or experimental cultures was determined using the method of Fedorak et al. (1986b). The samples were placed in sealed vessels then acidified with 4 M hydrochloric acid. The released sulfide was flushed with nitrogen gas through a gas dispersion tube into a receiving vessel containing 70 mL 0.1 M silver nitrate. The silver sulfide precipitate was then removed from the trapping solution by vacuum filtration. The amount of silver removed as silver sulfide was determined by adding 70 mL of 0.1 M potassium chloride and titration with 0.05 M silver nitrate. The Fajans titration procedure was used to quantitate excess chloride using dichlorofluorescein as the endpoint indicator.

Sulfide was calculated as 1/2 the molar amount of silver ion. Silver ion concentration was calculated as the amount of chloride ion that did not precipitate with the silver when the potassium chloride was added to the filtered trapping solution. All solutions were standardized against potassium chloride to determine their molarities.

3.4.10 Chemicals

The standards used in the identification of soluble intermediates were obtained as follows. Laboratory grade 2-hydroxy-4-methylbenzoic acid (4-methylsalicylic acid), p-hydroxyphenylacetic acid and glacial acetic acid were purchased from Aldrich Chemical Co. 2-Methylbenzoic acid was obtained from Matheson Coleman and Bell. 3-Methylbenzoic and 4-methylbenzoic acids were purchased from Pfaltz and Bauer. Phenylacetic acid was purchased from Sigma Chemicals. 2-Hydroxy-6-methylbenzoic (6-methylsalicylic acid) was kindly provided by Dr. G.M. Gaucher, University of Calgary, Calgary, Alberta.

The methyl esters of aromatic carboxylic acids were prepared using the method of

Fedorak and Westlake (1983). Cultures were acidified to pH 1 with 4 M H_2SO_4 and extracted with ether. One millilitre of the extract was put in a 25-mL round bottom flask. The solvent was removed under a slow stream of nitrogen gas. Two millilitres of HPLC grade methanol were then added and 1 drop of concentrated sulfuric acid. The sample was heated to boiling and kept at this temperature for 1 hour while refluxing through a coiled condenser. The sample was then cooled and diluted with 5 mL distilled water. The methyl esters were extracted with methylene chloride, washed twice with 0.2 M potassium bicarbonate and then water. The sample was concentrated by evaporation of the solvent under a slow stream of nitrogen gas.

4-Hydroxy-2-methyl benzoic acid was synthesized from m-cresol by the reaction described by Komiyama and Hirai (1984) without the use of cyclodextrins. The reaction was carried out with 3.0 g of m-cresol, 0.2 g of copper powder in 40 mL of 20% (w/v) aqueous sodium hydroxide. Six millilitres of carbon tetrachloride was added to start the reaction which was carried out under nitrogen at 80°C for 16 h. The reaction vessel was a three necked round bottom flask fitted with a coiled condenser. The other two necks were plugged with a glass stopper or a rubber stopper respectively. The rubber stopper was penetrated with a canula attached to a cylinder of nitrogen. Gas flow through the canula provided a nitrogen atmosphere in the flask. When the reaction was complete, the reaction mixture was adjusted to pH 8.5 with 6 M HCl, extracted with methylene chloride until the remaining m-cresol was removed. The aqueous phase was then adjusted to pH 1 with 6 M HCl before extraction with diethyl ether to remove the reaction products.

Sodium ^{14}C-bicarbonate, ring-U-^{14}C-m-cresol, ^{14}C-formate, 1-^{14}C-acetate and 2-^{14}C-acetate were obtained from Amersham Canada Ltd. (Oakville, Ontario). Custom synthesis of $^{14}CH_3$-m-cresol and $^{14}CH_3$-p-cresol were done by Pathfinder Laboratories (St. Louis, Missouri)

3.4.11 Electron Microscopy.

Scanning electron microscopy was performed by E. Schwaldt in the Medicine/Dentistry EM Unit, University of Alberta. The samples were first fixed with 2.5% glutaraldehyde in Milloning's buffer. The samples were then washed three times for 15 min each with Milloning's buffer and post fixed for 45-60 min in 1% osmium tetroxide, followed by three 10 min washes in double distilled water.

The samples were then dehydrated through a graded series of ethanol solutions. The samples in absolute ethanol were transferred to a Sevac critical point dryer. The samples were mounted with silver glue on aluminum stubs, sputter coated with gold (Edwards, model S150B sputter coater) and were then examined in a Phillips 505 scanning electron microscope at 20 kV.

3.4.12 Statistical methods.

The statistical methods used to evaluate the data obtained in this research were all available as APL functions written for the Apple Macintosh Computer. Most functions were adapted from the University of Alberta Computing Services APL Public Library.

Equations for best fit calibration curves for methane and substrate analyses were calculated by simple linear regression analysis. If the correlation coefficient was <0.99 the calibration method was repeated. Evaluation of data from experiments that did not require comparisons to control cultures were done using an ANOVA analysis and Duncan's multiple range test (Steel and Torrie 1980). When the effects of a series of test treatments were compared to a control treatment, the method of Dunnett (1955) was used. When the difference between two independant regressions was compared the t-test of Steel and Torrie (1980) was used The above analyses were all performed testing the H_0 hypothesis at $P<0.05$. This meant that there was a 5% chance that a correct hypothesis would be rejected

4. Culturing Methods and Characterization of m-Cresol-Degrading Consortia

Investigations concerning the ability of environmental sources to degrade m-cresol under anaerobic conditions have used a variety of inocula and culture conditions. Horowitz et al. (1982) and Boyd et al. (1983) used a 10% (v/v) sewage sludge inoculum to obtain m-cresol-degrading methanogenic consortia. Fedorak and Hrudey (1984) used a 50% (v/v) sewage sludge inoculum amended with 1 mM sodium sulfide. Smolenski and Suflita (1987) used aquifer solids and undiluted ground water amended with 1 mM sodium sulfide to obtain m-cresol degrading enrichment cultures.

Media described in the literature were often simple minimal nutrient media supplying B-vitamins, essential trace minerals, inorganic phosphate, and ammonium-nitrogen. The media are usually buffered with bicarbonate/carbon dioxide to a pH of 6.9-7.1 and prereduced with sulfide/cysteine, AFS or 1 mM sodium sulfide, before inoculation.

Although many environmental sources may contain facultative organisms which will utilize oxygen and quickly establish anaerobic conditions, the culture of methanogenic consortia relies on extreme care in excluding oxygen from the culture vessels. The culture vessels for both enrichment cultures and subsequent transfers of these enrichment cultures are flushed with nitrogen/carbon dioxide mixtures that have been scrubbed free of oxygen by passage through a heated copper column to provide anaerobic conditions. This not only provides conditions suitable for the growth of methanogens but ensures that the degradation of m-cresol observed will be due to anaerobic activities.

The work described in this chapter was performed to accomplish three goals, these were:

1. To survey various media and enrichment procedures to improve the growth of m-cresol-degrading consortia.

2. To determine whether established m-cresol-degrading consortia can utilize other electron acceptors.

3. To determine the response of m-cresol-degrading consortia to elevated m-cresol concentrations.

The detailed experimental procedures are given in Appendix 2.1. The results in Section 4.1 were obtained early in the project whereas those in Sections 4.2 and 4.3 were obtained late in the project.

4.1 Comparison of the Activities of m-Cresol-Degrading Consortia in Three Different Media

Initial enrichment cultures for m-cresol degradation were obtained using 98% (v/v) inoculum from the Edmonton Gold Bar Sewage Treatment Plant. The 90 mL cultures were amended with 1 mL of a 27,000 mg/L m-cresol solution (final concentration 300 mg/L) and 1 mL of a 0.01% resazurin solution as a redox indicator. After an initial adaptation period the resultant enrichment cultures were able to degrade m-cresol but were not able to consistently survive transfers to fresh medium for subsequent experimental batch cultures.

Therefore, three different media were examined for their ability to sustain transfers of m-cresol-degrading enrichment cultures. The abilities of two-fold serial dilutions of an m-cresol enrichment culture to degrade m-cresol were tested in the Medium WR86, and modified versions of Butlin's medium and *Methanosarcina* medium. Butlin's medium is commonly used to culture lactate-utilizing SRB. For this experiment the medium was modified to contain m-cresol rather than lactate as the carbon source. *Methanosarcina* medium has been used to culture *M. barkeri*. The organic substrate normally supplied in the medium was replaced with m-cresol as the major organic carbon source.

The results after 47 days of incubation showed that in Medium WR86 and

modified *Methanosarcina* medium only the first dilution (1/2) was capable of carrying out m-cresol degradation whereas dilutions up to and including the fifth dilution (1/32) in the modified Butlin's medium were capable of degrading m-cresol.

An examination of the differences between the modified Butlin's medium and the other two media indicated that sulfate salts and yeast extract were present in modified Butlin's medium and not in the others. New modified media were prepared, each lacking one of these compounds. Two-fold dilutions of enrichment inoculum were again made in each of the modified media.

The inoculum again grew well in all three of the media formulations. The last dilution able to degrade m-cresol after 56 days incubation was 1/128 in the complete modified Butlin's medium and the medium lacking sulfate, and 1/256 in the medium lacking yeast extract. These results did not explain the improved ability of the inoculum to survive dilution but did indicate that sulfate and yeast extract were not the beneficial ingredients. Eventually, all of the dilutions made in modified Butlin's medium minus sulfate had degraded all of the m-cresol present. The final dilution (1/1024) was maintained with m-cresol as the major carbon source. This was designated Consortium A.

Another difference among the three different media tested was the methods used to prereduce them to an Eh value which turned the resazurin indicator colorless. Iron nails were used to prereduce Butlin's medium whereas sulfide (1 mM final concentration) was used to prereduce Medium WR86 and a mixture of cysteine and sulfide (1.7 and 1.25 mM final concentrations respectively) to prereduce *Methanosarcina* medium. Therefore, the effects of the reducing methods were the focus of a subsequent experiment.

4.2 Comparison of the Effect of Commonly Used Reducing Agents on m-Cresol Degradation

In order to further investigate the effects of using different reducing agents to prereduce medium for transfers of m-cresol on m-cresol degradation, three different reducing agents were tested. Figure 4.1 shows that different methods of prereducing Medium WR86 influenced the m-cresol-degrading ability of transfers from enrichment cultures. In the transfer cultures reduced with 2 iron nails, the sterile medium was left in contact with the nails for 1 week prior to inoculation. This reduced the Eh of the medium to below the potential of resazurin. In contrast, the medium was reduced below the potential of resazurin in a matter of seconds when 0.1 ml of AFS or 0.1 or 0.05 ml of sodium sulfide were added as prereductants.

The onset of m-cresol degradation was quickest in the cultures containing iron nails as a reducing agent. Statistical analysis of the results showed that the concentrations of m-cresol in the medium were significantly lower ($P<0.05$) than those in the cultures prereduced with 1 mM sodium sulfide (control) on day 4 and subsequent analyses to the end of the incubation period. m-Cresol degradation started more quickly in medium reduced with AFS than in medium reduced with 0.5 mM sodium sulfide (Figure 4.1). Statistical analysis showed that m-cresol concentrations in cultures prereduced with AFS were significantly different ($P<0.05$) from the cultures prereduced with 1 mM sodium sulfide on day 11 and subsequent analyses to the end of the incubation. Cultures prereduced with 0.5 mM sodium sulfide did not contain significantly different ($P<0.05$) concentrations of m-cresol than the cultures prereduced with 1 mM sodium sulfide until after 14 days of incubation. Little degradation was observed in the medium reduced with 1 mM sodium sulfide. Methane production accompanied the m-cresol metabolism in the cultures reduced with nails, AFS and 0.5 mM sodium sulfide.

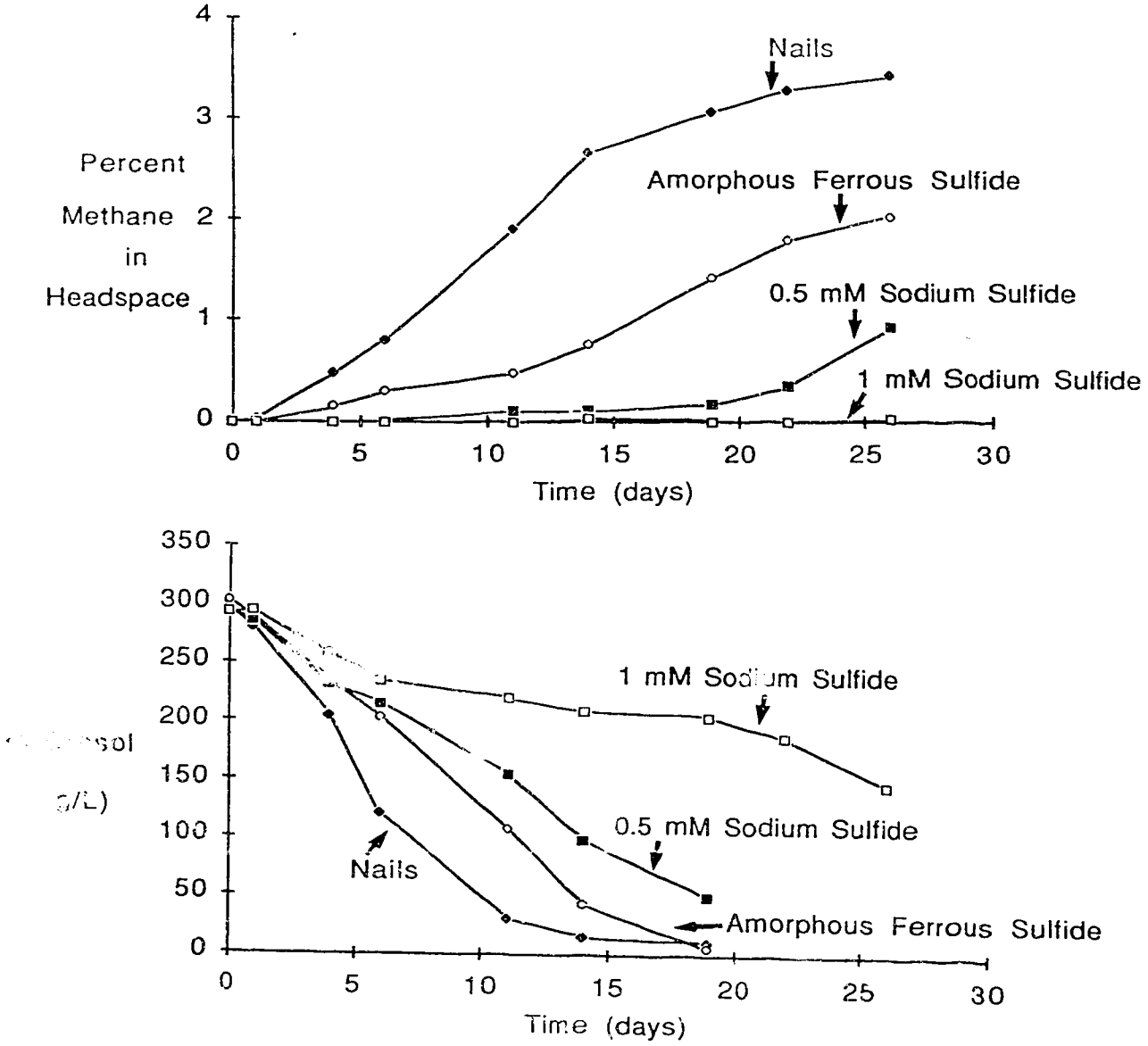


Figure 4.1. The effects of different methods of reducing Medium WR86 on the degradation of m-cresol and subsequent methane production. Triplicate cultures were prereduced with one of the reducing agents prior to inoculation with 5 mL of a 50% transfer of an m-cresol-degrading enrichment culture. m-Cresol concentration and methane volume were determined by GC methods.

4.3 Examination of Methods to Obtain Improved m-Cresol-Degrading Enrichment Cultures

The initial enrichment cultures obtained from the Edmonton Gold Bar Sewage Treatment Plant degraded their initial allotment of 300 mg/L m-cresol after a 51 d lag period. These cultures were then fed a second allotment of m-cresol which was degraded after a 3 week lag. Subsequent allotments of m-cresol were degraded quickly by the enrichment cultures, allowing the 80-mL cultures to be fed 300 mg/L m-cresol once per week. Transfers of these enrichment cultures to new medium for batch experiments were not consistently successful. When new enrichment cultures were required it was decided to examine the methods used to establish enrichment cultures to see if methods could be developed to obtain more stable enrichment cultures in a shorter time period.

4.3.1 Sulfate Addition to Sludge Inoculum

Smolenski and Suflita (1987) found that an inoculum from a sulfate-reducing site of an anoxic aquifer was able to degrade m-cresol with a shorter lag time (43 d) than an inoculum from a methanogenic site of the same aquifer (46-90 d). As an application of these observations, sodium sulfate was added to sewage sludge to determine if its presence would have any effect on the ability to obtain m-cresol-degrading enrichment cultures.

The addition of sulfate to enrichment cultures of anaerobic sewage sludge increased the acclimation time for m-cresol degradation (Table 4.1). After 73 d incubation, only 1 of 5 cultures which received sulfate had begun to degrade m-cresol whereas this substrate was metabolized in all 5 unsupplemented cultures. These observations are in contrast to those of Smolenski and Suflita (1987). Although an active sulfate-reducing population in our sludge increased the free and total sulfide concentrations significantly in the sulfate supplemented cultures (Table 4.1), the

active sulfate-reducing population in our sludge increased the free and total sulfide concentrations significantly in the sulfate supplemented cultures (Table 4.1), the

Table 4.1. The effect of sulfate addition on the degradation of m-cresol.[a]

Sulfate Added	Number of cultures degrading m-cresol	Free sulfide (mM)[b]	Total sulfide (mM)[b]
No	5	1.8 ± 0.5	4.8 ± 0.3
Yes	1	3.1 ± 0.4	6.7 ± 0.8

[a] Anaerobic sewage sludge was supplemented with 300 mg/L m-cresol. One set of five replicates received no sulfate, and the other received 14 mM sodium sulfate. Samples were analyzed by GC to determine m-cresol concentration and using the sulfide analysis procedure for sulfide analysis after 73 days of incubation.

[b] Values are mean ± SD.

reduction of sulfate was not coupled to m-cresol degradation.

4.3.2 Dilution of Sludge Inoculum

Many anaerobic habitats contain sulfide as a result of the action of SRB. The sulfide concentrations in sewage sludge digestors are typically not inhibitory to methanogenic consortia. In order to determine if the inoculum from the Edmonton Gold Bar Sewage Treatment Plant contained concentrations of sulfide that would inhibit m-cresol degradation, enrichment cultures were established with various volumes of anaerobic sewage sludge diluted in anaerobic distilled water to decrease the initial sulfide concentrations. Figure 4.2 shows the changes in m-cresol concentration in these cultures. Net methane production was determined for each inoculum size by subtracting the amount of methane produced by control cultures containing the same volume of sludge without m-cresol. The negative net methane volumes in Figure 4.2 indicate that the presence of 220 mg/L m-cresol initially inhibited the production of methane from the organic compounds in the sludge inoculum.

The 96% (v/v) sludge cultures required 61 d incubation before m-cresol degradation and subsequent methane production were observed. Sulfide analyses performed on these cultures showed that the total sulfide concentration was 0.8 mM. The acclimation times for m-cresol degradation were decreased by diluting the sludge. The cultures which contained 24% sludge (calculated sulfide = 0.2 mM) began m-cresol degradation 10 d before the 48% cultures (calculated sulfide = 0.4 mM) and 24 d before the 96% inoculum. Vigorous methane production was observed in each of the dilutions shortly after the onset of m-cresol degradation.

