12/88

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION

NOTICE OF PROJECT CLOSEOUT

Date 5/24/89

Project No. G-32-625 Center No. R6033-0A0

Project Director W. J. Jones School/Lab App. Bio.

Sponsor US Department of Energy

Contract/Grant No. DE-AC21-85FC10625 GTRC XX GIT ___

Prime Contract No. ___

Title Biological Degradation of Low-Rank Coal

Effective Completion Date 5/30/88 (Performance) 5/30/88 (Reports)

Closeout Actions Required:

___ None
___ Final Invoice or Copy of Last Invoice - Already submitted.
X Final Report of Inventions and/or Subcontracts - Patent questionnaire sent to PI
___ Government Property Inventory & Related Certificate - Already submitted.
___ Classified Material Certificate
___ Release and Assignment
___ Other ___

Includes Subproject No(s). E-20-G02(R6033-0A1)/Chian/CE

Subproject Under Main Project No. ___

Continues Project No. ___ Continued by Project No. ___

Distribution:

X Project Director
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Biological Degradation of Low-Rank Coal

Final Report

Submitted by
W. J. Jones
School of Applied Biology
Georgia Institute of Technology
Atlanta, Georgia 30332

Work performed under contract no. DE-AC18-85FC10625

Prepared for
U. S. Department of Energy
Morgantown Energy Technology Center
Morgantown, West Virginia

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Abstract

The principal objective of this research project was to investigate the potential for anaerobic bioconversion of low-rank coal. The research was divided into three phases, including: (a) assessment of biodegradation and coal chemistry, (b) anaerobic bioconversion of "model" low-rank coal constituents; and (c) anaerobic bioconversion of coal.

A literature review of coal chemistry and microbially-mediated processes related to coal bioconversion was performed. Only a few investigations were found dealing directly with coal bioconversion processes; however, several studies related to anaerobic bioconversion of simple (mono-aromatic organics) constituents possibly related to coal structure have been reported. Anaerobic bioconversion of complex aromatic constituents has not been reported in any detail.

Initial lab studies were conducted with selected "model" compounds, including simple aromatic constituents (phenol, cresol, catechol) as well as more complex aromatic compounds (naphthol, 9-phenanthrol, dibenzothiophene) which may be components of low-rank coal. Analytical procedures were developed for efficient extraction, separation and quantitation of the test "model" compounds. Subsequent bioconversion studies (strictly anaerobic conditions) with various natural anaerobic inocula revealed that the more complex aromatics constituents were more recalcitrant to anaerobic bioconversion than were the simple mono-aromatic compounds. Phenol, p-cresol and catechol were anaerobically transformed to CH_4 after six to eight weeks incubation at 30-37°C. However, little evidence of significant bioconversion of the complex aromatics was noted.

Additional studies with a benzene-derived extract of a low-rank coal sample were performed. Extraction and quantitation procedures were developed to assess bioconversion potential. Little or no evidence for biotransformation of the major benzene-derived constituents was noted. Further, oxidation (H_2O_2) of the benzene-derived extract provided no enhancement of biodegradation by the natural anaerobic inocula in this study.

Preliminary toxicity experiments with "model" compounds revealed partial inhibition of

growth of selected pure bacterial cultures as well as inhibition of microbial consortia at concentrations above those used in our test system. For most of the test compounds, little or no inhibition (toxicity) was noted.

Overall results suggest that complex aromatic constituents which may be representative of low-rank coal structure are relatively recalcitrant to microbial attack by natural microbial populations.

I. Assessment of Biodegradation of Low-Rank Coal

The Statement of Work for year one activities under contract DE-AC18-85FC10625 requested that an assessment of low-rank coal biodegradation be completed early in the contract period. This goal has been performed and the following text includes pertinent information, primarily from available literature, related to:

(1) coal chemistry, structure and composition, with primary emphasis on low-rank coals (primarily lignite and subbituminous);

(2) physical and/or chemical treatment of coal to enhance biodegradability; and

(3) anaerobic processes for bioconversion of low-rank coal or related constituents to fuel products such as methane.