The dilution of the sludge inoculum not only shortened the acclimation time for m-cresol degradation as shown in Figure 4.2, but it also yielded cultures which metabolized m-cresol more quickly after subsequent feedings. Five millilitre portions of 3,000 mg/L m-cresol in WR86 medium (prereduced with sodium sulfide to a final

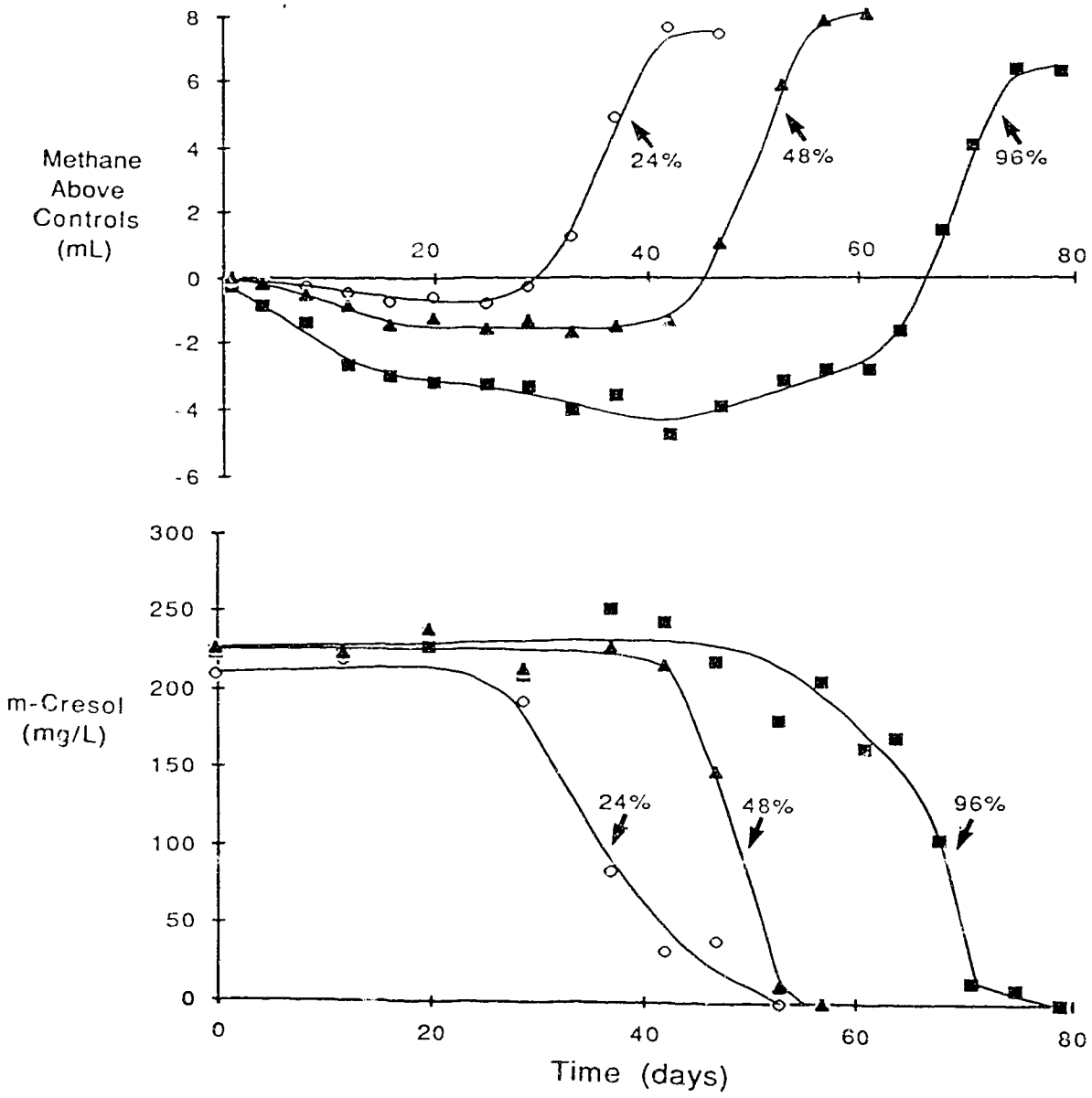


Figure 4.2. Net methane production and m-cresol concentrations in enrichment cultures that received various dilutions (expressed as percent by volume) of anaerobic sewage sludge. The total sulfide concentration in the 96% (v/v) sludge culture was 0.8 mM. The sludge was diluted with anaerobic distilled water.

concentration 0.5 mM) were added to duplicate cultures after methane production had ceased in the diluted enrichment cultures. The 24% sludge cultures completely consumed their fresh substrate in 14 d. In contrast, the duplicate 96% sludge cultures required 28 and 35 d to completely consume their substrate.

The dilution of sludge inoculum was seen to allow m-cresol degradation to begin in a shorter period of time. This may be due to the dilution of sulfide in the sludge inoculum or there could have been other inhibitory factors in the sludge inoculum that were decreased to below inhbitory levels by the dilution process.

4.3.3 Sulfide Addition to Sludge Inoculum

To provide further evidence that the presence of sulfide was inhibitory to m-cresol degradation, fresh sludge samples (93% inoculum) were amended with 2 mM sulfide, or left unamended, to demonstrate the direct effect of sulfide on the enrichment procedure. The cultures were monitored over the incubation period to determine m-cresol concentration and methane production. The results are presented in Figure 4.3.

The unamended cultures began to degrade m-cresol after 41 d incubation. The addition of 2 mM sulfide [illegible] m-cresol degradation over the 71 d that the cultures were monitored. The addition of 4 mM ferrous iron precipitated the added sulfide and restored the ability of the enrichment cultures to degrade m-cresol. Statistical analysis of the results showed that the amount of methane in the headspace gases was significantly lower ($P<0.05$) in the cultures that received 2 mM sulfide than in the unamended cultures (controls) after 56 d incubation. At that time the m-cresol present in the unamended cultures had been almost completely been degraded, whereas it was still present in the cultures amended with 2 mM sodium sulfide. This indicated that the difference in methane production was due to m-cresol degradation in the unamended cultures. The cultures that received 2 mM sulfide plus 4 mM ferrous iron were seen to

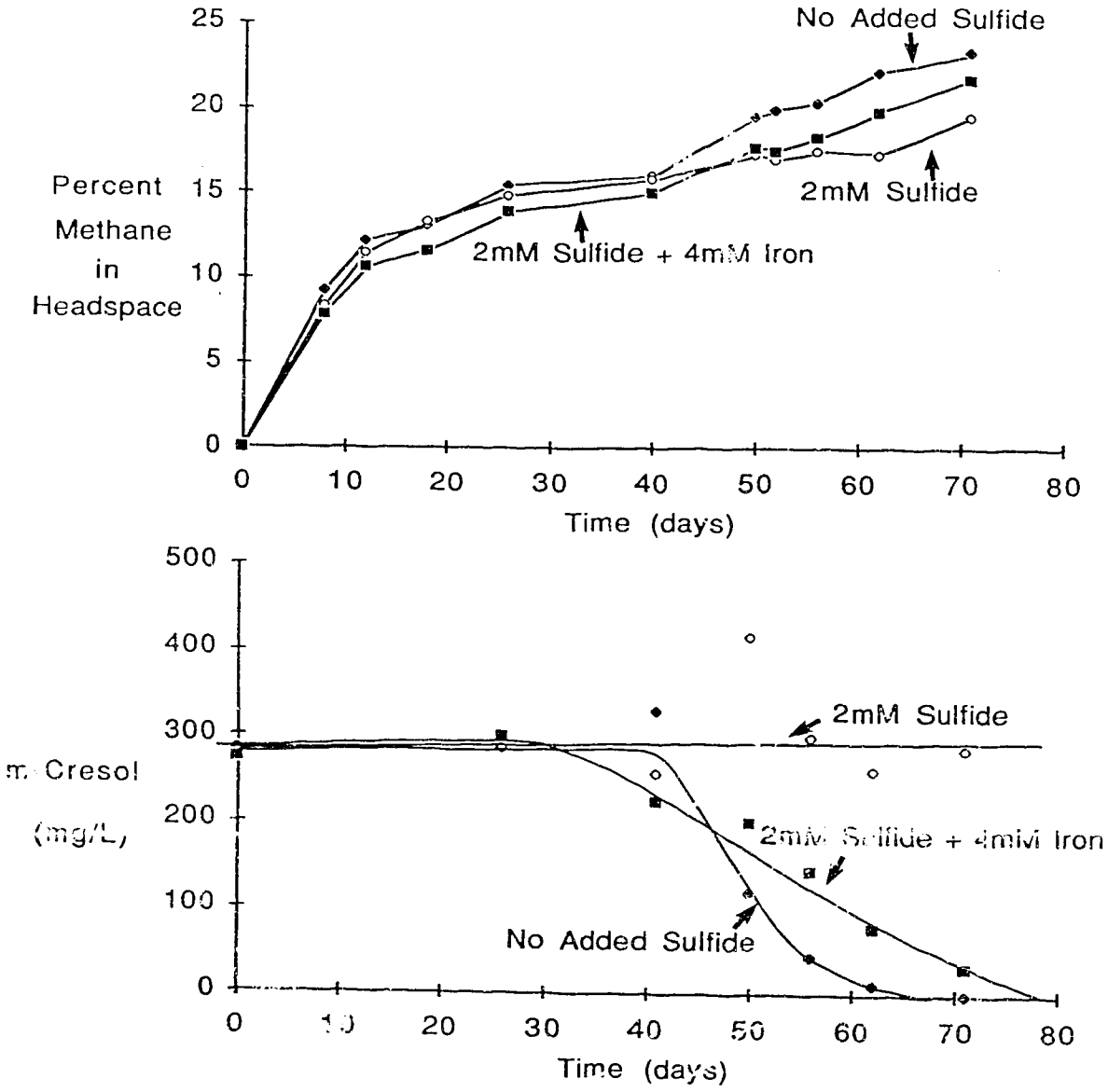


Figure 4.3. Inhibition of m-cresol degradation by sulfide addition in methanogenic enrichment cultures. Triplicate cultures of 93% sewage sludge were used for the experiment.

contain amounts of methane that were not significantly different ($P<0.05$) than those in the unamended cultures throughout the incubation. The total percent methane in the headspace gases was presented in Figure 4.3 to demonstrate the production of methane throughout the incubation period. This suggested the production of methane from organic compounds in the initial sludge inoculum was not inhibited by sulfide.

The unamended 93% inoculum used in these cultures required approximately 41 d before m-cresol degradation began (Figure 4.3). This acclimation time was much shorter than the acclimation time of approximately 55 days observed for the 96% inoculum of sewage sludge seen in Figure 4.2. The 48% inoculum of sewage sludge seen in Figure 4.2 required approximately 41 d before m-cresol degradation began, which was more comparable to the lag time observed for unamended sludge shown in Figure 4.3. These results demonstrate the heterogeneity of the inoculum from the Edmonton Goldbar Treatment Plant. The influent to the digestor is not consistent, but varies with time, therefore the condition of the sludge will vary with time as well. The inocula used for the experiments depicted in Figures 4.2 and 4.3 were obtained two months apart; volatile suspended solids were not analysed so a comparison of biomass in the inocula was not possible. Nor was the total sulfide concentration analyzed.

4.4 The Effect of m-Cresol Concentration on m-Cresol Degradation

Five millilitres portions of a 26-month-old m-cresol-degrading enrichment culture were used to inoculate triplicate cultures consisting of 4 mL of Medium WR86, 0.5 mL of a 400 mg/L solution of glacial acetic acid (final concentration 20 mg/L) and 0.5 mL of anaerobic distilled water, or 6,500, 8,400, 11,000, 14,000, or 15,000 mg/L m-cresol solutions (final concentrations 320, 420, 560, 710, and 740 mg/L, respectively). The concentrations of m-cresol and the amounts of methane in the culture were determined several times during the incubation period.

The results presented in Figure 4.2 suggested that the concentration of m-cresol in the enrichment culture may have had an adverse effect on the methanogenic population from the sewage digestor. This was further investigated using transfers of m-cresol-degrading enrichment cultures. Triplicate cultures were fed 320, 420, 560, or 710 mg/L m-cresol and monitored for m-cresol degradation and methane production. Figure 4.4 presents the results of m-cresol and methane analyses in these cultures.

The volume of methane, produced from substrates brought in with the inoculum, in control cultures (not containing m-cresol) over the initial 2 d incubation was significantly higher ($P<0.05$) than that in the cultures fed m-cresol. This indicated that the methanogenic bacteria were inhibited by the m-cresol present in the test cultures over the initial 2 d of incubation. The rates of methane production, as determined from the slopes of the linear portions of the curves, were 0.53, 0.38, and 0.32 mL methane/day for cultures receiving 320, 420, and 560 mg/L m-cresol, respectively. Statistical analyses of the data indicated that the rates of methane production decreased significantly ($P<0.05$) with increased initial m-cresol concentrations.

The rates of m-cresol degradation, as determined from the linear portions of the curves (Figure 4.4), were 42, 33 and 33 mg/L m-cresol/day for cultures receiving initial concentrations of m-cresol of 320, 420 and 560 mg/L, respectively. Statistical analyses of these data indicated that the rate of m-cresol degradation in cultures fed 320 mg/L was significantly different than that in cultures fed 420 and 560 mg/L m-cresol ($P<0.05$).

The rate for m-cresol degradation in the cultures fed 710 mg/L was determined to be 53 mg/L m-cresol/d for the first three days incubation. This rate was not significantly different from the rate of m-cresol degradation in cultures fed 320 mg/L m-cresol. The loss of ability to degrade m-cresol seen in the cultures fed 710 mg/L m-cresol was probably due to an accumulation of intermediates caused by the inhibition of the methanogenic bacteria by the m-cresol present. There was no methane produced.

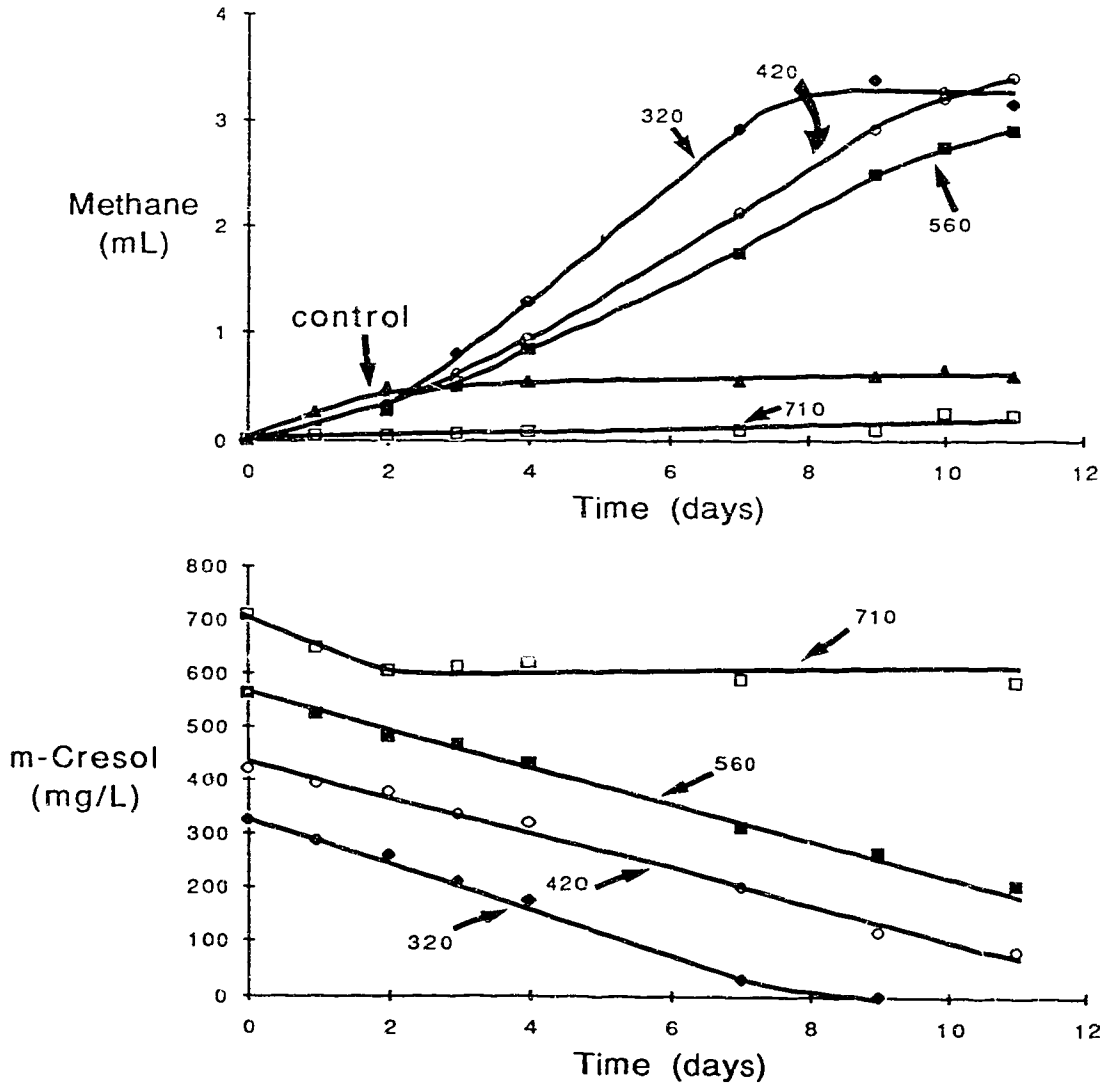


Figure 4.4. m-Cresol degradation and methane production in 50% transfers of methanogenic m-cresol enrichment cultures fed increasing levels of m-cresol. The curves are identified by the initial concentration of m-cresol (mg/L).

These results suggested that the methanogenic members of the enrichment culture were more sensitive to m-cresol concentration than were the m-cresol-degrading bacteria but that the latter could not completely mineralize m-cresol without an active methanogenic population. Fedorak et al. (1986a) also found that the rate of degradation of phenolic compounds could be slowed by inhibiting the methanogenic members of the cultures. The use of specific inhibitors such as BESA and cyanide, or the addition of acetate, to simulate product accumulation all inhibited the degradation of phenolic compounds (Fedorak et al. 1986a).

4.5 The Use of Alternate Terminal Electron Acceptors by m-Cresol-Degrading Cultures

The anaerobic degradation of aromatic compounds can be coupled to sulfate and nitrate reduction as well as methanogenesis. Research with cultures utilizing these terminal electron acceptors has allowed the isolation of pure cultures of aromatic compound-degrading organisms. For example *Desulfobacterium phenolicum,* which can degrade phenol and p-cresol as well as other non-phenolic aromatic compounds, was isolated under sulfate-reducing conditions (Bak and Widdel 1986). Tschech and Fuchs (1987) have isolated pure cultures of denitrifying pseudomonads capable of the degradation of phenol and m- and p-cresol among other aromatic compounds. Bossert et al. (1986) isolated a bacterial coculture capable of the oxidation of p-cresol under nitrate-reducing conditions.

There have also been reports concerning the isolation of organisms capable of using electron acceptors other than those on which the culture had been enriched. Ferry and Wolfe (1976) isolated a benzoate-utilizing facultative anaerobefrom a benzoate-degrading methanogenic enrichment. Evans (1977) isolated a nitratereducing, benzoate-utilizing organism from a methanogenic benzoate-degrading enrichment.

Early studies with sewage sludge inocula suggested that sulfate would not serve as a terminal electron acceptor during m-cresol degradation (Section 4.3.1) and that sulfide (the endproduct of sulfate reduction) was inhibitory to m-cresol degradation (Sections 4.2 and 4.3.3). Experiments were performed to confirm these results using a methanogenic culture enriched on m-cresol and to determine if this culture could utilize other alternate electron acceptors during m-cresol degradation. Triplicate 50% transfers of the second 2-L m-cresol-degrading enrichment culture were incubated in the presence of 20 mM sodium sulfate, sodium thiosulfate or potassium nitrate. As a positive control, a set of cultures were incubated with carbon dioxide as the terminal electron acceptor. BESA was added to the cultures containing the alternate electron acceptors to inhibit methanogenesis. A triplicate set of cultures was incubated aerobically to determine if the m-cresol-degrading organism might be a facultative anaerobe capable of using oxygen as the terminal electron acceptor. Thermodynamic considerations (explained in section 2.3 equations 2.1-2.3) suggest that the nitrate or sulfate present as alternate electron acceptors would be preferentially utilized over the carbon dioxide present as the buffer in the medium.

The results showed that m-cresol was not utilized by this inoculum when thiosulfate, nitrate and oxygen were present as terminal electron acceptors. m-Cresol was initially utilized by cultures containing sulfate as the terminal electron acceptor but a subsequent feeding of m-cresol was not utilized. There was no evidence of sulfate reduction (i.e. no black precipitate of FeS observed). Calculations showed that there was sufficient sulfate present to allow the degradation of a second allotment of m-cresol. Smolenski and Suflita (1987) have calculated that sulfate-reducing enrichment cultures degrading p-cresol used 3.4 mol sulfate/mol p-cresol. The cultures receiving sulfate as a terminal electron acceptor in the experiment performed in our lab used 0.016 mmol m-cresol present in the first aliquot of m-cresol. If the results from the studies of the degradation of p-cresol can be applied to m-cresol then 0.05 mmol of

sulfate would have been used. The cultures received 0.2 mmol of sulfate initially, indicating that there would be 0.15 mmol of sulfate remaining.

Methane was produced by the cultures incubated with carbon dioxide as a terminal electron acceptor in the absence of BESA but not by cultures incubated with any of the alternate electron acceptors or by the BESA-inhibited culture incubated with carbon dioxide as the terminal electron acceptor. The fermentation of the first allotment of m-cresol in the absence of methanogenesis, due to BESA inhibition, was observed throughout the study and will be presented in more detail in Chapter 5.

These results suggested that m-cresol degradation could not be coupled to the use of any electron acceptors other than carbon dioxide, which the culture had been enriched upon. Similarly, Suflita et al. (1989) tested the ability of phenol- and p-cresol-degrading sulfate-reducing enrichment cultures to utilize the alternate electron acceptors sulfite, thiosulfate, nitrate and carbon dioxide and found that no substrate depletion was evident with any treatment other than sulfate as a terminal electron acceptor.

4.6 Attempts to Isolate Specific Nutritional Types of Bacteria from the m-Cresol-Degrading Consortium

The members of consortia involved in the methanogenic metabolism of phenol and benzoate have been defined according to nutritional type. Phenol-degrading methanogenic consortia were proposed "to consist of three interacting physiological groups of bacteria: a phenol metabolizer, an hydrogen gas-utilizing methanogen, and an acetotrophic methanogen" (Dwyer et al. 1986). Knoll and Winter (1989) described their phenol-degrading consortium to consist of a phenol-carboxylating organism, a benzoate-degrading organism, and hydrogen gas-utilizing methanogens. Small numbers of *Desulfovibrio* were also present in the cultures. Acetate accumulated in the cultures as an endproduct of benzoate metabolism. Acetate-utilizing methanogens were

not present in the cultures.

Ferry and Wolfe (1976) observed three predominant methanogenic organisms in benzoate-degrading methanogenic enrichment cultures. Two of these, *Methanobacterium formicium* and *Methanospirillum hungateii*, were isolated from the cultures with hydrogen gas as the substrate. An acetate-utilizing methanogen could not be isolated. None of the methanogens were capable of the metabolism of benzoate. A facultative Gram-negative organism which utilized benzoate aerobically was also present, but it failed to grow in pure culture on benzoate anaerobically even in the presence of nitrate. Evans (1977) also described benzoate-degrading methanogenic cultures in the same manner as Ferry and Wolfe (1976). The Gram-negative facultative anaerobe from these cultures was able to metabolize benzoate anaerobically in the presence of nitrate.