Information acquired from this assessment will aid in the selection of model compounds, representative of organic compounds found as relevant constituents of low-rank coal, to be used in initial biodegradation experiments and will be invaluable in our attempt to establish microbial cultures capable of bioconversion of the model compounds to methane.

An accurate assessment of the biological degradation or conversion of low-rank coal by anaerobic processes must include information on coal chemistry, structure, and composition as well as information concerning anaerobic bioconversions related to low-rank coal constituents. The goal of this assessment is to provide information in these subject areas in order to establish an experimental plan for the development of bacterial cultures capable of conversion of low-rank coal to fuel related products such as methane.

A. Coal chemistry, structure and composition. Coal is an extraordinary, complex material. It is not our intention to discuss details of the various types of coals and their constituents, but rather to emphasize differences between "low-rank" and "high-rank" coals with relation to structure and composition. Of primary interest in our research are the organic constituents comprising low-rank coal which may be used as a substrate and metabolized by microorganisms.

To this end, it is desirable to identify major constituents of coal of different rank. An

interpretation of coal structure and coal composition has been made by several methods, including:

(a) identifying materials from which coal was originally formed and deducing what change may have occurred in the original material with time through various chemical, physical and biological processes (1), and

(b) identifying products formed from coal through solvent extraction, liquefaction and thermal degradation processes (2).

Each method only approximates original coal structure and composition and the methods have drawbacks in interpretations.

The original organic constituents of coal are predominantly celluloses, lignins and other minor plant components. The process in which these materials are converted to coal is termed coalification, and it is generally accepted that reactions such as deoxygenation followed by dehydrogenation occur as coal matures. If lignins are the most abundant starting material for coal formation, one can begin to visualize the structures which might be found in coal or which are condensed over time to form the final coal structure. The basic structural unit of lignin is considered to be phenyl-propane units held together by diaryl-ether linkages and carbon-carbon bonds, an illustration of which is given in Figure 1.

Thus, if the lignin structure remained intact in the coal particle, constituents released by heat treatment (Fig. 1) may be the structural compounds found in coal and these would include mono-aromatic structures containing oxygen. It is unlikely that the lignin structure remains intact since the coalification process involves deoxygenation and dehydration reactions (polycondensation). The extent of these reactions determines the "rank" or maturity of coal, and it has been shown that oxygen content in coal decreases with increasing coal rank (1). Thus, it is unlikely that mono-aromatic compounds are major constituents of low-rank or higher rank coals. This is in agreement with many chemists who are currently investigating coal structure and constituents of coal (Ben Benjamin, Oak Ridge National Labs, personal communication). Further, many functional oxygen groups ($-OCH_3$, -COOH, -OH, C=O) are believed to exist in coal constituents and these functional groups vary in type and concentra-

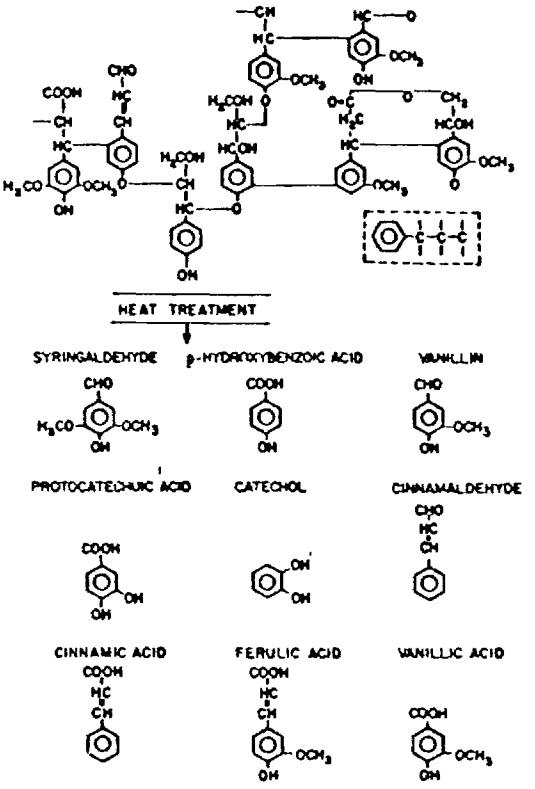


Figure 1. Representative structure of lignin and examples of aromatic compounds released by heat treatment. Taken from Healy and Young (63).