Although the main focus of this thesis project was directed toward the identification of the metabolites formed during m-cresol degradation, studies to define the m-cresol-metabolizing consortia were performed as a side interest. The work was performed under the hypothesis that the m-cresol-degrading consortia could be divided into three functional groups as observed for benzoate degradation by Ferry and Wolfe (1976) and Evans (1977). These functional groups were: the m-cresol-utilizing bacteria, the acetate-utilizing bacteria, and the hydrogen gas-utilizing bacteria.

4.6.1 Hydrogen Gas-Utilizing Methanogenic Bacteria.

Hydrogen gas-utilizing methanogens were obtained in pure culture from the first 2-L m-cresol-degrading enrichment culture. This was accomplished by an initial 5-fold dilution series in Medium WR86 plus hydrogen gas, growth of the last dilution of this and subsequent dilution into a second 5-fold dilution series in Medium WR86 plus hydrogen gas. The last dilution showing methane production of this second dilution series was grown until turbid and used to inoculate a 10-fold dilution series into roll

tubes of Medium WR86 plus hydrogen gas and 2 % agar.

Microscopic analysis of the final dilution (1×10^{-7}) of the initial dilution series showed three cellular morphologies. The major morphology was that of a slender, rod shaped bacteria in long, sheathed filaments. The minor cellular morphologies were short, motile rods and short, fat, non-motile rods. Microscopic analysis of the final dilution (1×10^{-7}) of the second dilution series showed one cellular morphology, that of the major cellular morphology described above.

Observations of roll tube cultures indicated one colonial morphology. Sub-surface colonies were slightly greyish, spherical and filamentous. Four of these colonies were picked and inoculated into Medium WR86 plus hydrogen gas. Only one grew and produced methane. Culture purity was checked by inoculating PCA plates and incubating them under aerobic and anaerobic conditions. No growth was observed after 4 days incubation under aerobic conditions or after 2 weeks under anaerobic conditions.

Microscopic observations of wet mounts of turbid cultures showed long, narrow, irregularly curved, non-motile rods. The majority of the rods were in flocs although some were seen as free organisms. Each successive transfer to fresh medium appeared to contain more flocs and fewer free organisms. A scanning electron micrograph of one such floc is presented in Plate 4.1.

The organisms shown in the electron micrograph were 0.3-0.4 μm in width and of various lengths. The presence of large amounts of extracellular material was evident in the plate. The hydrogen gas-utilizing methanogens isolated in this study were slightly narrower than any described in the literature. *Methanobacterium formicicum* was reported to be 0.4 to 0.8 μm in width depending on the strain (Bryant 1974, Mah and Smith 1981). This methanogen was reported to have slender cylindrical, unevenly crooked cells which could form chains and filaments. The colonies were white to grey, flat and filamentous when on the surface of agar and profusely filamented

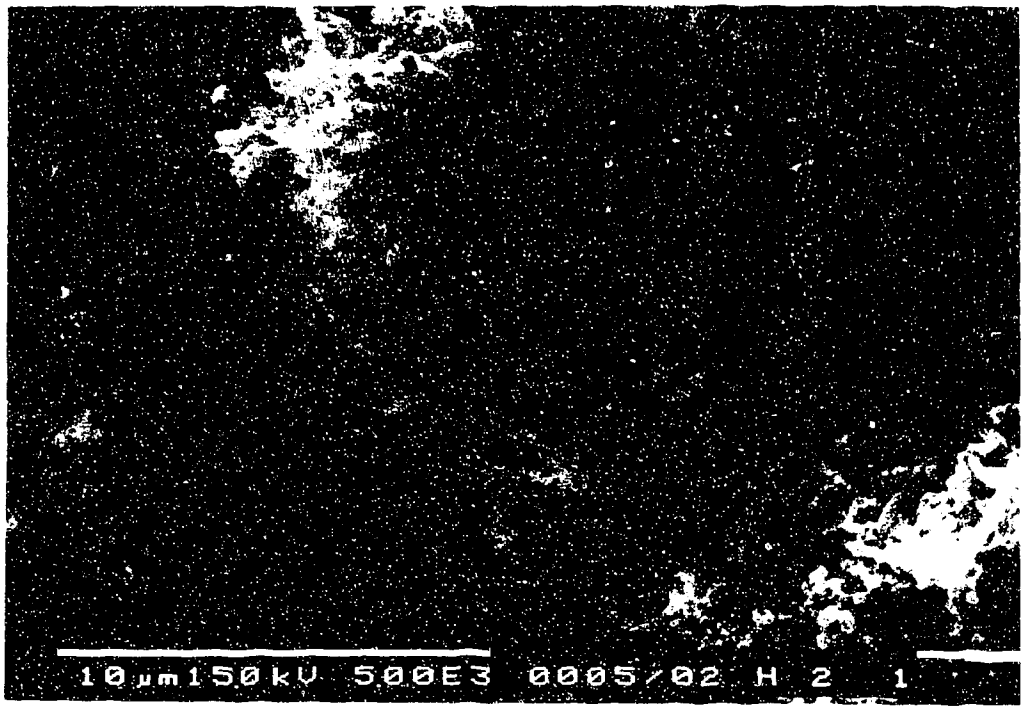


Plate 4.1. Scanning Electron Micrograph of a pure culture of hydrogen gas-utilizing methanogenic bacteria isolated from an m-cresol-degrading enrichment culture by the roll tube method.

spheroids when growing within the agar. This description was similar to that of the methanogen isolated in this study. There was no mention of production of extracellular material by *M. formicicum*. (Bryant 1974, Mah and Smith 1981).

The hydrogen gas-utilizing methanogens were not predominant members of the m-cresol-metabolizing consortium. Wet mount observations of a 1/5 dilution of Consortium A (designated Consortium B) showed very few bacteria with the same morphology as the hydrogen gas-utilizing methanogens. The culture had been maintained by feeding as necessary (low m-cresol concentration) without substrate removal for 19 months as Consortium A and then 23 months as Consortium B. Fluorescence microscopy of these organisms showed a strong green fluorescence when the culture was exposed to UV light.

A scanning electron micrograph of a sample of Consortium B is presented in Plate 4.2. There were only a few cells of the hydrogen gas-utilizing bacteria present in this field; many fields had no detectable hydrogen gas-utilizing bacteria. The cell indicated by an H had the same width and general appearance as the cells from the pure culture of hydrogen gas-utilizing bacteria (Plate 4.1).

A photomicrograph taken of a sample of Consortium B using a light microscope on bright-field with 1000x magnification under oil immersion is presented in Plate 4.3. The hydrogen gas-utilizing methanogens are again indicated with an H. The low numbers of hydrogen gas-utilizing methanogens suggested that there was very little hydrogen gas produced during m-cresol metabolism.

4.6.2 Acetate-Utilizing Methanogenic Bacteria

Attempts to obtain pure cultures of acetate-utilizing methanogens were not successful. Microscopic examination of the last dilution capable of methane production (1/625) of an initial dilution series of an m-cresol-degrading culture in Medium WR86 plus acetate showed five cellular morphologies. Two morphologies were similar to

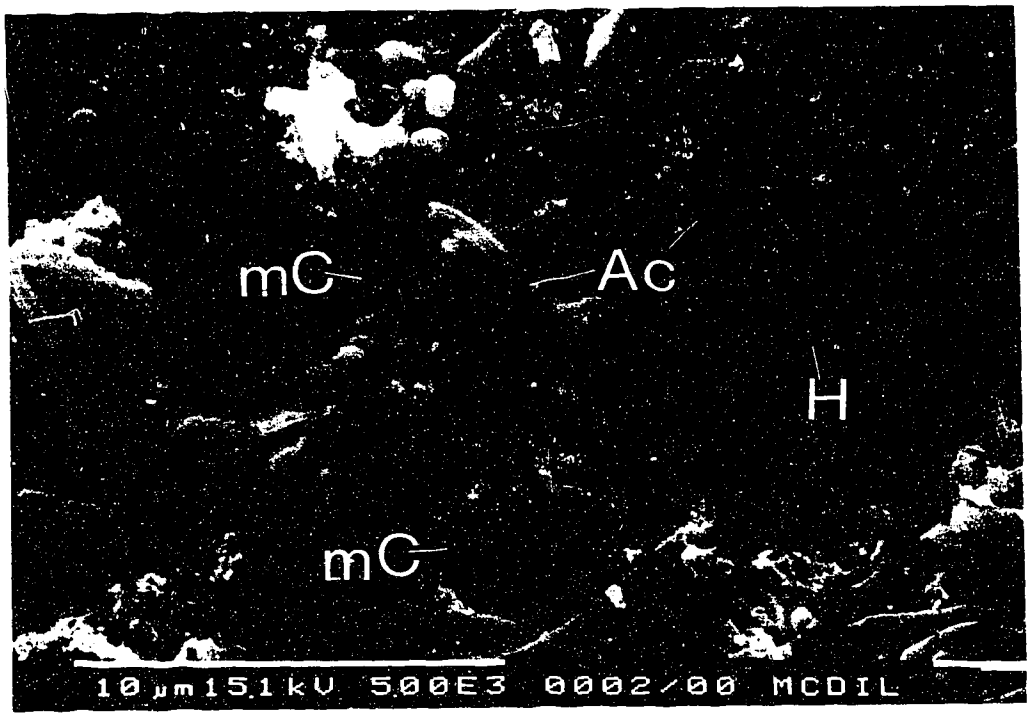


Plate 4.2. Scanning electron micrograph of a methanogenic culture maintained with m-cresol as the major source of organic carbon and diluted several times from sewage sludge (Consortium B). Ac- acetate-utilizing methanogen, H- hydrogen gas-utilizing methanogen, mC- m-cresol-utilizing bacteria.

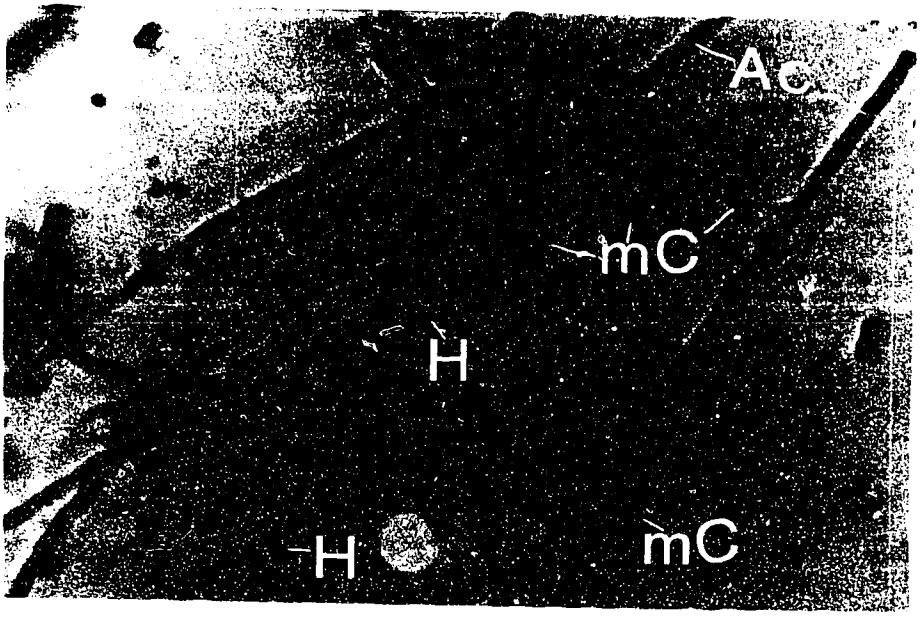


Plate 4.3. Photomicrograph of a methanogenic culture maintained with m-cresol as the major source of organic carbon and diluted several times from sewage sludge (Consortium B). Ac- acetate-utilizing methanogen, H- hydrogen gas-utilizing methanogen, mC- m-cresol-utilizing bacteria.
The photograph was taken of a specimen stained with crystal violet at 1000x magnification using oil immersion. Final magnification was not determined.

those reported for methanogenic bacteria; one was very similar to the hydrogen-utilizing methanogen whereas the other appeared to be individual rod shaped bacteria enclosed in very long, wide sheaths. Further dilution of this culture into Medium WR86 plus acetate failed to grow. Dilutions of this culture made into Medium WR86 containing yeast extract and acetate grew and produced methane.

Roll tubes of Medium WR86 plus acetate agar and Medium WR86 containing acetate and yeast extract agar were inoculated with the last dilution (1/3125) of the dilution series in Medium WR86 plus acetate and yeast extract. One colonial morphology was observed to grow in the roll tubes of Medium WR86 agar that did not contain yeast extract. These colonies were medium size, greyish and filamentous. Four colonial morphologies were observed in the roll tubes of medium WR86 agar that did contain yeast extract. Small, black colonies; small, gold, translucent colonies; large, filamentous, greyish spherical colonies; and very small pink colonies were observed. Colonies of each morphology were picked and inoculated into Medium WR86 containing acetate and yeast extract. Cultures from the three pigmented colonies became turbid after 2 d incubation but did not produce methane.

Methane was produced by cultures from colonies whose colonial morphology was similar to that of the hydrogen-utilizing methanogen. After 5 months of incubation very little methane had been produced. Hydrogen was added in an attempt to stimulate methane production This caused the methane production to increase significantly. An analysis of the acetate concentration in the culture showed that no acetate had been utilized. Microscopic examination of the culture revealed cellular morphologies identical to those of the hydrogen-utilizing methanogen. It was assumed that the hydrogen-utilizing methanogen had managed to survive on residual amounts of hydrogen present in the cultures.

Other workers have also been unable to isolate acetate-utilizing methanogens in pure culture using solid culture methods. Organisms believed to be the acetate-utilizing

methanogens have been observed to be the predominant organisms in sewage sludge (Patel 1984). The proposed acetate-utilizing methanogen has been described as the "fat rod" or "acetate organism" by many workers but attempts to isolate it have not often resulted in success. Zehnder et al. (1982) described the enrichment of *M. soehngenii*, an acetate-utilizing methanogen, from sewage sludge by dilution techniques. The organism was not obtained in pure culture but was the predominant organism in the culture. *M. soehngenii* grew with acetate as the only source of organic carbon. The organism was described as non-motile, non-sporeforming, rod shaped cells (0.8 x 2 μm) which were normally combined end to end in long filaments surrounded by a sheath like structure.

Patel (1984) was able to isolate the organism *Methanothrix concilii* by dilution in medium containing kanamycin to inhibit the growth of other acetate-utilizing bacteria. This organism was described as a fat, rod-shaped, non-motile bacteria. Cells were flat-ended and present in long filaments, separated by septa. The single cells were on the average 0.8 x 3.5 μm. Extracellular material was observed under phase contrast microscopy.

Plates 4.2 and 4.3 were dominated by organisms that resemble the description given for the two species of *Methanothrix*. No single cells were observed which had the dimensions given in the literature. The wide sheaths identified with an A[illegible] in Plates 4.2 and 4.3 were the predominant morphology in acetate- and m-cresol-metabolizing cultures. These sheaths had an average diameter of 0.6 μm, were blunt ended, and seemed to be coated with an extracellular material. The sheathed structure was slightly more slender than the diameters given for single cells of both *Methanothrix* species. There were some single cells with diameters the same as the diameter of the sheathed structures found in the cultures used in this study but it cannot be determined if these were actually unsheathed cells without pure culture studies.

Fluorescence microscopy of the mixed culture showed that the wide sheathed rods fluoresced slightly under UV light. The reports have indicated other acetate-utilizing methanogens had very poor UV-fluorescence capabilities (Patel 1984).

4.6.3 m-Cresol-Metabolizing Organisms

Consortia A and B contained many short fat rods (0.7 x 1.4 μm average dimensions) designated mC in Plates 4.2 and 4.3. These rods did not fluoresce under UV light, which distinguished them from methanogens. The rods had a characteristic halo that could be seen in wet mounts, suggesting the presence of a capsule. The non-motile, Gram-negative rods grew as individual cells and in large compact clumps, often attached to methanogenic bacteria.

Attempts to purify the m-cresol-metabolizing organism by coculture and pure culture techniques failed. Attempts to coculture m-cresol-utilizing bacteria with *W. succinogenes* present as a hydrogen-utilizing organism failed, probably because the rich medium used to grow *W. succinogenes* inhibited m-cresol degradation. Attempts with *M.barkeri* failed, probably due to the fact that *M. barkeri* preferentially uses hydrogen and then will take a long time to regain the ability to utilize acetate. *M. barkeri* did grow in the dilution tubes but no m-cresol degradation by the inoculum was observed. Coculture with an SRB was not attempted since the metabolism of m-cresol was inhibited by sulfide, the final product of sulfate reduction.

Transfers of Consortium A into medium with BESA and m-cresol were observed to utilize the first allotment of m-cresol very slowly but would not utilize any subsequent additions of m-cresol. Transfers of Consortium A into medium containing BESA and glucose grew turbid rapidly. Transfers of these cultures back into medium containing m-cresol as the organic carbon source failed to utilize the m-cresol within 1 year even after *M. barkeri* was added as a hydrogen or acetate scavenger.

Although the attempts at coculture technique failed to isolate an organism that

could be considered to be the m-cresol-utilizing organism the microscopic observation of dilutions of m-cresol-metabolizing cultures indicated that the short, fat, Gram-negative rod was likely the m-cresol-utilizing organism. This organism grew in prominance in the cultures as the dilution factor increased. It appeared to increase in number as the dilute cultures utilized m-cresol and was not present in the most dilute cultures of acetate- and hydrogen-utilizing methanogens.

4.7 Summary

The results from this portion of the study clearly showed that m-cresol degradation in methanogenic consortia was adversely affected by low concentrations of sulfide in the culture. Sulfide concentrations as low as 0.4 mM were observed to delay the onset of m-cresol degradation by sewage sludge inoculum. The prereduction of medium with 0.5 mM sodium sulfide delayed the onset of m-cresol degradation by transfers of enrichment cultures. The non-methanogenic members of the m-cresol-degrading consortium were likely the organisms which were sensitive to the addition of sulfide because its presence did not inhibit the production of methane from the organic compounds which were in the sludge inoculum (Figure 5.3).

Others have found methanogens resistant to the concentrations of sulfide that were used in these experiments, again implying that the non-methanogenic members of the consortia were the bacteria inhibited by sulfide. Zehnder et al. (1982) reported that most investigators found inhibition of methane formation in mixed and pure cultures started at total sulfide ($H_2S + HS^- + S^=$) concentration of about 3 mM. After testing the effects of pH and sulfide concentration on anaerobic digestion, Hilton and Oleszkiewicz (1988) concluded that at high levels of total sulfide (1,000 mg/L, 31 mM) and elevated pH ($\geq$ 7.4), methanogenic utilization of acetate was not inhibited but at lower pH values methanogenesis was inhibited. It was shown that the inhibition of

methanogens was due to the concentration of H_2S in the medium rather than total sulfide concentration.

The concentration of H_2S is in a pH dependant equilibrium with ionized sulfide. Based on the data reported by Sawyer and McCarty (1967), concentrations of H_2S in our cultures (pH 6.9-7.1) would be 45-55% of the total sulfide. Thus, the highest concentration of H_2S present in this study was approximately 1 mM, arising in cultures which contained 2 mM total soluble sulfide. This was well below the concentrations of H_2S found to be inhibitory to methanogens.

In order to maintain a sufficiently low redox potential to allow the growth of methanogens it was decided to use 0.5 mM sulfide to prereduce transfer medium and Medium WR86 for future culture of m-cresol-degrading methanogenic consortia. Although this concentration of sulfide was found to delay the onset of m-cresol degradation by transfers of m-cresol-degrading enrichment cultures in initial experiments (Section 4.2) it was found that when the enrichment cultures were maintained under low sulfide conditions the prereduction of medium with 0.5 mM sodium sulfide was no longer inhibitory. The degradation of m-cresol began almost immediately in most experiments using these methods. The decreased amount of sulfide used to prereduce Medium WR86 allowed the concentration of sulfide in the enrichment cultures to remain at low levels. No other factors affecting the degradation of m-cresol were identified during the course of the study.

The m-cresol-degrading cultures enriched for by the above procedures were not able to utilize sulfate, thiosulfate, nitrate or oxygen as terminal electron acceptors. The m-cresol-degrading bacteria could not be isolated as cocultures with *W. succinogenes* or *M. barkeri*. Observations of dilute m-cresol-degrading cultures indicated that the m-cresol-degrading organism was a short, fat, non-motile, Gram-negative, rod. A hydrogen-utilizing methanogen was isolated from m-cresol-degrading enrichment

cultures. This bacterium was a slender, UV-fluorescent, long rod. Acetate-utilizing methanogenic bacteria could not be isolated in pure culture but were observed to be contained in very long, fat, sheaths very much like *Metha. thrix* sp. The acetate-utilizing methanogens were the predominant members of the consortia.

5. Products of the Metabolism of m-Cresol by Methanogenic Consortia

Tarvin and Buswell (1934) studied the complete conversion to methane and carbon dioxide, of several aromatic compounds by methanogenic enrichment cultures to methane and carbon dioxide. The results of these studies led to Eqn. 5.1, which can be used to predict the theoretical amounts of methane and carbon dioxide from any organic compound.

$$C_nH_aO_b + [n - a/4 - b/2]\ H_2O \rightarrow [n/2 - a/8 + b/4]\ CO_2 + [n/2 + a/8 - b/4]\ CH_4$$

Eqn. 5.1

Based on Equation 5.1 the complete conversion of m-cresol to methane and carbon dioxide by methanogenic consortia is summarized in Eqn. 5.2. A total of 7 mol of gas would be expected from the complete mineralization of 1 mol of m-cresol, 4.25 mol of this would be methane.

$$C_7H_8O + 4.5\ H_2O \rightarrow 4.25\ CH_4 + 2.75\ CO_2$$

Eqn. 5.2

The fate of the carbon atoms of organic molecules can be studied using ^{14}C-labeled substrates. These studies can be used to determine the amount of carbon atoms released as $^{14}CO_2$ or $^{14}CH_4$, as well as the amount of ^{14}C-label incorporated into cell carbon or not completely metabolized by the culture. The use of radioactive compounds with the ^{14}C-label in specific positions, such as $^{14}CH_3$-m-cresol, can be used as an aid in the determination of the pathway of metabolism, as well as the determination of products. The detection of a specific carbon atom as CH_4 or CO_2 may be used as an indication of whether the carbon is oxidized or reduced during metabolism.

No studies on the fate of the carbon atoms of m-cresol have been reported. Although the fate of the methyl carbon of p-cresol has not been published, the results of studies on the initial steps in p-cresol metabolism have indicated that the methyl carbon of p-cresol was oxidized while still on the aromatic ring to produce p-hydroxybenzoic acid (see literature review). This would result in the production of $^{14}CO_2$ from $^{14}CH_3$-p-cresol. Studies were performed using ring-U-^{14}C-m-cresol $^{14}CH_3$-m-cresol, $^{14}CH_3$-p-cresol , and $H^{14}CO_3^-$, to further define the fates of the labeled carbon atoms in methanogenic consortia. The detailed experimental procedures are given in appendix 2.2.

5.1 Determination of Methane and Carbon Dioxide Yields from Unlabeled m-Cresol

Transfers of m-cresol-degrading enrichment cultures were incubated with 200-300 mg/L of m-cresol in order to determine the amounts of total gas and methane produced by methanogenic consortia. The results showed that 4.13 ± 0.34 (n=24) mol of methane were formed per mol of m-cresol metabolized and that 5.74 ± 1.17 (n=24) mol of total gas were produced per mol of m-cresol used. These values are 97 and 82% of the values predicted from Eqn. 5.2, respectively.

The standard deviation observed for total gas production was much higher than that for methane production. Also, the total gas recovery was lower than the methane recovery. These results are most likely due to the problems encountered in measuring the amount of carbon dioxide produced. There are many factors effecting the distribution of carbon dioxide in the cultures. Umbriet et al (1972) report the distribution of carbon dioxide to be dependant upon the equilibrium between CO_2 (g) with CO_2 (aq), which is temperature and pressure dependant; as well as upon the chemical equilibria among CO_2, H_2CO_3 and HCO_3^-, which is dependant upon the pH.

The distribution of carbon dioxide between the aqueous and gaseous phases changed rapidly near physiological pH values and was illustrated by Fedorak et al (1982). Small variations in the pH of the cultures would cause differences in the solubility of carbon dioxide and thus differences in measured gas production. The pH values of the cultures at the end of the incubation were not measured. In addition carbon dioxide may also be present in the cells thereby affecting its overall distribution and the measured amount of total gas produced.

5.2 Products from ^{14}C-Labeled m-Cresol and ^{14}C-Bicarbonate

Radiotracer studies were performed using a m-cresol-metabolizing inoculum incubated with $^{14}CH_3$-m-cresol, ring-U-^{14}C-m-cresol, or $H^{14}CO_3^-$ and unlabeled m-cresol, to determine the fates of the labeled carbon atoms during m-cresol metabolism. To determine the fate of the methyl carbon of m-cresol transfers of enrichment cultures were made into medium containing 1 x 10^6 dpm of $^{14}CH_3$-m-cresol and 200 mg/L of unlabeled m-cresol. The loss of m-cresol and subsequent production of $^{14}CH_4$ from $^{14}CH_3$-m-cresol are shown in Figure 5.1. These cultures reduced the methyl label to $^{14}CH_4$ with only small amounts of $^{14}CO_2$ being detected. The cultures exhibited a short lag period before m-cresol degradation reached the maximum rate and a longer lag period before the production of $^{14}CH_4$ reached the maximum rate. The majority of the substrate m-cresol had been degraded before the $^{14}CH_4$ began to appear (Figure 5.1).

Table 5.1 summarizes the final distribution of the ^{14}C-label in cultures incubated with 1 x 10^6 dpm of $^{14}CH_3$-m-cresol or ring-U-^{14}C-m-cresol or 5 x 10^5 dpm of $H^{14}CO_3^-$ incubated for 20, 73, and 20 d respectively. The results clearly showed that

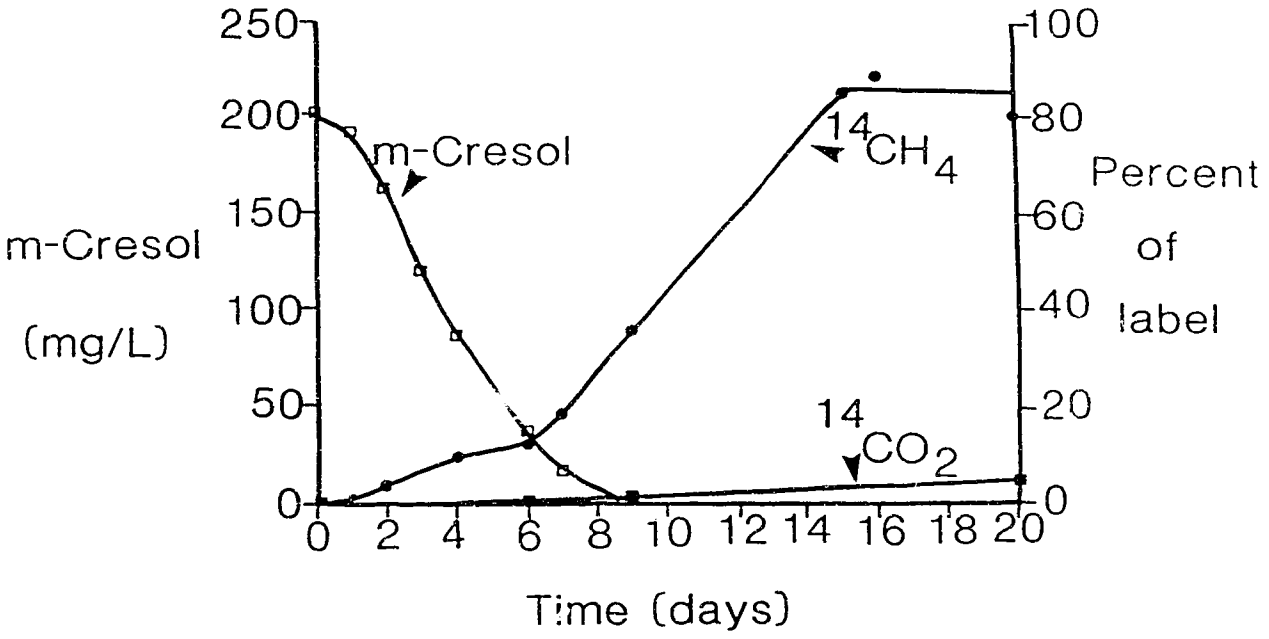


Figure 5.1. m-Cresol degradation and subsequent release of $^{14}CH_4$ and $^{14}CO_2$ in cultures that received 1 x 10^6 dpm of $^{14}CH_3$-m-cresol plus 200 mg/L of unlabeled m-cresol. Percent $^{14}CO_2$ was estimated from the amount of $^{14}CO_2$ found in the headspace gases divided by 0.31 which was the calculated proportion of $^{14}CO_2$ partitioned into the headspace at the end of the incubation period.

Table 5.1. Mean recoveries of ^{14}C (± SD) from m-cresol-degrading methanogenic consortia given various ^{14}C-labeled substrates.

	Percent of added label recovered as				
Substrates	$^{14}CO_2$[a]	$^{14}CH_4$[b]	^{14}C in cells[c]	^{14}C in filtrate	Total
$^{14}CH_3$-m-cresol[d]	4.8 (0.85)	85.9 (6.2)	2.2 (0.22)	6.2 (0.34)	99.9 (6.3)
ring-U-^{14}C-m-cresol[e]	32.9 (3.4)	46.3 (2.0)	3.2 (0.32)	13.6 (1.5)	96.0 (4.6)
$H^{14}CO_3^-$ + m-cresol[f]	96.5 (2.0)	ND[h]	0.32 (0.15)	0.18 (0.07)	97.0 (1.7)
$H^{14}CO_3^-$ no m-cresol[g]	96.9 (5.5)	ND[h]	0.11 (0.06)	0.18 (0.03)	97.1 (5.5)

[a] Acidified cultures were flushed with Nitrogen gas into Carbosorb and ACS for trapping and quantitation of $^{14}CO_2$.

[b] Measured by GC/GPC analysis of samples of headspace gases.

[c] Retained on 0.22 µm filters after washes with water, TCA and methanol.

[d] Means are the results of 9 batch cultures of m-cresol enrichment inoculum incubated with 1 x 10^6dpm of $^{14}CH_3$-m-cresol and 200 mg/L unlabeled m-cresol.

[e] Means are the results of 6 batch cultures of m-cresol enrichment inoculum incubated with 1 x 10^6 dpm of ring-U-^{14}C-m-cresol and 200 mg/L unlabeled m-cresol.

[f] Means are the results of 3 batch cultures of m-cresol enrichment inoculum incubated with 5 x 10^5 dpm of $H^{14}CO_3^-$ and 200 mg/L unlabeled m-cresol.

[g] Means are the results of 3 batch cultures of m-cresol enrichment inoculum incubated with 5 x 10^5 dpm of $H^{14}CO_3^-$ with no m-cresol present.

[h] Not detectable above background.

the majority of the methyl carbon atom of m-cresol was metabolized to methane while the ring carbon atoms were metabolized to both carbon dioxide and methane. There was no detectable production of $^{14}CH_4$ from $H^{14}CO_3^-$ during m-cresol metabolism indicating very little production of hydrogen gas as a result of m-cresol metabolism.

A significantly larger proportion of the ^{14}C-label was recovered as cell material from ^{14}C-U-ring-m-cresol than $^{14}CH_3$-m-cresol. The results for the incorporation of $H^{14}CO_3^-$ into cell material (lines 3 and 4, Table 5.1) suggested that although no net reduction of $H^{14}CO_3^-$ to $^{14}CH_4$ occurred, the cultures were able to incorporate $H^{14}CO_3^-$ into cell carbon.

5.3 The Fate of the Radioactivity from $^{14}CH_3$-p-Cresol

Studies were performed to determine the fate of the methyl carbon atom of p-cresol to serve as a comparison to the results described for the fate of the methyl carbon atom of m-cresol. An enrichment culture that had been maintained on p-cresol for 9 months was used as the inoculum for this study. Figure 5.2 shows the loss of p-cresol from batch cultures which received $^{14}CH_3$-p-cresol and the ^{14}C-labeled gases in the headspace. No lag period was noticeable before p-cresol degradation began and the degradation of p-cresol was accompanied by a rapid production of $^{14}CO_2$. The presence of small amounts of $^{14}CH_4$ was also evident after a considerable amount of $^{14}CO_2$ had been produced.

Table 5.2 summarizes the distribution of ^{14}C from p-cresol-degrading consortia given one of the following; $^{14}CH_3$-p-cresol, $H^{14}CO_3^-$ plus unlabeled p-cresol or $H^{14}CO_3^-$ without p-cresol. The distribution of ^{14}C after gas production had reached its maximum in cultures given $^{14}CH_3$-p-cresol is shown in the first line. Clearly, CO_2

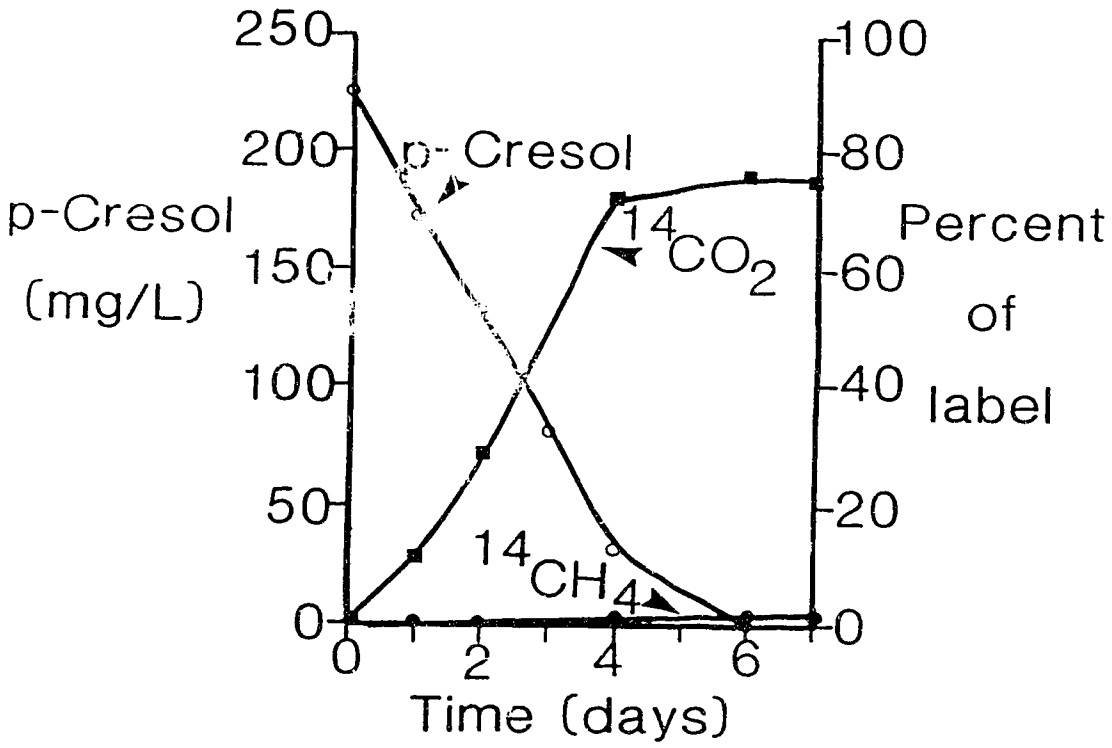


Figure 5.2. p-Cresol degradation and subsequent release of $^{14}CH_4$ and $^{14}CO_2$ in cultures that received 1 x 10^6 dpm of $^{14}CH_3$-p-cresol plus 230 mg/L of unlabeled p-cresol. Percent $^{14}CO_2$ was estimated as outlined in Figure 5.1.

Table 5.2. Mean recoveries of ^{14}C (± SD) from p-cresol-degrading methanogenic consortia given various ^{14}C-labeled substrates.

	Percent of added label recovered as:				
Substrates	$^{14}CO_2$[a]	$^{14}CH_4$[b]	^{14}C in cells[c]	^{14}C in filtrate	Total
$^{14}CH_3$-p-cresol[d]	85.8 (2.7)	1.9 (0.48)	1.4 (0.03)	4.7 (0.1)	93.7 (2.4)
$H^{14}CO_3^-$ + p-cresol[e]	97.0 (1.1)	1.6 (0.42)	0.42 (0.04)	0.1 (0.06)	99.2 (1.6)
$H^{14}CO_3^-$ no p-cresol[f]	98.0 (1.4)	ND[g]	0.07 (0.02)	0.04 (0.02)	98.1 (1.4)

[a] Acidified cultures were flushed with nitrogen gas into Carbosorb and ACS for trapping and quantitation of $^{14}CO_2$.

[b] Measured by GC/GPC analysis of samples of headspace gases.

[c] Retained on 0.22 µm filters after washes with water, TCA and methanol.

[d] Means are the results of 6 batch cultures of p-cresol enrichment inoculum incubated with 1 x 10^6dpm of $^{14}CH_3$-p-cresol and 230 mg/L unlabeled p-cresol.

[e] Means are the results of 3 batch cultures of p-cresol enrichment inoculum incubated with 5 x 10^5 dpm of $H^{14}CO_3^-$ and 230 mg/L unlabeled p-cresol.

[f] Means are the results of 3 batch cultures of p-cresol enrichment inoculum incubated with 5 x 10^5 dpm of $H^{14}CO_3^-$ with no p-cresol present.

[g] Not detectable above background.

was the major metabolic product of the methyl carbon atom of p-cresol.

The small proportion of label recovered as $^{14}CH_4$ (1.9%) was thought to arise from the reduction of $^{14}CO_2$ using hydrogen gas generated during the fermentation of p-cresol. To verify this, $H^{14}CO_3^-$ was added to cultures which contained the same amount of p-cresol as those cultures which received $^{14}CH_3$-p-cresol. Control cultures received $H^{14}CO_3^-$ but no p-cresol. The rate of production of $^{14}CH_4$ from $H^{14}CO_3^-$ (results not presented) was similar to the rate of $^{14}CH_4$ formation from $^{14}CH_3$-p-cresol shown in Figure 5.2.

The proportion of label converted to $^{14}CH_4$ (1.6%) from $H^{14}CO_3^-$ was the same as that found in the cultures which received $^{14}CH_3$-p-cresol (1.9%) ($P<0.05$). There was no detectable $^{14}CH_4$ found in the culture which received $H^{14}CO_3^-$ with no p-cresol. These observations are consistent with the hypothesis that the $^{14}CH_4$ produced in the culture containing $^{14}CH_3$-p-cresol was derived from the reduction of $^{14}CO_2$ using hydrogen gas produced during p-cresol degradation.

The recovery of the label from $^{14}CH_3$-p-cresol as $^{14}CO_2$ is in agreement with the results of Senior and Balba (1984) indicating that the methyl carbon atom of p-cresol is oxidized to give p-hydroxybenzoic acid during p-cresol metabolism by methanogenic consortia. The results from the study of p-cresol metabolism under nitrate-reducing conditions (Bossert and Young 1986) and sulfate-reducing conditions (Suflita et al. 1989) also indicated that the methyl carbon atom of p-cresol was oxidized to give p-hydroxybenzoic acid.

5.4 Summary.

The complete metabolism of m-cresol by methanogenic consortia resulted in the production of 4.13 mol of methane and 5.74 mol of total gas per mol of m-cresol metabolized. This observation of 97% of the theoretical methane production and 82% of the predicted total gas production from m-cresol by transfers of enrichment cultures indicated an almost complete mineralization of the compound and was comparable to the results in the literature. Shelton and Tiedje (1984a) found 87.6% of the theoretical methane production from m-cresol in sewage sludge cultures capable of m-cresol degradation. They also found 84% of the theoretical methane production from p-cresol in sewage sludge cultures. Boyd et al. (1983) reported >90% recovery of the predicted methane production in p-cresol-degrading enrichment cultures. No values for total gas recovery were given. Healy and Young (1978) found 88.7% of the theoretical methane production and 79% of the predicted total gas from phenol degradation. Fedorak (1984) found recoveries of 90.4 and 89.3% of predicted methane production and 80 and 76.6% of predicted total gas production from two phenol-degrading cultures. Recoveries of 86.3% theoretical methane production and 79.8% of the theoretical total gas production were obtained from a p-cresol-degrading culture.

Studies using $^{14}CH_3$-m-cresol indicated that the methyl carbon atom of m-cresol was metabolized to methane whereas the ring carbon atoms were metabolized to both methane and carbon dioxide. Although the majority of the carbon atoms of m-cresol were mineralized small amounts of carbon were incorporated into cell material during m-cresol metabolism. Some incorporation of carbon dioxide also occurred during m-cresol metabolism. Very little hydrogen gas was produced during m-cresol metabolism as evidenced by a lack of reduction of $H^{14}CO_3^-$ to $^{14}CH_4$.

These results were in contrast to those determined for the metabolism of p-cresol by methanogenic consortia. The methyl carbon atom of p-cresol was converted to carbon dioxide and then a small portion was reduced to methane. This indicated

different pathways existed for the metabolism of the two isomers. In p-cresol-metabolizing cultures, p-cresol metabolism and gas production occurred almost simultaneously while in the m-cresol metabolizing consortium, little radioactivity was observed in the headspace gases until after the majority of the m-cresol had been removed from the medium. This temporal separation of methanogenesis from m-cresol metabolism allowed ^{14}C-compounds to accumulate in the culture fluids when m-cresol metabolism was almost complete but methanogenesis had not begun.

the supernatant at the time of analysis. The remainder of the radioactivity was present as unmetabolized $^{14}CH_3$-m-cresol.

6.2 Time Course Analyses of Acetate Production During m-Cresol Metabolism

Figure 6.1 presents the results of experiments designed to examine the production of acetate in m-cresol-metabolizing cultures that did not contain BESA. Triplicate cultures were incubated with 190 mg/L (17.4 µmol per culture) m-cresol and monitored periodically to determine acetate and m-cresol concentrations. The rate of m-cresol degradation by these cultures was 5.5 µmol/d, the rate of methane production was 16.3 µmol/d. Very low concentrations of acetate (24 mg/L, 0.23 µmol per culture) had accumulated in these cultures after 1.5 d incubation and had disappeared by 3 d incubation. These results showed that acetate accumulated only transiently during m-cresol degradation.

Figure 6.2 represents the results of a similar analysis of culture fluids from triplicate cultures incubated in the presence of BESA to inhibit methanogenesis. The rate of degradation of m-cresol was 2.1 µmol/d (as calculated from the slope of the linear portion of the curve). Statistical analysis of these data showed the rate of m-cresol degradation was significantly slower ($P<0.05$) in the cultures containing BESA than in cultures not containing BESA. The complete allotment of m-cresol was not metabolized by the BESA-inhibited cultures. Acetate accumulated at a rate of 6.4 µmol/d. Methane was not produced by these cultures.

The observation of the ability of the consortia to degrade m-cresol when the methanogenic members of the consortia were inhibited with BESA has been reported several times during this study. The cultures were able to degrade between 10 and 15 µmol m-cresol per culture before m-cresol degradation stopped. Acetate was found to accumulate in all of these cultures. Hydrogen gas accumulation was not determined.