tion and are dependent on the rank of the coal. Generally, lignite coal has a higher percentage of $-OCH_3$ and -COOH groups than higher rank coals, and a greater content of aliphatic side chains (3), but the concentration of the functional groups declines rapidly during coalification. Functional groups such as -OH and -C=O, however, tend to be more evenly dispersed among coals of differing rank yet do decrease (to a lesser extent) during coalification. In addition, solubility of coal decreases with increasing rank and this is evident in that lignite coal is very soluble in alkali solutions while high rank coal is insoluble (4). Cellulose, the other major constituent of plant material, is an oxygen rich organic compound that probably has little bearing on coal structure. Cellulose is degraded by aerobic and anaerobic microorganisms rather rapidly after deposition (compared to lignin) and therefore does not accumulate to any appreciable extent as does lignin. Thus, cellulose probably does not contribute to coal structure.

The current view of coal structure is based on the chemical and physical alteration (coalification) of the original source material, lignin. A schematic representation of bituminous coal is presented in Fig. 2. Our assessment of coal biodegradability involves "low-rank" coal (lignite and subbituminous coals) and model compounds thus selected for biodegradation studies would consist of polycyclic aromatic compounds of low complexity (smaller aromatic clusters) with a greater quantity of oxygen containing functional groups, an example of which is presented in Fig. 3. Included in the examples of "model compounds" for potential use in biodegradation studies are nitrogen-containing and sulfur-containing polycyclic aromatic compounds. These types of structures are present in differing abundance in low-rank coals (2) and may be important in biodegradation events.

Some recent reports (5,6) corroborate the idea that low-rank coals have oxygen-containing substituents on aromatic ring clusters. It has been reported that certain subbituminous coals, after mild alkali extraction procedures, contain mono- and di-hydroxy substituents on benzene, naphthalene and anthracene aromatic clusters, and these constituents are prevelant aromatic structural units of coal. Mild alkaline treatment cleaves linkages between aromatic compounds bound by ether or ester linkages to reveal "backbone" constituents of coal in