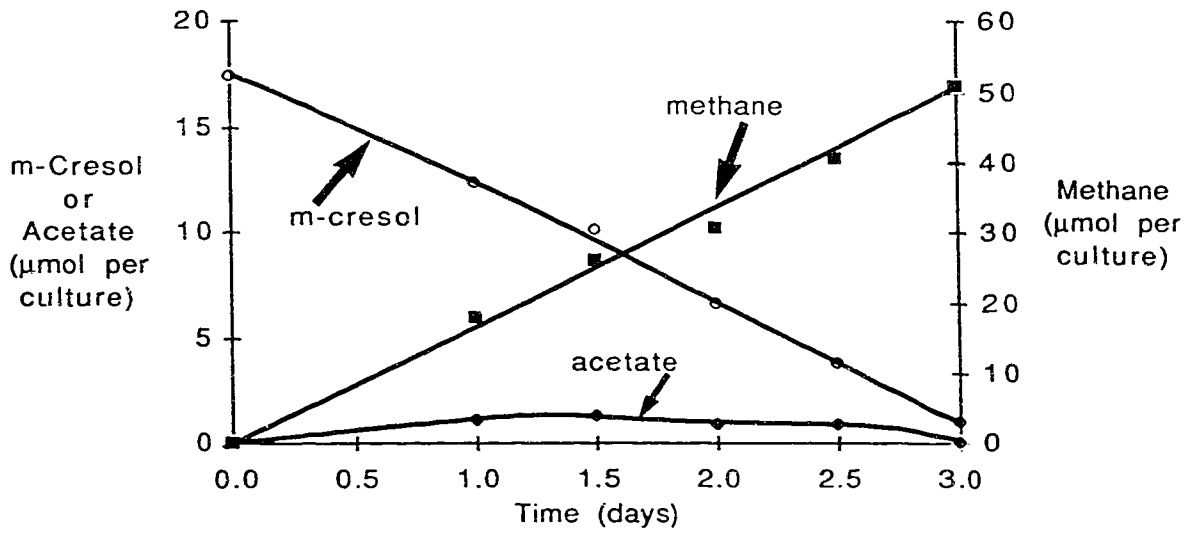


Figure 6.1. The transient accumulation of acetate in m-cresol-metabolizing cultures. Results are the means from triplicate cultures containing a 50% inoculum from an m-cresol enrichment culture. m-Cresol was degraded at a rate of 5.5 µmol/d, methane was produced at rate of 16.3 µmol/d.

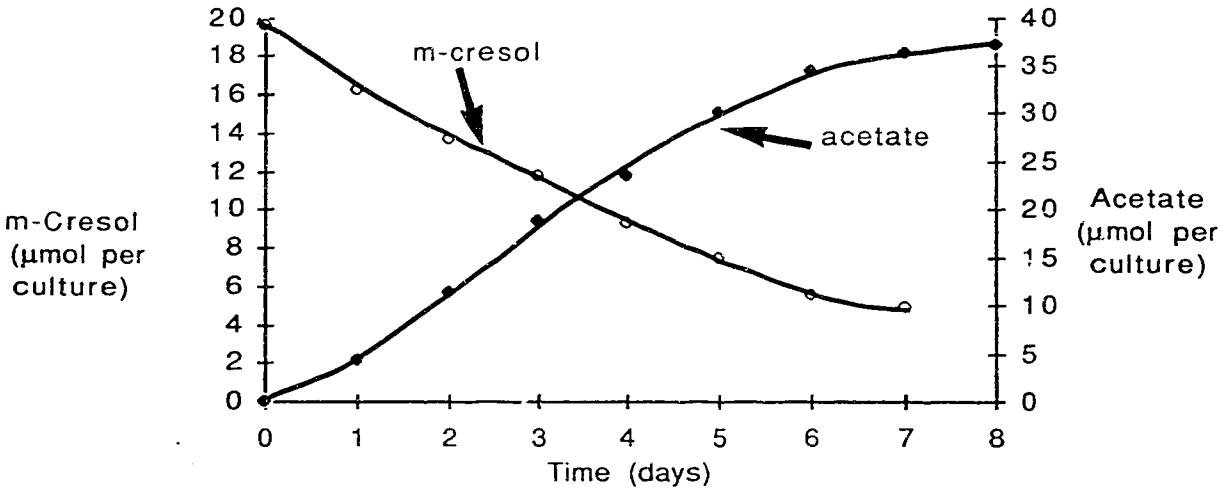


Figure 6.2. The accumulation of acetate in m-cresol metabolizing cultures incubated with 50 mM BESA. Results are the means of triplicate cultures containing a 50% inoculum from an m-cresol enrichment culture. m-Cresol was degraded at a rate of 2.1 µmol/d, acetate was produced at a rate of 6.4 µmol/d.

These results suggested that m-cresol degradation could occur to a minor extent even though the products of the non-methanogenic fermentations were not immediately removed. Whether the cessation of m-cresol degradation was due to unfavorable thermodynamic equilibria or to biological causes such as feedback inhibition was not determined.

6.3 Determination of the Total Amount of Acetate Produced by BESA-Inhibited m-Cresol-Degrading Cultures

In order to determine the total amount of acetate produced during m-cresol degradation, BESA was added to inhibit the methanogenic population, preventing the removal of the acetate. One set of six and three sets of five cultures were inoculated with 50% transfers of the second 2-L enrichment culture or batch enrichment cultures.

After determining the initial concentrations of m-cresol and acetate the cultures were incubated until GC analysis showed acetate production had ceased. This required 6 or 7 d for the different repetitions of the procedures. The remaining m-cresol concentration was then determined and subtracted from the original concentration of m-cresol to obtain the figure for the amount of m-cresol utilized by the cultures. A set of typical data are presented in Table 6.1. Control cultures not fed m-cresol did not accumulate acetate.

Summarizing all of the results, there were 4 ± 0.8 (n=21) mol of acetate produced per mol of m-cresol used. m-Cresol can only account for seven of the eight carbon atoms found in the 4 mol of acetate produced from 1 mol of m-cresol. This suggested that another carbon atom was incorporated during m-cresol metabolism.

Table 6.1. Sample data for the determination of the amount of acetate produced per mol of m-cresol degraded in cultures inhibited with 50 mM BESA.[a]

Trial	µmol m-cresol consumed[b]	µmol acetate produced[c]	Ratio acetate/m-cresol
1	11.9	43.8	3.7
2	9.5	43.7	4.6
3	18.3	56.7	3.1
4	9.9	46	4.6

[a] Replicate 10-mL cultures containing 5 mL of m-cresol enrichment culture inoculum 200 mg/L m-cresol and 50 mM BESA in Medium WR86. Methane production did not eexceed that observed in control cultures containing no m-cresol.

[b] Mean determined by subtraction of the final concentration of m-cresol from the initial concentration of m-cresol in each culture. Concentrations were determined by GC analyses.

[c] Mean amount of acetate present at the end of the incubation. No acetate was detected in the cultures at the time of inoculation. Control cultures without m-cresol did not produce acetate.

These results clearly showed that carbon dioxide incorporation occurred during m-cresol metabolism and that this resulted in the accumulation of the majority of the label from $H^{14}CO_3^-$ as ^{14}C-acetate in BESA-inhibited cultures. In cultures without m-cresol, very little incorporation of $H^{14}CO_3^-$ occurred resulting in only half of the label from $H^{14}CO_3^-$ incorportation accumulating as ^{14}C-acetate.

The incorporation of CO_2 into cell carbon by methanogenic cultures has been reported previously. Knoll and Winter (1987) reported finding small amounts of $H^{14}CO_3^-$ incorporated into benzoate and acetate during phenol degradation by methanogenic consortia. Pure cultures of methanogenic bacteria have also been found to incorporate $H^{14}CO_3^-$ into acetate and cell carbon. This may be the explanation for the incorporation of $H^{14}CO_3^-$ in the absence of m-cresol. Stupperich and Fuchs (1983, 1984a, 1984b) found that *Methanobacterium thermoautotrophicum* incorporated $^{14}CO_2$ into both carbon atoms of acetate during autotrophic growth. This incorporation was believed to be independant of methanogenesis, since it required enzymes and cofactors not involved in the methanogenic pathway. *Methanothrix concilii* has also been found to incorporate $^{14}CO_2$ into cell carbon (Patel 1984). The pathway for CO_2 incorporation by these methanogens was unknown.

6.5 Determination of the Positions of the ^{14}C-Label in Acetate from Incorporation of $^{14}CH_3$-m-Cresol or $H^{14}CO_3^-$

Ion exchange chromatography was used to purify acetate from the supernatants of BESA-inhibited cultures incubated in the presence of unlabeled m-cresol and $H^{14}CO_3^-$ and from those incubated with $^{14}CH_3$-m-cresol. The Schmidt degradation was performed on the purified acetate to determine the position of the ^{14}C incorporated into

Table 6.2. The position of the ^{14}C-label in acetate produced by anaerobic consortia as determined by the Schmidt degradation.

Culture Substrates and Source of label	Average Percent of ^{14}C recovered (±SD)	
	as CO_2 from the carboxyl carbon	as methylamine from the methyl carbon
$H^{14}CO_3^-$ and m-cresol[a]	89 (4)	11 (4)
$H^{14}CO_3^-$ alone[b]	62 (5)	38 (5)
$^{14}CH_3$-m-cresol and unlabeled bicarbonate[c]	2.3 (1.6)	98 (1.6)

[a] data from 5 replicates of samples from batch cultures of m-cresol enrichment culture inoculum fed 1 x 10^7 dpm $H^{14}CO_3^-$ and 200 mg/L unlabeled m-cresol.

[b] data from 5 replicates of samples from batch cultures of m-cresol enrichment inoculum fed 1 x 10^7 dpm $H^{14}CO_3^-$ with no m-cresol present.

[c] data from 13 replicates of samples from batch cultures of m-cresol enrichment inoculum fed 1 x 10^6 dpm $^{14}CH_3$-m-cresol and 200 mg/L unlabeled m-cresol. Unlabeled bicarbonate was present as the buffer for Medium WR86

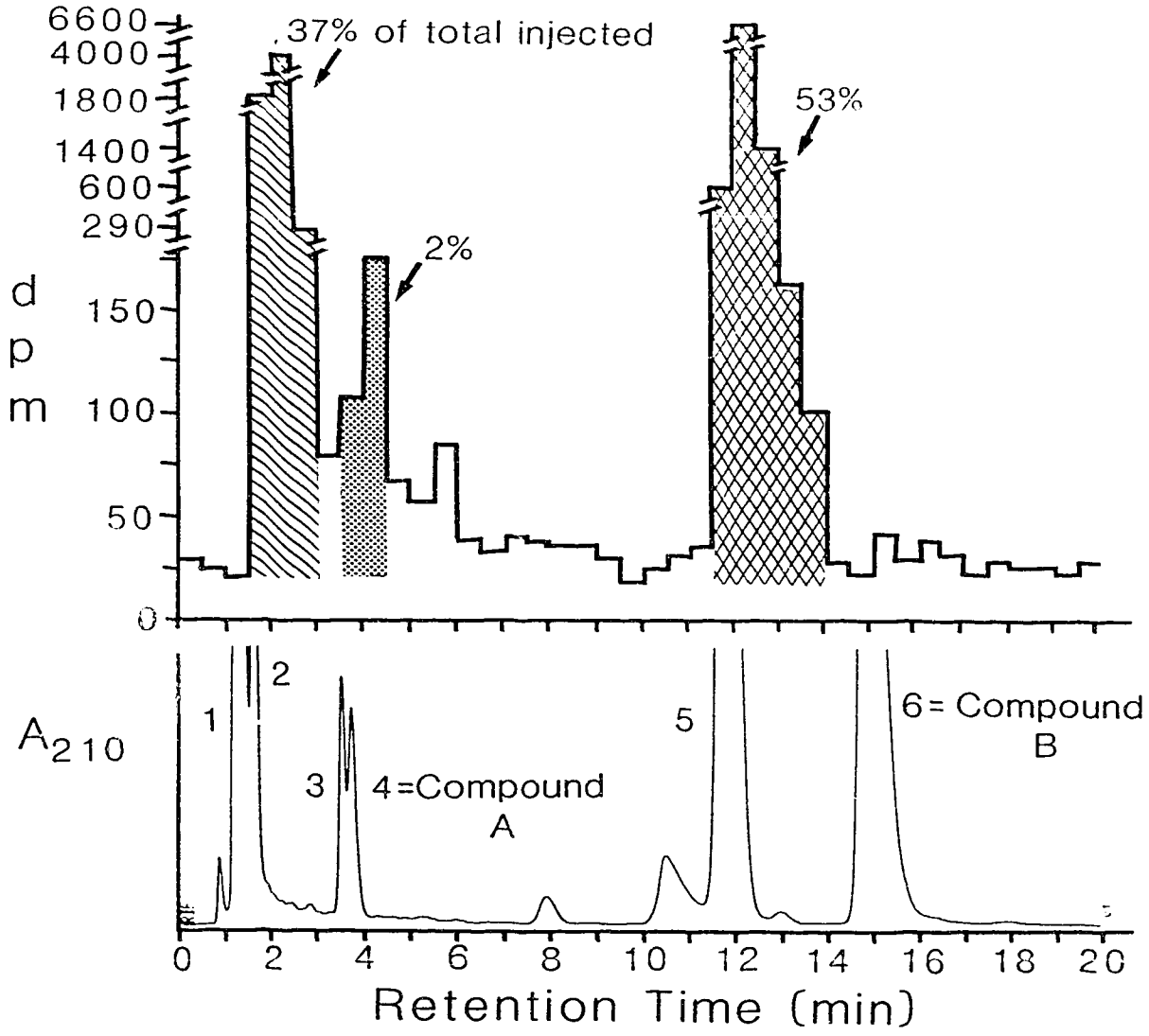


Figure 6.3. HPLC/LSC analysis using mobile phase 1 of the supernatant from a BESA-inhibited m-cresol-metabolizing culture containing 1 x 10^6 dpm of $^{14}CH_3$-m-cresol. The culture was incubated for 4 days. The column effluent was collected (1 fraction per min) and the amounts of radioactivity in these were determined by LSC.

Peak Identifications: 1. acetate, 2. BESA, 3. unidentified, 4. Compound A, 5. m-cresol, 6. Compound B.

Peak 6, designated Compound B, was quite abundant in the culture supernatants but was not labeled to a significant extent in most samples. However, low levels of radioactivity were detected in this peak from some cultures.

Peaks 3 and 4 were not well resolved using mobile phase 1 (Figure 6.3). Together these accounted for 2% of the recovered radioactivity. These peaks were resolved using mobile phase 2. The first 20 min of the analysis of the culture supernatant from cultures fed $^{14}CH_3$-m-cresol is shown in Figure 6.4. The radioactivity recovered was associated only with the peak that eluted at about 15 min. The material in this fraction was collected and reanalyzed using mobile phase 1 and it eluted at the same time as peak 4 in Figure 6.3. Similarly, analysis of the material collected from the 17.5 min peak in Figure 6.4 showed that it had the same retention time as peak 3 in Figure 6.3 when mobile phase 1 was used.

Peak 3 was commonly observed at low concentrations in the HPLC analyses of culture supernatants. However it had no radioactivity associated with it regardless of the labeled substrates used in the cultures ($^{14}CH_3$-m-cresol, ^{14}C-U-ring-m-cresol or $H^{14}CO_3^-$). No attempts were made to identify this compound.

Peak 4, designated Compound A, was labeled in experiments with cultures incubated in the presence of $^{14}CH_3$-m-cresol (Figure 6.4), as well as $H^{14}CO_3^-$ plus m-cresol or ^{14}C-U-ring-m-cresol (results not included). This compound was not labeled in samples of control cultures that contained $H^{14}CO_3^-$ but no m-cresol nor was it found in control cultures that had been sterilized by autoclaving and received ^{14}C-U-ring-m-cresol. These results suggested that the presence of m-cresol and an active biological population were required for Compound A to be formed.

Initial radiotracer studies indicated that Compound A accumulated in the culture fluids during early m-cresol metabolism but decreased in amount during the later stages of m-cresol metabolism. To verify this, time course analyses of the supernatants of

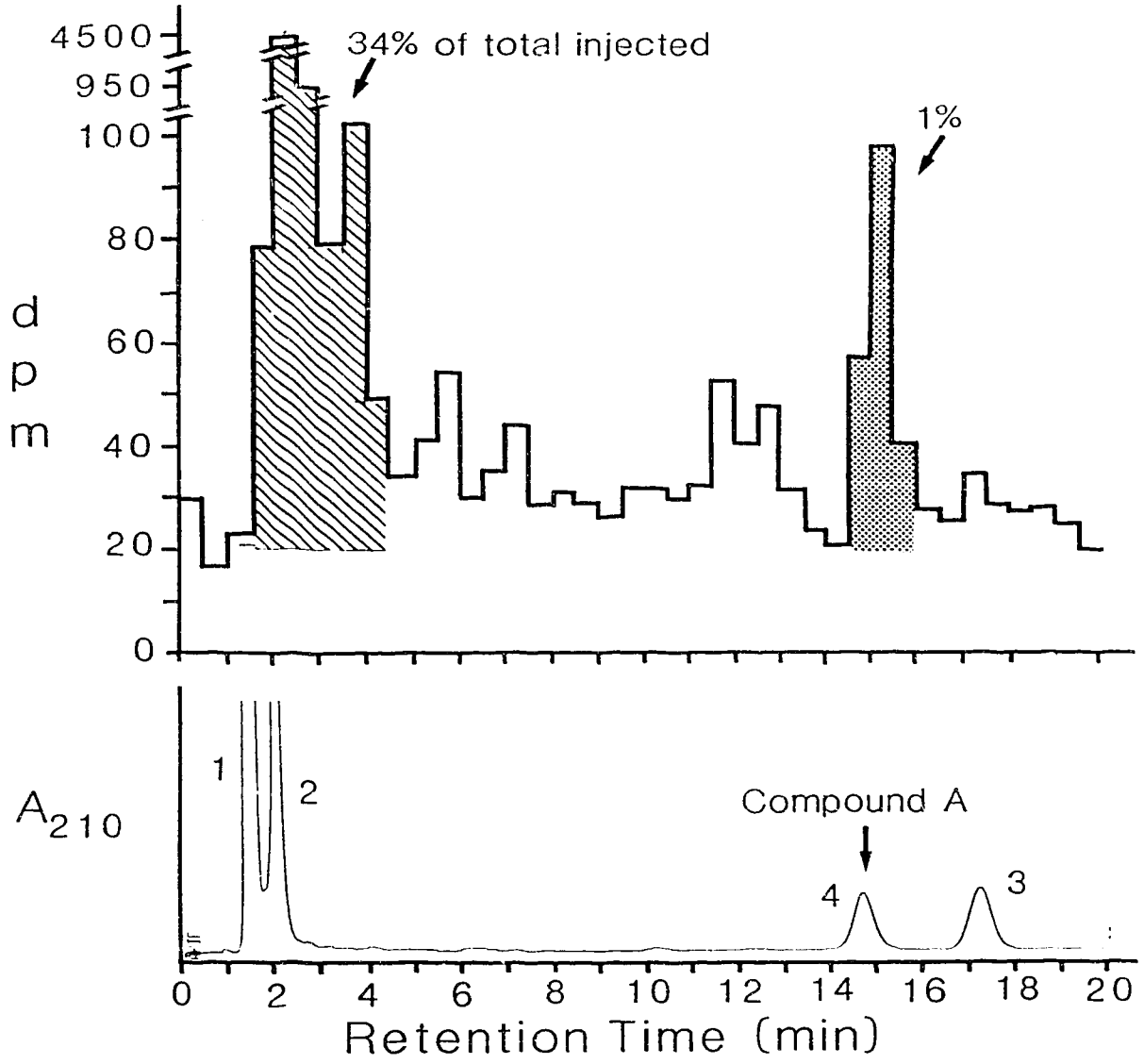


Figure 6.4. HPLC/LSC analysis using mobile phase 2 of the supernatant from a BESA-inhibited m-cresol-metabolizing culture containing 1 x 10^6 dpm of $^{14}CH_3$-m-cresol. The culture was analyzed after 4 days incubation. The column effluent was collected and analyzed as described in Figure 6.3. The results from the first 20 min of the analysis are presented. See Figure 6.3 for peak identifications.

m-cresol metabolizing cultures were performed. The quantitation of Compound A was accomplished by a comparison of the peak areas obtained from HPLC analysis with those of the internal standard phenol. Figure 6.5 shows the peak area ratios of m-cresol and Compound A throughout the incubation. Compound A appeared after m-cresol degradation began and reached a maximum concentration when most of the m-cresol had been transformed. Thereafter, the amount of Compound A in the culture decreased. This, in addition to the results of radiotracer studies, suggested that Compound A was a transient intermediate of m-cresol metabolism.

6.7 Identification of Compound A

Compound A was seen to contain the ^{14}C label when produced by cultures incubated with $^{14}CH_3$-m-cresol, ^{14}C-U-ring-m-cresol, and $H^{14}CO_3^-$ (when unlabeled m-cresol was being degraded). These results suggested that the compound contained a carbon atom from carbon dioxide, the methyl carbon atom of m-cresol and the ring carbon atoms of m-cresol. A UV scan of the fraction from HPLC analysis containing this compound showed absorption maxima of 204 and 256 nm. The absorbance at 256 nm was interpreted to indicate that the compound was aromatic in nature.

Compound A could not be extracted into methylene chloride from the aqueous phase at pH 8.5 nor did it extract into methylene chloride at pH 1. The compound did extract into diethylether at pH 1. These results were consistent with Compound A being a carboxylic acid.