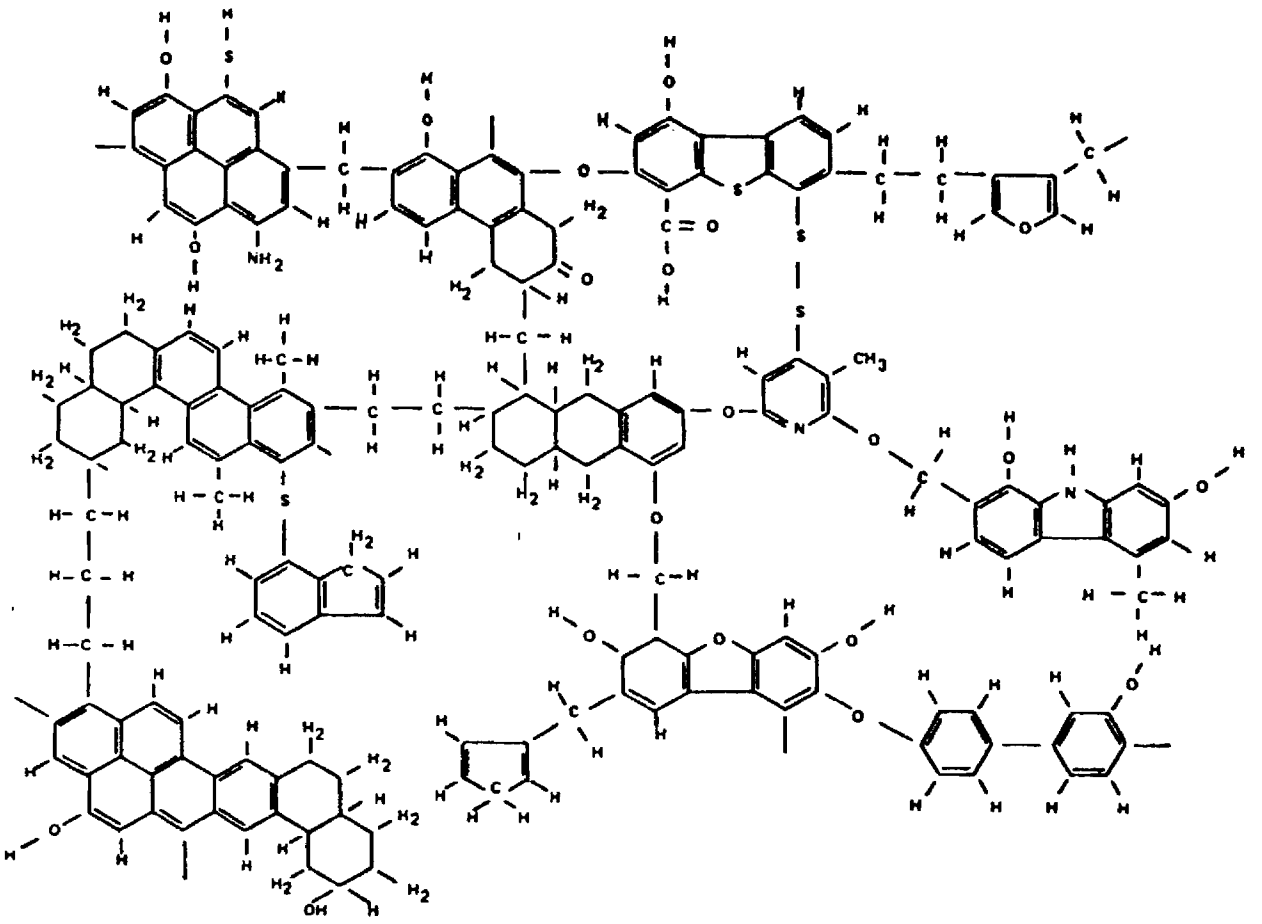


Figure 2. Representative structure of bituminous coal. Taken from Larsen (1).

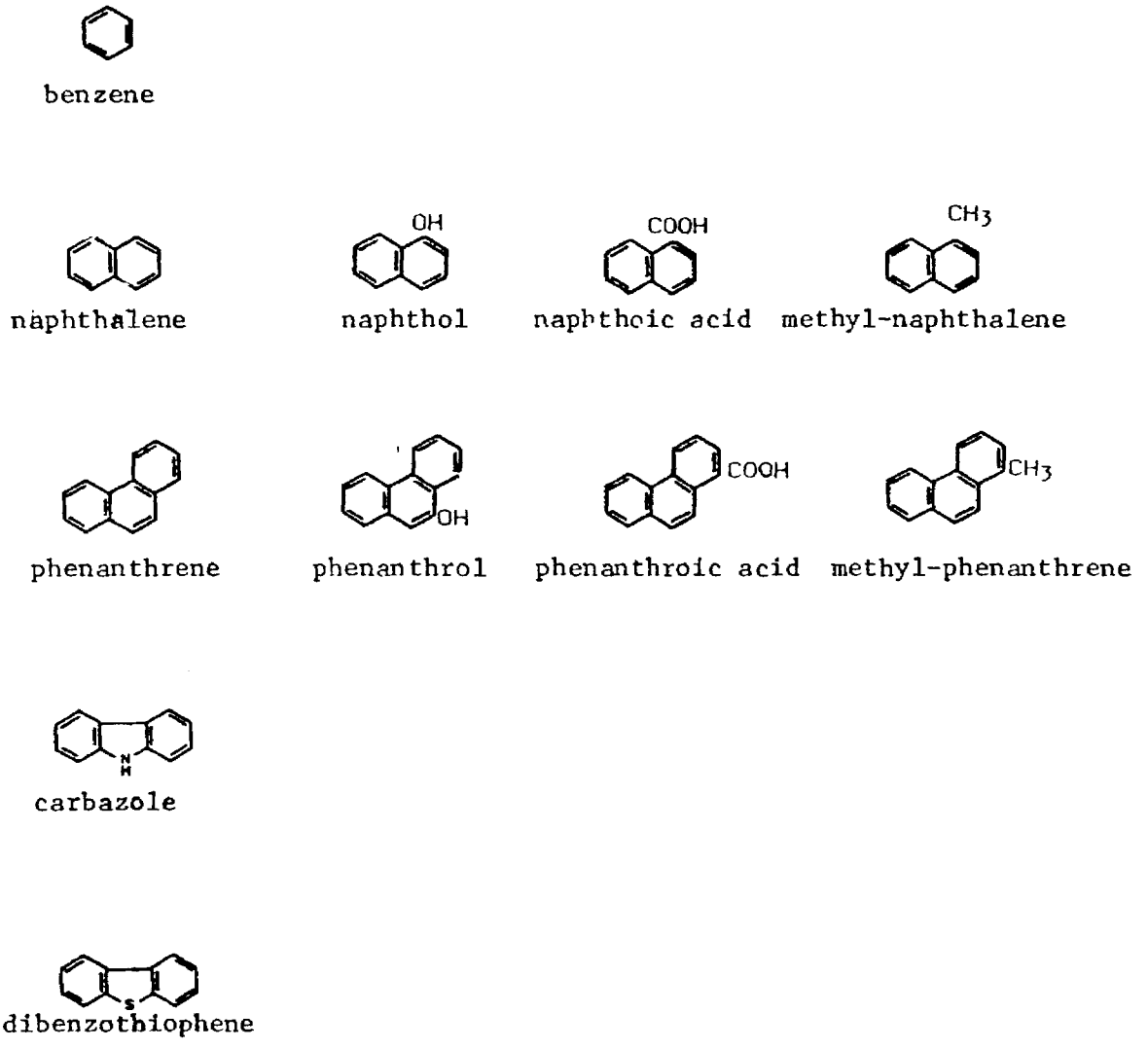


Figure 3. Examples of potential "model" compounds, representing relevant constituents of low-rank coal, for use in biodegradation studies. Benzene, naphthalene and phenanthrene are shown only as parent members of their respective groups.

addition to numerous other hydrocarbons. Fused ring constituents (2 and 3 aromatic ring clusters) were proposed to dominate over single aromatic substituents containing hydroxyl, carboxyl or ether groups as side chain functionalities.

B. Potential for the anaerobic bioconversion of low-rank coal. As discussed in the previous section, the generally accepted view is that low-rank coal primarily consists of various substituted polycyclic aromatic compounds. The following text is a review of pertinent literature concerned with the biological conversion of coal and coal-related constituents (aromatic compounds) with an emphasis on anaerobic bioconversion processes.

The biological conversion of organic molecules is known to occur by three primary means: aerobic respiration, with O_2 as terminal electron acceptor; anaerobic respiration, in which inorganic compounds such as nitrate, sulfate, and carbon dioxide serve as electron acceptors to form nitrogen gas, sulfide or methane; and fermentation, in which no external electron acceptor is required. In nature, the type of metabolism is usually dictated by the physical and chemical restrictions of the habitat; for example, marine sediments covered by water are often colonized by anaerobic, heterotrophic sulfate-reducing bacteria due to the physical restriction of O_2 entrance to the sediment and the abundance of sulfate in seawater, thus allowing anaerobic respiration of organic matter to proceed. A variety of metabolic schemes are therefore available for bioconversion of organic compounds.