GC/MS analysis of an ether extract of acidified culture supernatant produced a peak with the mass spectrum shown in Figure 6.6a. The base peak was the molecular ion at m/z=152 which was consistent with an isomer of hydroxymethylbenzoic acid or hydroxyphenylacetic acid. The peaks at m/z=135 and m/z=107 corresponded to the loss of -OH and -COOH groups respectively. Two commercially available

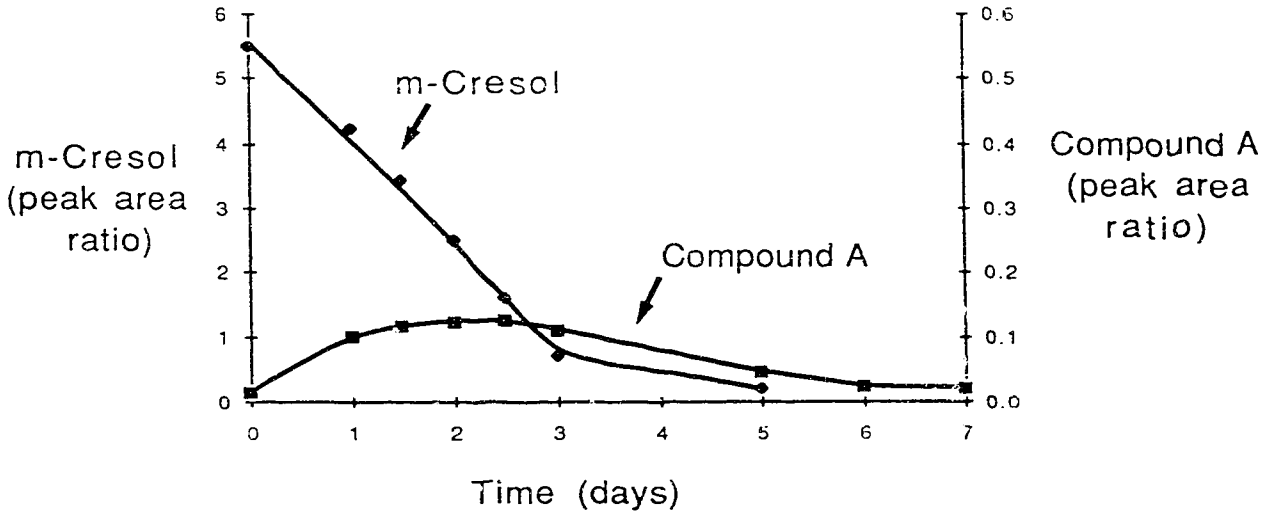


Figure 6.5. The transient accumulation of Compound A during methanogenic m-cresol degradation. Samples of culture supernatants from batch cultures of m-cresol enrichment inoculum were analyzed by HPLC using mobile phase 1 with phenol as an internal standard. Peak areas are expressed relative to the internal standard.

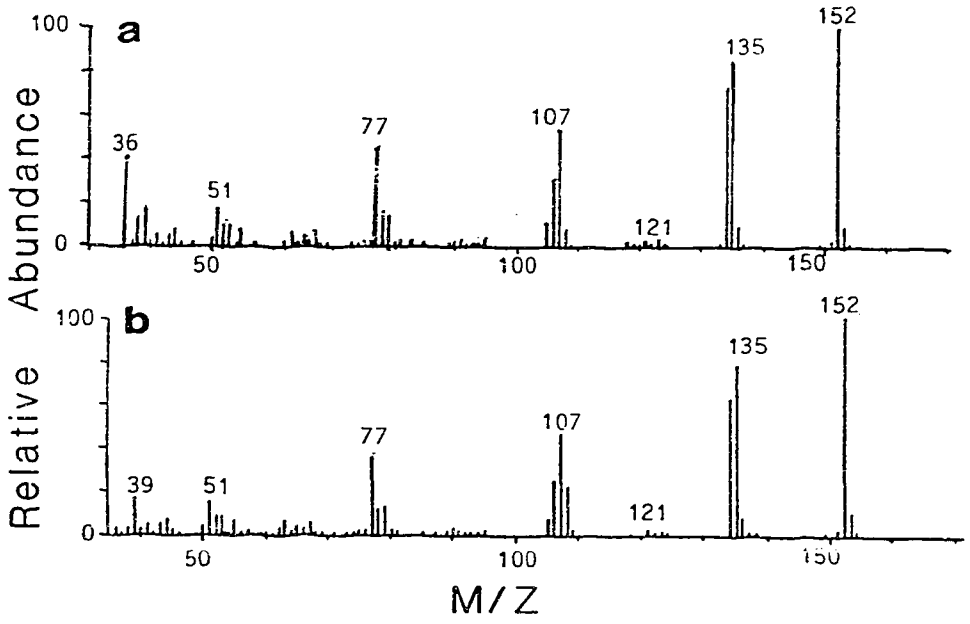


Figure 6.6. Mass spectra of (a) Compound A extracted from acidified supernatants of m-cresol-metabolizing cultures with diethyl ether and (b) authentic 4-hydroxy-2-methylbenzoic acid. Samples were analyzed by GC/MS.

hydroxymethylbenzoic acids (2-hydroxy-4-methylbenzoic acid and 2-hydroxy-3-methylbenzoic acid) as well as 3-hydroxyphenylacetic acid were analyzed by HPLC but none had the same retention time as Compound A.

Komiyama and Hirai (1984) reported a method for the carboxylation of phenolic compounds to produce benzoic acids. Starting with m-cresol, the reaction was reported to yield the two ortho isomers, 2-hydroxy-4-methylbenzoic acid and 2-hydroxy-6-methylbenzoic acid; and the para isomer, 4-hydroxy-2-methylbenzoic acid. The authors stated a 68% selectivity for the para oriented substitution.

This synthesis was used and the HPLC analysis of the reaction products indicated that three compounds were formed as previously described (Komiyama and Hirai 1984). Based on capillary GC analysis using a flame ionization detector, the most abundant compound was approximately 10 times more prevalent than the second most abundant compound. The least abundant compound was not readily quantitated by this method. The most abundant compound was presumed to be the para substituted isomer, 4-hydroxy-2-methylbenzoic acid.

HPLC analysis of authentic samples of the two ortho isomers 2-hydroxy-4-methylbenzoic acid and 2-hydroxy-6-methylbenzoic acid showed that the former had the same retention time as the second most abundant reaction product and that the latter had the same retention time as the least abundant reaction product. The GC and HPLC retention times as well as the absorption maxima obtained from UV/Visible scans (190-600 nm) of the sample and standard compounds are summarized in Table 6.3.

The mass spectrum of the most abundant isomer in the reaction mixture is shown in Figure 6.6b. Sen et al. (1987) reported the presence of the ions m/z=152 (molecular ion, 100%), m/z=135 and m/z=107 in the mass spectrum of 4-hydroxy-2-methyl benzoic acid. The mass spectrum in Figure 6.6b contains these major ions. These results consistently support the presumption that the most abundant compound in the synthesis was 4-hydroxy-2-methylbenzoic acid.

Table 6.3. Identification parameters for Compound A and hydroxymethylbenzoic acid isomers.

Compound	Relative HPLC Retention Time[a]	Relative GC Retention Time[b]	Absorbance Maxima (nm)
Compound A[c]	1	1	204, 256
4-hydroxy-2-methyl benzoic acid[d]	1	1	204, 256
2-hydroxy-4-methyl benzoic acid[e]	6.1	0.77	211, 246, 303
2-hydroxy-6-methyl benzoic acid[f]	3.7	0.49	195, 249, 314

[a] Analyses were performed using mobile phase 1.

[b] Analyses were performed using a 30-m DB-5 fused silica capillary column operated isothermally at 143° C.

[c] Extracted from m-cresol enrichment culture supernatants with diethyl ether.

[d] Synthesized by the method of Komiyama and Hirai (1984).

[e] Purchased from Aldrich Chemical Co.

[f] Kindly provided by Dr. G.M. Gaucher, University of Calgary, Calgary, Alberta.

The mass spectra in Figure 6.6 were very similar and suggested that Compound A was 4-hydroxy-2-methylbenzoic acid. In addition, the HPLC and capillary GC retention times of Compound A matched those of 4-hydroxy-2-methylbenzoic acid. The UV spectra of the two compounds were identical with absorption maxima at 204 and 256 nm (Table 6.3).

6.8 Utilization of 4-Hydroxy-2-Methylbenzoic Acid by m-Cresol-Metabolizing Cultures

Figure 6.7 demonstrates the metabolism of 4-hydroxy-2-methylbenzoic acid by cultures actively metabolizing m-cresol. The cultures were fed some of the reaction mixture containing all three hydroxymethylbenzoate isomers and were observed to degrade the 4-hydroxy-2-methylbenzoic acid but not the other two isomers. There was a 9 to 16-fold increase in the rate of metabolism of 4-hydroxy-2-methylbenzoic acid, when m-cresol was present in the culture originally, over the rate of 4-hydroxy-2-methylbenzoic acid degradation when m-cresol was not present in the culture (data not shown) or after the original allotment of m-cresol had been metabolized (Figure 6.7). Cultures fed the reaction mixture produced slightly more methane than controls not fed additional substrate. The original concentration of 4-hydroxy-2-methylbenzoic acid in the cultures was not known so a prediction of the amount of methane expected could not be made.

6.9 The Identification of Compound B

Compound B was easily extracted into methylene chloride from a culture supernatant acidified to pH 1. GC/MS analysis of this extract showed a molecular ion of m/z=136 with a base peak of m/z=91 (Figure 6.8a). These are consistent with Compound B being an isomer of methylbenzoic acid or phenylacetic acid. The base peak would be the result of the loss of -COOH. The peak at m/z=118 would

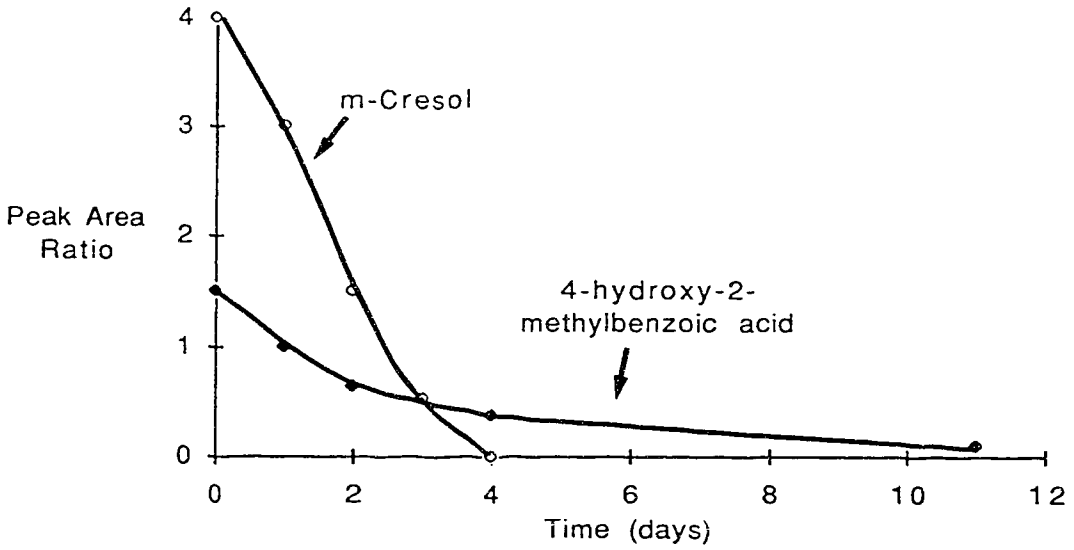


Figure 6.7. The metabolism of 4-hydroxy-2-methylbenzoic acid by active m-cresol metabolizing methanogenic cultures. Triplicate 50% transfers of an m-cresol enrichment culture were incubated in the presence of the extracted products from the reaction of Komiyama and Hirai (1984). Samples of culture supernatant were analyzed by HPLC using mobile phase 1 and phenol as an internal standard. Peak areas are expressed relative to the internal standard.

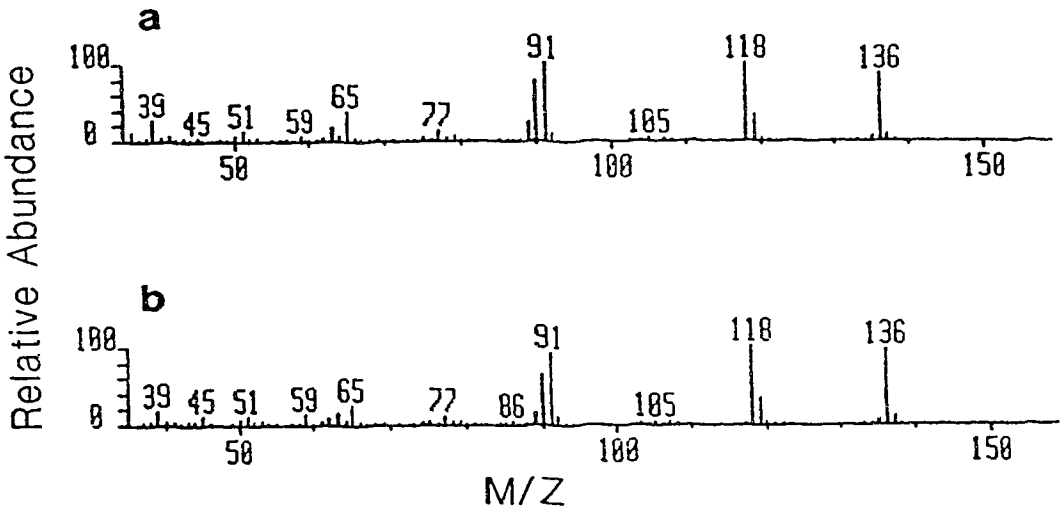


Figure 6.8. Mass spectra of (a) Compound B obtained by methylene chloride extraction of an acidified culture supernatant and (b) 2-methylbenzoic acid produced by a library search as the closest fit to that of (a). The sample was analyzed by GC/MS.

correspond to the loss of H_2O. A computer library search produced the mass spectrum of 2-methylbenzoic acid as the compound with the closest mass spectrum to that of Compound B (Figure 6.8b). The match quality was 958 out of a possible 999.

The methyl ester of Compound B was prepared and analyzed by GC/MS. The analysis showed a molecular ion of m/z=150, a base peak of m/z=119 corresponding to the loss of $-OCH_3$, and another abundant peak of m/z=91 corresponding to the loss of $-COOCH_3$ (Figure 6.9a). A computer library search produced the mass spectrum of the methyl ester of 2-methylbenzoic acid as the compound with the closest mass spectrum to the methyl ester of Compound B (Figure 6.9b). The match quality was 932 out of a possible 999. These results further supported the presumption that Compound B was an isomer of methylbenzoic acid.

Authentic standards of each of the three isomers of methylbenzoic acid and of phenylacetic acid were analyzed by HPLC using mobile phase 1. Only the retention time for 2-methylbenzoic acid matched that of Compound B. Similarly the HPLC retention time of the methyl ester of 2-methylbenzoic acid matched that of the methyl ester of Compound B. In addition, when analyzed by capillary GC, the retention time for 2-methylbenzoic acid and its methyl ester matched those of Compound B and its methyl ester, respectively. Based on these results, Compound B was identified as 2-methylbenzoic acid.

6.10 Utilization of 2-Methylbenzoic Acid by m-Cresol-Metabolizing Cultures

The abundance of 2-methylbenzoic acid in the culture fluids was demonstrated in Figure 6.3. HPLC analysis showed that large amounts of 2-methylbenzoic acid had accumulated in the 2-L enrichment culture over the two year period of maintenance. Although the accumulation of 2-methylbenzoic acid was not always observed in batch cultures, it

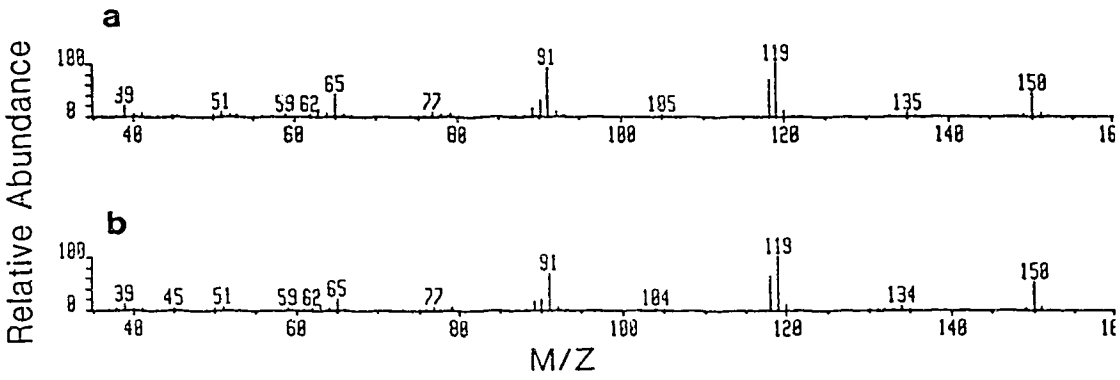


Figure 6.9. Mass spectra of (a) the methyl ester of compound B and (b) the methyl ester of 2-methylbenzoic acid produced by a library search as the closest fit to that of (a). The sample was analyzed by GC/MS.

was occasionally found to be labeled from $^{14}CH_3$-m-cresol or ^{14}C-U-ring-m-cresol. The concentration of 2-methylbenzoic acid remained relatively constant during m-cresol metabolism in batch cultures. Cultures actively metabolizing m-cresol were unable to metabolize authentic 2-methylbenzoic acid. These results suggested that even though large amounts of 2-methylbenzoic acid were present in the 2-L enrichment culture 2-methylbenzoic acid was a dead-end product that was formed only occasionally by m-cresol-degrading methanogenic cultures.

6.11 Summary

The evidence presented in this chapter is summarized in the partial metabolic pathway presented in Figure 6.10. All of the intermediates included have been detected in m-cresol-metabolizing cultures and the positions of the ^{14}C-label from $H^{14}CO_3^-$ and $^{14}CH_3$-m-cresol as determined by single-labeled experiments are indicated by a • and an †, respectively.

The balanced chemical equations for the reactions required to produce the intermediates of m-cresol degradation observed in this study are presented below. The equation for the production of 2-methylbenzoic acid is not included because of the low frequency of this reaction.

$$4\ CO_2 + 4\ H_2O \rightarrow 4HCO_3^- + 4\ H^+ \quad \text{Eqn. 6.1}$$

$$4\ C_7H_8O + 4\ HCO_3^- \rightarrow 4\ C_8H_7O_3^- + 4\ H_2O \quad \text{Eqn. 6.2}$$

$$4\ C_8H_7O_3^- + 20\ H_2O \rightarrow 16\ CH_3COO^- + 12\ H^+ + 4\ H_2 \quad \text{Eqn. 6.3}$$

$$4\ C_7H_8O + 4\ CO_2 + 20\ H_2O \rightarrow 16\ CH_3COO^- + 16\ H^+ + 4\ H_2 \quad \text{Eqn. 6.4}$$

$$16\ CH_3COO^- + 16\ H^+ \rightarrow 16\ CH_4 + 16\ CO_2 \quad \text{Eqn. 6.5}$$

$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \quad \text{Eqn. 6.6}$$

$$4\ C_7H_8O + 18\ H_2O \rightarrow 17\ CH_4 + 11\ CO_2 \quad \text{Eqn. 6.7}$$

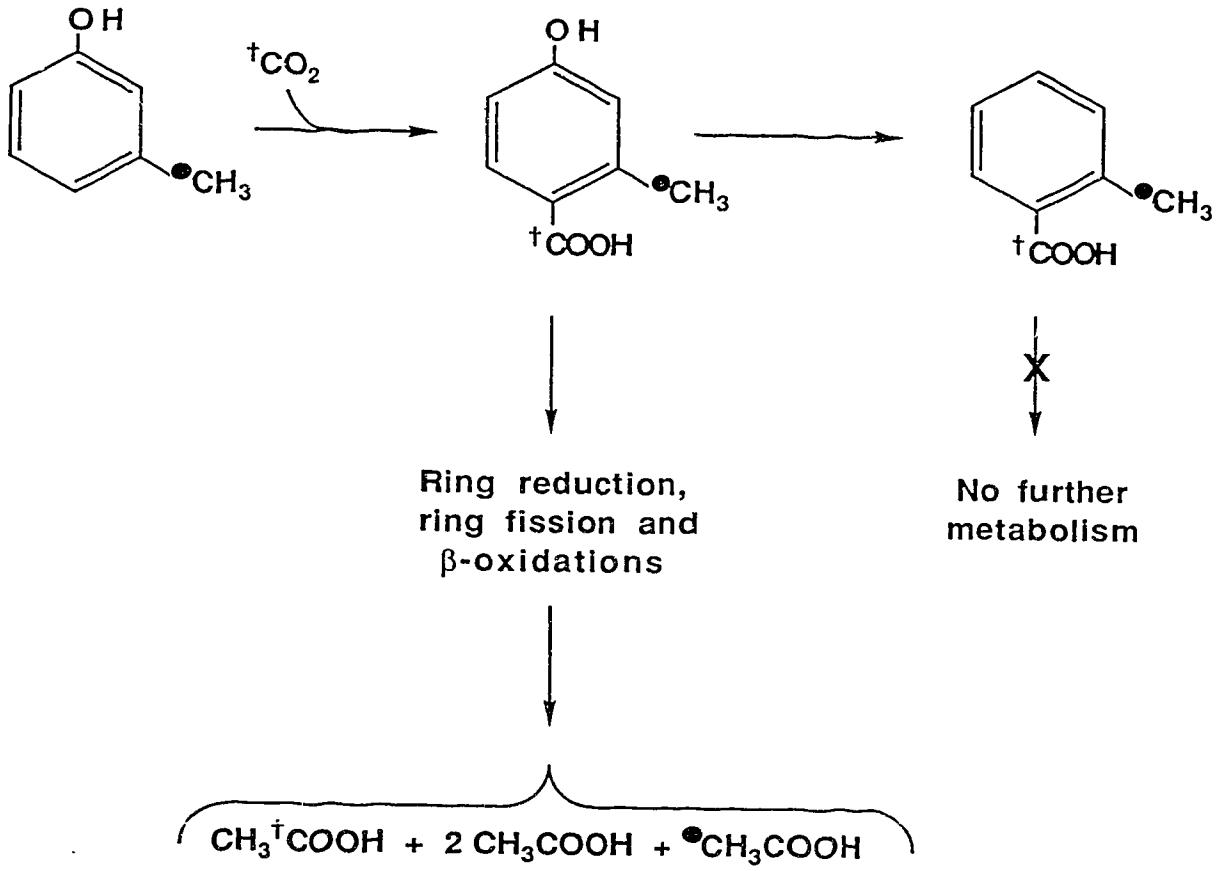


Figure 6.10. The proposed pathway of the non-methanogenic metabolism of m-cresol. No other intermediates or products were detected during this study. The positions of the ^{14}C-carbon atoms used in single-labeled experiments are shown as • and †.