For more than 50 years, it has been recognized that microorganisms are capable of the aerobic metabolism of a variety of aromatic compounds (7,8,9,10), including (a) benzenes, (b) alkyl substituted benzenes, (c) biphenyls, and (d) various polycyclic aromatic hydrocarbons including naphthalene, phenanthrene and anthracene (11-16). Many of these compounds may be chemically and structurally analogous to constituents which comprise low-rank coals. Many of the microorganisms responsible for the aerobic bioconversion of aromatic compounds have been isolated primarily from soil samples and include organisms such as *Nocardia* (17), *Aerobacter* (18), *Pseudomonas* (19), *Achromobacter* (20), and *Flavobacterium* (21), to name a few. The pathways for benzene ring cleavage involve oxygenases, and molecular oxygen is

essential; these processes have been reviewed extensively (22,23). There seems to be a ubiquitous distribution of bacteria in soil capable of bioconversion of aromatic compounds (24); this phenomenon is not unlikely since aromatic compounds are widely distributed in soil (25) and are hypothesized to originate from the deposition of airborne particulates arising from the pyrolysis of organic material (26-29), including fossil fuels, and to a much less extent through biogenic sources (30). Additionally, energy-related chemical manufacturing processes, such as coal-gasification, coal liquefaction, and refinery of petroleum, account for production and environmental contamination of polycyclic aromatic hydrocarbons (31). The variety and distribution of aromatic compounds found in soils and sediments has only recently been determined due to the advent of advanced analytical techniques; surprisingly, a soil sample may contain hundreds or thousands of aromatic compounds (32-33), and the presence of microorganisms, both aerobic and anaerobic, capable of the bioconversion of these compounds, is likely (34). Microorganisms are impressively versatile in their ability to metabolize both natural and synthetic compounds (34).

Documentation of the aerobic bioconversion of various aromatic compounds has been well established (22,35). However, only very few studies have demonstrated the bioconversion of aromatic compounds under strict anaerobic conditions (35-37), the primary subject of this text. In fact, the majority of the published studies have dealt only with bioconversion of substituted monoaromatic compounds. However, the results from these investigations should provide insight on the anaerobic fate of more complex aromatic compounds hypothesized to be important constituents of low-rank coals.

One of the earliest investigations of anaerobic bioconversion of aromatic compounds was performed using mixed microbial cultures. Tarvin and Buswell (38) reported the bioconversion of benzoate and other substituted aromatic compounds to products such as CH_4 and CO_2. The use of methanogenic microbial consortia (mixed cultures) was further investigated by Clark and Fina (39) in 1952 who demonstrated degradation of ^{14}C-benzoate to $^{14}CH_4$ by anaerobic digestor sludge. These investigations and others (40-41) paved the way for further studies involving anaerobic metabolism of aromatic compounds, including metabolism under

non-methanogenic conditions such as anaerobic photometabolism, conditions of sulfate and nitrate reduction, and via fermentation. These will be reviewed after a discussion of the anaerobic metabolism of benzoate, one of the earliest and most widely studied aromatic substrates.

Aside from the earliest studies of Tarvin and Buswell (38) and Clark and Fina (39), other investigators have examined benzoate degradation under various anaerobic conditions. Oshima (42) in 1965 reported bacterial cultures from soil that degraded aromatic substrates (benzoate) only in the presence of nitrate. Later, Taylor et al. (43) isolated a *Pseudomonas* species capable of nitrate-dependent metabolism of benzoate and showed that the pathway was distinct from the aerobic pathway. The pseudomonad, designated strain PN-1, was isolated from soil in minimal media plus p-hydroxybenzoate and nitrate. In addition, benzoate and o-hydroxybenzoate were metabolized by PN-1. A non-reductive pathway was postulated for benzoate catabolism (in contrast to the reductive pathway proposed by Dutton and Evans; 44) since cyclohexane carboxylate, an intermediate in the reductive pathway of photosynthetic benzoate degraders, was not metabolized in the presence of nitrate by the pseudomonad. Isolate PN-1 apparently possesses an oxygenase enzyme for benzoate catabolism that doesn't require oxygen per se as an inducer (45). In contrast, Williams and Evans (46) isolated a pseudomonad (*Pseudomonas stutzeri*) that grew anaerobically on benzoate-nitrate medium and produced hydroxycyclohexane carboxylate. No oxygenase activity was detected and results supported the reductive pathway for aromatic degradation. The same investigators also isolated a species of *Moraxella* which metabolized benzoate anaerobically with nitrate as sole electron acceptor and results of pathway intermediates, such as cyclohexane carboxylate and hydroxycyclohexane carboxylate, supported a reductive pathway of benzoate catabolism (47). Cultures of *Rhodopseudomonas* were shown to degrade benzoate under photosynthetic anaerobic conditions (44) and mutant strains were used to support the idea of a reductive pathway for benzoate degradation (48). Dutton and Evans (44) proposed a general degradative scheme for anaerobic benzoate photocatabolism. Initial steps involve ring hydrogenation as part of the reductive pathway (Figure 4). Following ring reduction, cleavage of the ring is