Eqns. 6.1 to 6.3 describe the initial reactions of m-cresol degradation presented in Figure 6.10. The carboxylation reaction producing 4-hydroxy-2-methylbenzoic acid is given in Eqn. 6.2 and the metabolism of this compound to produce acetate is given in Eqn. 6.3. Eqn. 6.4 summarizes the non-methanogenic steps in the metabolic pathway. Eqns. 6.5 and 6.6 are the methanogenic steps in the metabolic pathway, Eqn 6.7 is a summary of the overall pathway obtained by adding equation 6.4, 6.5 and 6.6. This equation can be compared to Eqn. 5.2 discussed earlier. Dividing Eqn. 6.7 by 4 results in Eqn. 5.2 which was obtained using the equation of Buswell and Tarvin (1934). Eqn 6.7 is the same equation given by Godsy and Goerlitz (1984), reported as equation 2.17. The free energies of the reactions given above were not calculated because the ΔG_f° for 4-hydroxy-2-methylbenzoic acid was not available in the literature.

Eqn. 6.4 demonstrates the very low amounts of hydrogen produced during m-cresol degradation. Four mol of m-cresol must be metabolized to provide enough hydrogen to reduce one mol of carbon dioxide. The equations proposed by Godsy and Goerlitz (1984) for the metabolism of m-cresol predicted a total of 28 mol of hydrogen from 4 mol of m-cresol. Twenty of these from the initial reaction (Eqn. 2.13) and eight from the reduction of formate to methane (Eqn. 2.15). This results in a total of 7 mol of hydrogen per mol of m-cresol.

The equations for the anaerobic metabolism of benzoate reported in section 2 predict a total of 12 mol of hydrogen from the 4 mol of benzoate. Eight of these from the initial reaction (Eqn. 2.8) and four from the reduction of formate to methane (Eqn. 2.10). This results in a total of 3 mol of hydrogen per mol of benzoate.

The two aromatic metabolites identified in this study would be formed by CO_2-incorporation giving 4-hydroxy-2-methylbenzoic acid and a dehydroxylation of this giving 2-methylbenzoic acid. The latter reaction appeared to occur to a minor degree yielding a dead-end product. Although no other intermediates of 4-hydroxy-2-methylbenzoic acid metabolism were found, it presumably undergoes ring

reduction, ring fission and β-oxidation.

BESA-inhibited cultures gave 4 mol of acetate per mol of m-cresol. If the cultures contained $H^{14}CO_3^-$, carboxyl-labeled acetate was found. If they contained $^{14}CH_3$-m-cresol, methyl-labeled acetate was found. These results are consistent with ring fission occurring between C-1 and C-2 of 4-hydroxy-2-methylbenzoic acid yielding a C_8-carboxylic acid. The product of the first β-oxidation would be acetate with its carboxyl group derived from the incorporated carbon dioxide. The fourth acetate molecule (resulting from the final β-oxidation of the proposed C_8-carboxylic acid) would have its methyl group derived from the methyl group of m-cresol. These modes of ring cleavage and β-oxidation would yield $^{14}CH_4$ from $^{14}CH_3$-m-cresol as was observed in section 5.2.

The reduction and subsequent fission of aromatic rings containing hydroxyl substituents has been reported previously. Tschech and Schink (1986) found that 2-hydroxybenzoic acid was not dehydroxylated before ring fission and they pointed out that the hydroxyl substituent was in the correct position for further β-oxidations to occur after ring reduction. In the case of 4-hydroxy-2-methylbenzoic acid, the hydroxyl group is also in the correct position for subsequent β-oxidation reactions after ring reduction and fission between C-1 and C-2.

The findings of the current study and a survey of the literature suggest that the presence of a carboxyl moiety on an aromatic ring may facilitate its further metabolism under anaerobic conditions. Knoll and Winter (1987, 1989) have documented the production of benzoic acid in phenol-degrading methanogenic consortia. This was an exergonic reaction and was found to require molecular hydrogen. Tschech and Fuchs (1987) found that the carbon atom of $^{14}CO_2$ was incorporated into 4-hydroxybenzoic

acid by cells of denitrifying pseudomonads when they were grown on phenol. This did not occur when the cells were grown on 4-hydroxybenzoic acid.

The results presented in this thesis are the first to suggest that a methyl group on an aromatic ring was not oxidized during metabolism under anaerobic conditions. Studies with aromatic compound-degrading anaerobic cultures reported in the literature have consistently shown that aryl methyl groups were oxidized to carboxyl groups prior to ring cleavage. For example, Kuhn et al. (1988) studied the degradation of toluene by denitrifying enrichment cultures and found benzoic acid and 4-hydroxybenzoic acid in the culture fluids. The results were inconclusive in regards to the actual pathway of toluene degradation but the evidence was firm that benzoic acid was an intermediate, probably due to an oxidation of the methyl group.

Studies concerning the degradation of p-cresol have shown that 4-hydroxybenzoic acid was an intermediate. This reaction has been seen to occur under aerobic (Bayly et al. 1966 and Tabak et al. 1964), nitrate-reducing (Bossert and Young 1986), sulfate-reducing (Suflita et al. 1989) and methanogenic conditions (Senior and Balba 1984).

Suflita et al. (1989) reported that benzoic acid and a hydroxybenzoate isomer were found in o-cresol-degrading sulfate-reducing enrichment cultures. This was interpreted to be a result of the oxidation of the methyl group of o-cresol. They could find no indication of the oxidation of the methyl group of m-cresol and suggested that an alternate pathway for anaerobic m-cresol decomposition might exist.

The conversion of aryl compounds to benzoic acids by anaerobic consortia may be a fundamental step in their metabolism. Studies of the degradation of benzoic acid have indicated that the first step in these degradations was the activation of benzoic acid by conversion to benzoyl-CoA (Evans and Fuchs 1988). This thioesterification couples the degradation of benzoic acid to the most common energy generating system under anaerobic conditions. This also increases the reactivity of benzoic acid (an α-β

unsaturated acid) towards reducing agents because of the resonance effects between the double bond and the S-CO group (Evans and Fuchs 1988).

The inability of the culture to metabolize 2-methylbenzoic acid suggested the hydroxyl group of 4-hydroxy-2-methylbenzoic acid was required for the metabolism of the latter compound. m-Cresol-metabolizing cultures did not transform 2-hydroxy-4-methylbenzoic acid or 2-hydroxy-6-methylbenzoic acid. This indicated the relative positioning of the hydroxyl and methyl groups was also important. The observation that 4-hydroxy-2-methylbenzoic acid was only slowly metabolized in cultures not currently metabolizing m-cresol suggested that the enzyme or enzyme system responsible for the metabolism may be induced by m-cresol and not 4-hydroxy-2-methylbenzoic acid.

It was unlikely that the hydroxybenzoate isomers were not metabolized due to inabilities of the cells to transport these compounds into the cell. Harwood and Gibson (1986) examined the uptake of benzoate by *Rhodopseudomonas palustris* and proposed that the uptake of benzoate and probably other simple aromatic compounds was due to passive diffusion facilitated by the degradation of benzoate within the cell.

The intermediates identified during this research were found in direct samples of culture fluids. There were no attempts to physically release the compounds from the cells. The fact that intermediates were detected indicated that these intermediates were released from the cells, probably due to passive diffusion when concentrations inside the cell increased.

It has been proposed that the ring cleavage products from the anaerobic metabolism of aromatic compounds are fatty acids and that these are metabolized by β-oxidation reactions to produce methanogenic precursors (Evans and Fuchs 1988). β-Oxidation reactions produce acyl-CoA molecules as products. This thioesterification of reaction products may explain why no intermediates of β-oxidation were detected during m-cresol degradation. These products would not diffuse freely from the cell nor

would they be detected by the methods used in this study. Energy would be generated to carry out cell functions during the cleavage of the thioester bond of acetyl CoA producing acetate, CoA and ATP (Thauer et al. 1977). The acetate could then diffuse from the cell and be used as a substrate by the methanogens.

Other pathways of m-cresol degradation by anaerobic bacteria may exist. The evidence presented in this thesis suggests the consortium converts m-cresol to 4-hydroxy-2-methylbenzoic acid before ring reduction and ring cleavage occur producing 4 mol of acetate per mol of m-cresol. Evidence presented by Godsy and Goerlitz (1984) has indicated that another pathway could exist. They found that formate and acetate accumulated in BESA-inhibited, methanogenic m-cresol-degrading enrichment cultures derived from anaerobic sewage sludge. The molar ratios of formate to acetate were near 1.4. β-Oxidation of a straight chain fatty acid would not be expected to produce these compounds in the observed ratio.

Aerobic metabolism of m-cresol was shown to proceed via two pathways. In the meta pathway the methyl group of m-cresol was left intact, whereas the methyl group was oxidized to form a carboxylic acid in the gentisate pathway. The metabolism of the ring under aerobic conditions was an oxidative process. The products of ring fission were unsaturated fatty acids whereas the proposed ring fission products under anaerobic conditions were saturated fatty acids. β-Oxidation reactions proposed for anaerobic metabolism of ring fission products were not observed to occur under aerobic conditions.

The evidence presented in this section has led to the hypothesis that the initial reaction of m-cresol metabolism was a carboxylation reaction producing 4-hydroxy-2-methylbenzoic acid. The 4-hydroxy-2-methylbenzoic acid then apparently underwent ring reduction and ring fission between C-1 and C-2 before β-oxidation reactions produced 4 mol of acetate for each mol of m-cresol metabolized. The acetate and hydrogen gas produced during the non-methanogenic metabolism of m-cresol were

consumed by methanogenic bacteria which produced 4.13 mol of CH_4 per mol of m-cresol utilized.

7. Overall Summary, Conclusions and Suggestions for Further Research.

The information presented in the Literature Review showed that m-cresol is amenable to aerobic and anaerobic metabolism. The anaerobic degradation of m-cresol is not completely understood and has not been found to occur consistently in all inocula tested.

This study examined some current culture methods used to obtain m-cresol-degrading enrichment cultures and the methods used to maintain and transfer these cultures to fresh medium. The results showed that the m-cresol-degrading bacteria were sensitive to concentrations of sulfide used to prereduce the medium. As little as 1.5 mM sulfide inhibited m-cresol degradation. This extreme sensitivity to sulfide has not been previously reported during other studies of anaerobic cultures. An application of these results to the culture of anaerobic m-cresol-degrading cultures would be to keep the concentration of sulfide in culture fluids or industrial waste-waters for the degradation of m-cresol as low as possible. If high concentrations of sulfide are found in the medium or waste-water that the consortium will be fed, iron could be added to precipitate the soluble sulfide thus removing the toxicity.

The m-cresol-degrading consortium consisted of three nutritional groups of bacteria, each represented by one major morphological type of bacterium. The proposed m-cresol-utilizing organisms were short, fat, non-motile, Gram-negative rods. These organisms were quite prevalent in older, more dilute m-cresol-degrading cultures. The hydrogen gas-utilizing methanogens were long, narrow, unevenly crooked bacteria that fluoresced green under UV light. These bacteria were not very prevalent in the older, more dilute m-cresol-degrading cultures. The proposed acetate-utilizing methanogens were found in chains encased in very long, wide sheaths. These bacteria fluoresced very weakly under UV light and were the most prevalent organisms in older, more dilute m-cresol-degrading cultures. A bacterial consortium that had been maintained on

m-cresol for a long period were not inhibited by this substrate at concentrations of up to 710 mg/L. In contrast the methanogenic population in this same consortium was moderately inhibited by m-cresol concentrations as low as 320 mg/L. The description of this culture compares well to other descriptions of methanogenic cultures that degrade aromatic compounds. The inability to obtain pure cultures of m-cresol-degrading organisms and acetate-utilizing methanogens has also been experienced by other workers.

Results of metabolic studies indicated that the first step in the metabolism of m-cresol was a carboxylation reaction producing 4-hydroxy-2-methylbenzoic acid. This indicated that the consortium had a requirement for carbon dioxide in the medium which was easily supplied by a carbon dioxide/bicarbonate buffer system. This is the first report of the direct carboxylation of a methylated aromatic compound under anaerobic conditions. The carboxylation of m-cresol may be an important step in the pathway as this reaction results in a compound that is more susceptable to ring reduction reactions. The creation of a carboxyl moiety on the aromatic ring is seen to be a common phenomenon in the recent literature concerning the anaerobic degradation of aromatic compounds.

Another compound observed in m-cresol-metabolizing cultures was 2-methylbenzoic acid, presumably, resulting from a dehydroxylation of 4-hydroxy-2-methylbenzoic acid. This compound was not metabolized by the cultures and accumulated very slowly suggesting that it was a dead-end product. The accumulation of dead-end products such as 2-methylbenzoic acid might best be prevented by maintaining a mixed consortium on a mixed substrate waste-water rather than selecting for m-cresol metabolism alone using Medium WR86 containing only m-cresol. This method may allow the population to be diverse enough to maintain the ability to degrade 2-methylbenzoic acid or other dead-end products that arise.

The non-methanogenic metabolism of m-cresol through 4-hydroxy-2-methyl-

benzoic acid produced 4 mol of acetate and, presumably, 1 mol of hydrogen per mol of m-cresol. The methanogenic metabolism of acetate and hydrogen produced during m-cresol metabolism gave rise to 4.13 mol of methane per mol of m-cresol which was 97% of the 4.25 mol predicted by Buswell's equation. These results indicate that m-cresol was completely metabolized to methane and carbon dioxide by the m-cresol enrichment cultures.

Further studies could be done to test the improved culture methods and further define m-cresol metabolism. The application of the improved culture methods could be tested by establishing enrichment cultures on a synthetic mixed substrate wastewater or on an actual wastewater and maintenance of these enrichment cultures over long periods of time under low sulfide conditions. This would best be done by repeating the work of Fedorak and Hrudey (1986a) under low sulfide conditions.

Further studies to determine the complete metabolic pathway of m-cresol metabolism could be performed. These would have to incorporate cell free technique, which is not commonly performed on mixed cultures. Initial investigations could look for intermediates containing thioester bonds such as 4-hydroxy-2-methylbenzoyl-CoA and 4-hydroxy-2-methylcyclohexanoyl-CoA or the acetyl-phosphate esters of these compounds. The identification of β-oxidation intermediates would also rely on the ability to release the acyl-CoA intermediates from the cells.

Methods could be adapted from the studies of Harwood and Gibson (1986). These workers identified the intermediates of anaerobic benzoic acid metabolism by pseudomonads. It is possible that studies requiring cell free systems or at least broken cells can be adapted to mixed culture systems. It may be possible to take advantage of the differences in composition between archaebacterial cell envelopes and eubacterial cell envelopes to devise a system in which only cells from certain members of the mixed culture are broken. This will allow a further definition of the functions of the members of the mixed culture.

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Appendix 1. Media for the Cultivation of Anaerobes

Appendix 1.1. Medium WR86 (Fedorak and Hrudey 1986)

Stock components used in the medium.

	Compound	Amount (g/L of distilled water)
Mineral Solution 1*	NaCl	50
	NH_4Cl	50
	$CaCl_2 \cdot 2H_2O$	10
	$MgCl_2 \cdot 6H_2O$	10
Mineral Solution 2	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	10
	$ZnSO_4 \cdot 7H_2O$	0.1
	H_3BO_3	0.3
	$FeCl_2 \cdot 4H_2O$	1.5
	$CoCl_2 \cdot 6H_2O$	10
	Mn · $4H_2O$	0.03
	$NiCl_2 \cdot 6H_2O$	0.03
	$AlK(SO_4)_2 \cdot 12H_2O$	0.1
Vitamin B Solution	Nicotinic acid	0.1
	Cyanocobalamine	0.1
	Thiamine	0.05
	p-Aminobenzoic acid	0.05
	Pyridoxine	0.25
	Pantothenic acid	0.025
Phosphate Solution	KH_2PO_4	50

* made in 0.01 M HCl rather than distilled water.

Final Composition of Medium WR86.

Component	Amount
Mineral Solution 1	1.0 mL
Mineral Solution 2	0.1 mL
Vitamin B solution	0.1 mL
Phoshate Solution	1.0 mL
Resazurin (0.01%)	1.0 mL
Sodium Bicarbonate	0.57 g
Distilled Water	97.0 mL
Sulfide (2.5% $Na_2S \cdot 9H_2O$)	1.0 mL

Mix all ingredients except sulfide and bicarbonate. Bring to boil, continue boiling for 2 min. Cool slightly then add bicarbonate, sparge with 30% CO_2/N_2 through a gas dispersion tube until the pH is 6.9-7.1. Tube or bottle anaerobically. Autoclave the bottled medium and sulfide solution separately. Add 1% (vol/vol) of sterilized sulfide solution just prior to inoculation. Substrates can be added to the medium before the bottles are autoclaved or as sterile solutions after the medium has been autoclaved.

Appendix 1.2. Butlin's Medium (Butlin et al. 1949)

Compound	Amount	Modifications
K_2HPO_4	0.5 g	
NH_4Cl	1.0 g	
Na_2SO_4	2.0 g	1
NaCl	0.56 g	
$CaCl_2 \cdot 2H_2O$	0.067 g	
$MgSO_4 \cdot 7H_2O$	1.0 g	2
Sodium-Lactate (60%)	2.5 mL	3
Yeast Extract	1.0 g	4
$FeSO_4 \cdot 7H_2O$	0.0036 g	5
Resazurin (0.01%)	10 mL	
Distilled water	1000 mL	

Mix all ingredients. Adjust the pH to 7.5 with 1N NaOH, tube or bottle anaerobically, adding two iron nails, that have been washed with methylene chloride then distilled water, to each tube. Sterilize by autoclaving.

Modifications

1 This ingredient was omitted for Butlin's minus sulfate medium.

2 This ingredient was replaced with $MgCl_2 \cdot 6H_2O$ in Butlin's minus sulfate medium.

3 This ingredient was omitted from all media. m-Cresol (300 mg/L final concentration) was added to the medium as a substrate.

4 This ingredient was omitted for Butlin's minus yeast extract medium.

5 This ingredient was replaced with $FeCl_2 \cdot 4H_2O$ in Butlin's minus sulfate medium.

Complete 1043 Medium

Mix the ingredients listed below in 950 mL distilled water and autoclave for 15 min. Cool under 3% H_2 in N_2.

Compound	Amount
K_2HPO_4	348 mg
KH_2PO_4	227 mg
NH_4Cl	500 mg
$MgSO_4 \cdot 7H_2O$	500 mg
$CaCl_2 \cdot 2H_2O$	250 mg
$FeSO_4 \cdot 7H_2O$	2.0 mg
Yeast extract	2.0 g
Casitone	2.0 mg
NaCl	2.25 mg
Trace element SL-6	3.0 mL
Resazurin (0.025%)	4.0 mL

When the above mixture is cool then add these ingredients to the mixture in this order

Vitamin solution	10.0 mL
$NaHCO_3$ solution	20.0 mL
Methanol*	10.0 mL
Cysteine/Na_2S solution	20.0 mL

Tube anaerobically and aseptically.

*The medium was modified by replacing the methanol with 200-300 mg/L m-cresol for the cultivation of m-cresol-degrading enrichments.

Appendix 2. Individual Experimental Methods

The detailed methods for the experiments described in Chapters 4, 5 and 6 are outlined in this appendix. The experiments are identified by the title of the section in which they appear in their respective chapters.

Appendix 2.1 Methods for Experiments Described in Chapter 4

Comparison of the Activities of m-Cresol-Degrading Consortia in Three Different Media

Trial #1: Medium WR86, modified Butlin's medium or *Methanosarcina* medium were prepared as outlined in Appendix 1. Medium WR86 was prereduced with 0.1 mL of a 2.5% solution of sodium sulfide (final concentration 1 mM). *Methanosarcina* medium was prereduced with 0.1 mL of a cysteine and sulfide solution (1.7 and 2.5 mM final concentrations, respectively). Butlin's medium was prereduced with 2 iron nails per culture. Inoculum from a 3-month-old batch enrichment culture was used to inoculate serial two-fold dilutions into 5 mL of each of the media. m-Cresol was added to an initial concentration of 300 mg/L (1 mL of a 1,500 mg/L solution) after the dilutions were made. m-Cresol concentrations in the culture supernatants were monitored weekly.

Trial #2: Modified Butlin's medium, modified Butlin's medium minus sulfate or modified Butlin's medium minus yeast extract were prepared as outlined in Appendix 1.2. Inoculum from a 5-month-old batch enrichment culture was used to inoculate serial two-fold dilutions into 5 mL of each of the media. m-Cresol was added to an initial concentration of 200 mg/L (1 mL of a 1,000 mg/L solution) after the dilutions were made. m-Cresol concentrations in the culture supernatants were monitored weekly.

The final diltuion (1/1024) in Butlin's medium minus sulfate was maintained by feeding 1/10 of the culture volume of a solution of 3,000 mg/L m-cresol in Medium WR86 as necessary. This culture was designated as Consortium A.

Comparison of the Effect of Commonly Used Reducing Agents on m-Cresol Degradation

AFS was prepared as per Brock and O'Dea (1977). Ferrous ammonium sulfate (1.96 g) and sodium sulfide (1.2 g) were boiled separately in 25 mL distilled water, each. The two solutions were combined in a 59-mL serum bottle and the headspace

The Use of Alternate Terminal Electron Acceptors by m-Cresol-Degrading Cultures

Trial # 1: 5 millilitre portions of the second 2-L enrichment culture were used to inoculate triplicate cultures consisting of 4.5 mL of Medium WR86 and 0.5 mL of m-cresol solution (2,000 mg/L, final concentration 200 mg/L) in 59 mL serum bottles. Na_2SO_4 (28.4 mg) or KNO_3 (30 mg) were added to two triplicate sets of serum bottles before the medium (sulfate and nitrate as terminal electron acceptors, respectively). One triplicate set received no additions (carbon dioxide as terminal electron acceptor) and another was incubated aerobically (oxygen as terminal electron acceptor). Control cultures to show indigenous methane production received 0.5 mL of anaerobic distilled water in place of m-cresol solution. The concentrations of m-cresol and the amounts of methane in the culture were determined several times during the incubation period.

Trial #2: 5 millilitre portions of the second 2-L enrichment culture were used to inoculate triplicate cultures consisting of 4.0 mL of Medium WR86 and 0.5 mL of m-cresol solution (2,000 mg/L, final concentration 200 mg/L). Two triplicate sets received 0.5 mL of anaerobic distilled water but no other additions. One of these was incubated aerobically (oxygen as the terminal electron acceptor), the other was incubated in the usual manner (carbon dioxide as terminal electron acceptor). Other cultures received 0.5 mL of BESA solution (50 mM final concentration) to inhibit methanogenesis and 28.4 mg of Na_2SO_4, 49.6 mg of $Na_2S_2O_3$, or 20.2 mg of KNO_3 (sulfate, thiosulfate or nitrate as terminal electron acceptors, respectively). The concentrations of m-cresol and the amounts of methane in the culture were determined several times during the incubation period. The cultures were fed an additional allotment of m-cresol solution (200 mg/L final concentration) after the first allotment of m-cresol was degraded.

Attempts to Isolate Specific Nutritional Types of Bacteria from the m-Cresol-Degrading Consortium

Pure and coculture techniques were performed in order to isolate bacteria from an m-cresol-degrading consortium. The isolations were performed according to the function of the organisms in the consortium.

Hydrogen Gas-Utilizing Methanogenic Bacteria

Serial 1/5 dilutions from 1 mL inoculum from the first 2-L m-cresol-degrading

enrichment culture were made into 4 mL of Medium WR86. The bicarbonate buffer provided the carbon source and 10 mL of hydrogen gas was added as a source of reducing power. When methane analysis showed that the production of methane had reached a plateau in the last positive dilution (1×10^{-7}), it was fed 10 mL of filter sterilized hydrogen gas using a sterile syringe. This was repeated each time methane production reached a plateau, until the culture appeared turbid. The dilution process was repeated once using inoculum from the turbid culture. The 1×10^{-7} dilution of the second dilution series was fed 10 mL of hydrogen gas until it was turbid and was then used to inoculate a ten-fold dilution series in roll tubes containing Medium WR86 plus hydrogen gas and 2% agar. Colonies that grew and gave evidence of gas production were picked and added to Hungate tubes containing 2 mL of Medium WR86 and 10 mL of hydrogen gas. One of these transfers survived. This culture was fed 10 mL of hydrogen gas as necessary.

When the resultant culture was turbid it was used to inoculate two Hungate culture tubes containing 4 mL Medium of WR86 and 10 mL of hydrogen gas. These cultures were maintained by periodic 1/5 transfers into fresh medium plus hydrogen gas. The purity of the culture was monitored by microscopic analysis and by occasionally streaking some of the culture onto plate count agar (PCA) and incubating both aerobically and anaerobically.

Acetate-Utilizing Methanogenic Bacteria

Attempts to obtain pure cultures of acetate-degrading methanogens were performed in the same manner as those for hydrogen-utilizing methanogens except that 1,000 mg/L of glacial acetic acid was added to Medium WR86 in place of hydrogen gas. After the first set of dilutions 1,000 mg/L of yeast extract was also included in the medium.

m-Cresol-Utilizing Members of the Consortium

Trial #1: Inoculum from the culture described in section 4.1.1 designated Consortium A was used to inoculate a ten-fold dilution series in Medium WR86 and Medium WR86 plus 1% beef extract. The growth tubes were amended with 1 mL of a fumarate solution (final concentration 25 mM), 0.5 mL of a formate solution (final concentration 5 mM) and 0.5 mL of a turbid suspension of *W. succinogenes* (ATCC #29543). One milliliter of a 2,000 mg/L m-cresol solution (final concentration 200 mg/L) was added after the dilutions were made. The concentration of m-cresol in the culture supernatant was monitored periodically over the 3-month incubation period.

Trial #2: Inoculum from the culture designated Consortium A was used to inoculate a two-fold dilution series. Five millilitres of the inoculum was diluted into 3 mL of Medium WR86 plus 2 mL of anaerobic distilled water. This was used to inoculate two dilution tubes containing 3 mL of Medium WR86, 1mL of BESA solution (5 mM final concentration) and either 1 mL of m-cresol solution (2,000 mg/L, 200mg/L final concentration) or 1 mL of glucose solution (2,000 mg/L, 200 mg/L final concentration). When these cultures had either degraded the m-cresol present or become turbid, they were used to inoculate another 50% transfer into another dilution tube of the same composition. The culture containing glucose was maintained to the 1/64 dilution and then transferred back into medium containing m-cresol.

Trial #3: Two 5-fold dilution series of inoculum from the culture designated Consortium A were made into dilution tubes containing turbid growth of *M. barkeri*.. The dilution tubes were set up with 3 or 4 mL of Medium WR86, 0.1 mL of a 40,000 mg/L acetate solution (final concentration 1,000 mg/L) and 1 mL of a turbid suspension of *M. barkeri*. Cultures that degraded the initial allotment of m-cresol were fed another 200 mg/L. If this was not degraded the cultures were fed 1 mL of a turbid culture of *M. barkeri*.

Appendix 2.2 Methods for Experiments Described in Chapter 5

Determination of Methane and Carbon Dioxide Yields from Unlabeled m-Cresol

Five experimental trials were performed to determine the yields of methane and carbon dioxide from m-cresol. These consisted of four trials of quintuplicate and one trial of quadruplicate cultures. All cultures were established using 5 mL of inoculum from batch enrichment cultures or one of the 2-L enrichment cultures. The cultures also received 4.5 mL of Medium WR86 and 0.5 mL of m-cresol solution (4,000-6,000 mg/L, final concentrations 200-300 mg/L). The same number of control cultures as test cultures in each trial received anaerobic distilled water in place of the m-cresol solution.

GC and pressure transducer analyses of the volumes of total gas and methane in the headspace of the cultures were performed daily for controls and tests until the volume of methane reached a constant amount in each culture. m-Cresol concentration was determined by GC analysis initially and after the incubation was complete. Total gas and methane production per mol of m-cresol were determined by subtracting the volumes measured in control cultures from those measured in test cultures and dividing the net volume produced by the amount of m-cresol consumed.

Products from ^{14}C-Labeled m-Cresol and ^{14}C-Bicarbonate

Test and control cultures were established in 34 mL serum bottles. These contained 5 mL of inoculum from m-cresol batch cultures or either of the 2-L enrichment cultures, 4 mL of Medium WR86 and 1 mL of a 2,000 mg/L m-cresol solution (final concentration 200 mg/L). Control cultures received 1 mL of anaerobic distilled water in place of the m-cresol solution. After the cultures were inoculated, the radioactive substrates were added in microlitre amounts.

$^{14}CH_3$-m-cresol: A triplicate set of cultures fed 1 x 10^6 dpm of $^{14}CH_3$-m-cresol and 200 mg/L of unlabeled m-cresol was monitored periodically by GC analysis to determine m-cresol concentration in the supernatant and by GC/GPC analysis to determine the activities of radioactive gases in the headspace. The total percent of $^{14}CO_2$ found in the cultures during these analyses was estimated from the amount of $^{14}CO_2$ found in the headspace gases divided by 0.31. This was the proportion of $^{14}CO_2$ partitioned into the headspace gases at the end of the incubation period, calculated by comparing the $^{14}CO_2$ in the headspace, as determined by GC/GPC analysis, with the total $^{14}CO_2$ in the culture, as determined by flushing acidified

cultures and trapping the $^{14}CO_2$ released in Carbosorb before LSC (Section 3.4.5). Six cultures fed 1 x 10^6 dpm of $^{14}CH_3$-m-cresol were left unanalyzed throughout the incubation. These were analyzed to determine the final distribution of label as $^{14}CH_4$, $^{14}CO_2$, and ^{14}C in cells and filtrate after the triplicate set had completed the metabolism of m-cresol, as indicated by a plateau in gas production (20 d incubation). The procedures for the analyses are presented in sections 3.4.5 and 3.4.8.

Ring-U-^{14}C-m-cresol: Two triplicate sets of cultures fed 1 x 10^6 dpm of ^{14}C-U-ring-m-cresol and 200 mg/L of unlabeled m-cresol were established. One of these sets was monitored throughout the incubation as for cultures fed $^{14}CH_3$-m-cresol whereas the second set was left unanalyzed until the end of the incubation period (73 d incubation). All six cultures were analyzed to determine the final distribution of the ^{14}C-label from ring-U-^{14}C-m-cresol as described for the cultures fed $^{14}CH_3$-m-cresol.

$H^{14}CO_3^-$: Two triplicate sets of cultures were fed 5 x 10^5 dpm of $H^{14}CO_3^-$ and 200 mg/L of unlabeled m-cresol. One triplicate set of control cultures containing $H^{14}CO_3^-$ but no m-cresol was also established. The cultures were incubated and analyzed as described for the cultures fed $^{14}CH_3$-m-cresol. Control cultures not fed m-cresol were analyzed for the final distribution of label at the same time as test cultures (20 d incubation).

The Fate of the Radioactivity from $^{14}CH_3$-p-Cresol

Test and control cultures were established in 34 mL serum bottles. These contained 5 mL of inoculum from a 2-L p-cresol enrichment culture, 4 mL of Medium WR86 and 1 mL of a 2,500 mg/L p-cresol solution (final concentration 250 mg/L). Control cultures received 1 mL of anaerobic distilled water in place of the p-cresol solution. After the cultures were inoculated, $^{14}CH_3$-p-cresol or $H^{14}CO_3^-$ were added in microlitre amounts to give final activities of 1 x 10^6 and 5 x10^5 dpm in the cultures, respectively.

Triplicate cultures receiving $^{14}CH_3$-p-cresol or $H^{14}CO_3^-$ plus unlabeled p-cresol were monitored periodically by GC analysis to determine the p-cresol concentration in the supernatant and GC/GPC analysis to determine the activities of radioactive gases in the headspace. Another set of triplicate cultures receiving either $^{14}CH_3$-p-cresol, $H^{14}CO_3^-$ plus unlabeled p-cresol or $H^{14}CO_3^-$ with no p-cresol were left unanalyzed

during the incubation. After p-cresol metabolism was complete in the first triplicate sets of cultures, as indicated by a plateau in gas production (14 d incubation) all of the cultures were analyzed to determine the final distribution of label in $^{14}CH_4$, $^{14}CO_2$ and as ^{14}C in cells and filtrate. The methods for these analyses are discussed in sections 3.4.5 and 3.4.8.

Appendix 2.3 Methods for Experiments Described in Chapter 6

Detection of Acetate from $^{14}CH_3$-m-Cresol Metabolism

Triplicate cultures, in 34 mL serum bottles, consisting of 4 mL of Medium WR86, 1 mL of a 2,000 mg/L m-cresol solution and 5 mL of inoculum from a 5-month-old batch enrichment culture were fed 1 x 10^6 dpm of $^{14}CH_3$-m-cresol after inoculation. The cultures were incubated for 52 days, at which time as described above and in Section 5.2.2 the majority of the m-cresol had been removed as indicated by GC analysis but no radioactive gases could be detected by GC/GPC analysis. The cultures were used for volatile organic acid analysis using the GC/GPC, acetate purification by anion exchange chromatography and the Schmidt degradation.

Time Course Analyses of Acetate Production During m-Cresol Metabolism

Cultures were established in 34 mL serum bottles. These cultures consisted of 4.5 mL of Medium WR86, 5 mL of inoculum from the second 2-L enrichment culture and 0.1 mL of a 20,000 mg/L m-cresol solution. One set of triplicate cultures also received 0.1 mL of BESA solution (50 mM final concentration) to inhibit methanogenesis while another set of triplicate cultures was left unamended. The culture volumes were brought to 10 mL with anaerobic distilled water. The concentration of m-cresol and acetate in the culture supernatants were followed by GC analyses. The production of methane by the cultures was followed by GC analyses of the headspace gases. HPLC analyses were performed to provide time course analyses of the accumulation of soluble intermediates.

Determination of the Total Amount of Acetate Produced by BESA-Inhibited m-Cresol-Degrading Cultures

Four experimental trials were performed to determine yields of acetate from BESA-inhibited m-cresol-degrading cultures. These trials consisted of three trials of quintuplicate cultures and one trial of six cultures. The cultures, in 58 mL serum bottles, consisted of 4 mL of Medium WR86, 0.5 mL of m-cresol solution (4,000 mg/L, final concentration 200 mg/L) and 0.5 mL of BESA solution (50 mM BESA final concentration) and 5 mL of inoculum from m-cresol batch enrichment cultures or the second 2-L enrichment culture. Equal numbers of control cultures were established with anaerobic distilled water in place of m-cresol solution in each trial to determine endogenous acetate production.

The initial concentrations of m-cresol and acetate were determined and the cultures were incubated until acetate concentrations reached a plateau. The final concentration of m-cresol was determined and subtracted from the initial concentration to obtain the amount of m-cresol used. The initial concentration of acetate was subtracted from its final concentration to determine the amount of acetate produced. These two values were used to determine the amount of acetate produced compared to the amount of m-cresol used. Control cultures not fed m-cresol were analyzed to determine if acetate accumulated in the absence of m-cresol.

Incorporation of $H^{14}CO_3^-$ into Acetate During m-Cresol Metabolism

Trial #1: Cultures were established in 34 mL serum bottles. These consisted of 3 mL of Medium WR86, 1 mL of m-cresol solution (2,000 mg/L), 1 mL of BESA solution (50 mM final concentration) and 5 mL of inoculum from a 9-month-old batch enrichment culture. Control cultures received anaerobic distilled water in place of the m-cresol solution. The cultures received 1 x 10^7 dpm of $H^{14}CO_3^-$ after inoculation. m-Cresol concentration in the culture supernatants was determined by GC analyses. The cultures were incubated until m-cresol degradation was complete before samples were subjected to acetate purification by anion exchange chromatography and the Schmidt degradation.

Trial #2: Cultures were established as in Trial #1 using inoculum from the second 2-L enrichment culture. The culture supernatants were analyzed by GC to determine m-cresol concentrations and by HPLC/LSC analyses to detect soluble radioactive intermediates. Acetate was purified by anion exchange chromatography and subjected to the Schmidt degradation.

Trial #3: Cultures were established as in Trial #1 using a N_2 atmosphere rather than a CO_2/N_2 atmosphere to increase the specific activity of $H^{14}CO_3^-$. The inoculum was from the second 2-L enrichment culture. Analyses were performed as Trial #2.

Determination of the Positions of the ^{14}C-Label in Acetate from Incorporation of $^{14}CH_3$-m-Cresol or $H^{14}CO_3^-$

Studies using $H^{14}CO_3^-$:

Samples from the cultures described above were used for acetate purification and the Schmidt degradation.

Studies Using $^{14}CH_3$-m-cresol:

Trial#1: This trial was described in the previous section.

Trial #2: Cultures were established in 34 mL serum bottles. These cultures consisted of 4.5 mL of Medium WR86, 5 mL of inoculum from the second 2-L enrichment culture and 0.1 mL of a 20,000 mg/L m-cresol solution. One set of quadruplicate cultures received 0.1 mL of BESA solution (50 mM final concentration) to inhibit methanogenesis while another set of quadruplicate cultures was left unamended. The volume of the cultures was brought to 10 mL with anaerobic distilled water. The first three BESA-inhibited cultures received 1 x 10^5 dpm of $^{14}CH_3$-m-cresol while a fourth BESA-inhibited culture and the uninhibited set were fed 5 x 10^6 dpm of $^{14}CH_3$-m-cresol after inoculation. m-Cresol concentration in the culture supernatants was monitored by GC analyses.

The uninhibited culture that received 5 x 10^6 dpm of $^{14}CH_3$-m-cresol was frozen when 50% of the m-cresol had been degraded and later used for acetate purification and the Schmidt degradation. The BESA-inhibited cultures receiving 1 x 10^5 dpm of $^{14}CH_3$-m-cresol were incubated until m-cresol degradation had stopped (22 d) and were then used for the Schmidt degradation. The BESA-inhibited culture receiving 5 x 10^6 dpm of $^{14}CH_3$-m-cresol was sampled periodically for HPLC/LSC analysis.

Trial #3: Two sets of triplicate cultures were established in 34 mL serum bottles using inoculum from a 15-month-old batch enrichment culture (5 mL). The cultures received 4 mL of Medium WR86 and 0.5 mL of a 4,000 mg/L m-cresol solution (200 mg/L final concentration). One set of triplicate cultures received 0.5 mL of BESA solution (50 mM final concentration) whereas the second set received 0.5 mL of anaerobic distilled water. The concentration of m-cresol in the culture supernatants was followed by GC analyses. The uninhibited cultures were frozen when 50% of the m-cresol had been degraded and used for HPLC/LSC analyses, acetate purification and the Schmidt degradation.

Trial #4: Cultures were established and analyzed as in Trial #3 using inoculum from the second 2-L enrichment culture.

HPLC/LSC Analysis of Supernatants from Cultures Fed $^{14}CH_3$-m-Cresol, Ring-U-^{14}C-m-Cresol or $H^{14}CO_3^-$

Studies using $^{14}CH_3$-m-cresol:

Samples from cultures described in the previuos sections were used for HPLC/LSC analyses.

Studies Using ring-U-^{14}C-m-cresol:

Cultures were established in 34 mL serum bottles. These consisted of 4.5 mL of

Medium WR86, 0.5 mL of a 4,000 mg/L m-cresol solution and 5 mL of inoculum. Six cultures were inoculated with 5 mL of an 18-month-old batch enrichment culture whereas six cultures received inoculum from the second 2-L enrichment culture. A triplicate set from each of these inocula was sterilized by autoclaving. The cultures were fed 1 x 10^8 dpm of ring-U-^{14}C-m-cresol after inoculation. The cultures were monitored periodically by HPLC/LSC analysis to determine soluble radioactive intermediates.

Studies using $H^{14}CO_3^-$: Samples from the cultures described in previous sections were used for HPLC/LSC analyses.

The results for the time course analyses of Compound A in culture supernatants were obtained by HPLC analyses of the cultures that did not contain BESA described in Trial #1 of the section entitled "Incorporation of $H^{14}CO_3^-$ into Acetate During m-Cresol Metabolism.

Identification of Compound A.

Several supernatant samples removed from the first 2-L enrichment culture during the draw and feed procedures were combined. Neutral compounds, including m-cresol, were removed by adjusting the pH to 8.5 then extracting with methylene chloride. Some acidic compounds present in the culture fluids were removed by acidification of the aqueous phase to pH 1 before another extraction with methylene chloride. The remaining extractable acids including Compound A were extracted into diethyl ether. The extracts were used for GC then GC/MS analysis. The extraction procedure was repeated on other samples throughout the study period. The details of the identification of Compound A was discussed in Chapter 6.

Utilization of 4-Hydroxy-2-Methylbenzoic Acid by m-Cresol-Metabolizing Cultures

Trial #1: Triplicate cultures were established in 59 mL serum bottles using 5 mL of inoculum from the 2-L enrichment culture. The cultures consisted of 4.5 mL of Medium WR86 and one of 0.5 mL of m-cresol solution (4,000 mg/L), 0.5 mL of a sample of the reaction mixture described in Section 3.4.10 or 0.5 mL of anaerobic distilled water. The cultures were analyzed to determine the concentrations of the compounds from the reaction mixture or m-cresol in the supernatant by HPLC and the volume of methane in the headspace gases by GC. When the concentration of m-cresol in the m-cresol fed cultures was 3/4 of the original concentration, two of the three cultures were fed an aliquot of the reaction mixture.

Trial #2: Cultures were established as in Trial #1. Inoculum was obtained from a batch enrichment culture (16-months-old) that had not completely degraded its last allotment of m-cresol. The inoculum carried m-cresol into the experimental cultures. A single culture was sterilized by autoclaving then fed a portion of the reaction mixture described in section 3.4.10. The exact amount of 4-hydroxy-2-methylbenzoic acid added was not known because no pure standard was available. Analyses were performed as described for Trial #1.

The Identification of Compound B

Compound B was extracted from the sample described in the section on identification of Compound A by extraction at pH 1 with methylene chloride. The details of the identification procedure was presented in Chapter 6.

Utilization of 2-Methylbenzoic Acid by m-Cresol-Metabolizing Cultures

Triplicate cultures were established in 59 mL serum bottles using inoculum from the second 2-L enrichment culture. The cultures consisted of 4 mL of Medium WR86 and one of 0.5 mL of m-cresol solution (4,000 mg/L), 0.5 mL of 2-methylbenzoic acid solution (4,000 mg/L), or 0.5 mL of anaerobic distilled water. One set of cultures received both m-cresol and 2-methylbenzoic acid. The cultures were analyzed by HPLC to determine the concentration of m-cresol and 2-methylbenzoic acid in the culture supernatant and by GC to determine the volume of methane in the headspace gases.