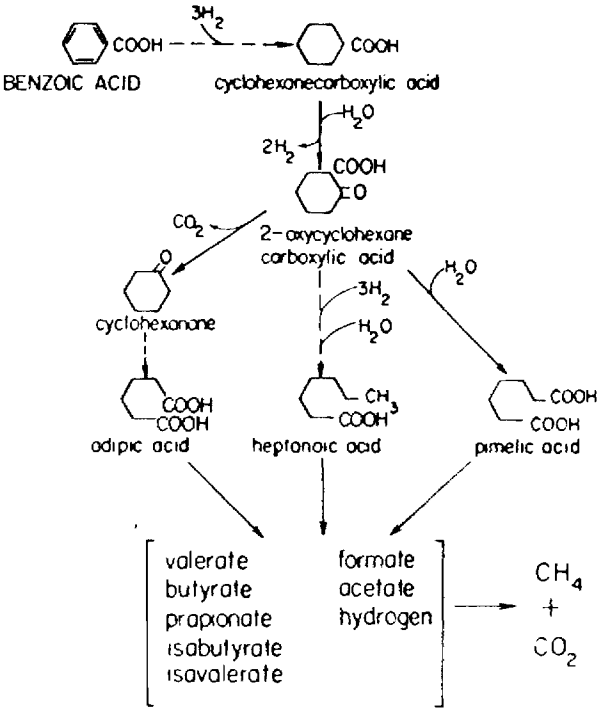


Figure 4. Proposed pathway for the anaerobic bioconversion of benzoic acid to CH_4 and CO_2. Taken from Healy et al.(49).

achieved via ring hydration. More recently, Grbic-Galic and co-workers (50) presented evidence that reductive ring cleavage is not the only mechanism of anaerobic "benzenoid" metabolism. These investigators have postulated an oxidation pathway and showed that the anaerobic degradation of benzene and toluene proceeded via formation of phenol and cresol by way of a hydroxylation reaction (50). In an analogous manner, heterocyclic aromatic compounds (for example, nitrogen containing aromatics such as nicotinic acid) are known to be metabolized anaerobically by ring hydroxylation followed by ring-reduction and hydrolytic cleavage (51). Although the pathways are similar, homocyclic aromatic (benzene) cleavage initially involves ring reduction while heterocyclic ring cleavage initially involves ring hydroxylation. In other studies, Widdel (52) demonstrated benzoate degradation using sulfate as an electron acceptor, and Mountfort and Bryant (53) have isolated a benzoate degrader in coculture with a sulfate-respiring bacterium. In addition to these investigations, numerous recent papers describe benzoate degradation under methanogenic conditions (35,54-55) and descriptions of intermediates and tracer analyses are reported. Ferry and Wolfe (56) have described three dominant organisms in a benzoate-methanogenic consortium and Grbic-Galic and Young (57) suggest that benzoate degradation may be uncoupled from methane production, but they did not suggest an alternative hydrogen sink. From the above-mentioned studies, it is evident that benzoate, and probably other aromatic compounds, may be metabolized under a variety of anaerobic conditions.

The majority of recent investigations concerned with metabolism of aromatic compounds have involved methanogenic consortia; i.e., the use of mixed anaerobic cultures from a variety of anaerobic habitats. The use of these "consortia" or mixed cultures for the microbial bioconversion of aromatic compounds has merit in that otherwise thermodynamically poor or unfavorable reactions become more favorable in mixed culture reactions compared to pure culture metabolism. This phenomenon of syntrophic interaction has been demonstrated in several metabolic schemes. For example, the oxidation of propionic and butyric acid anaerobically to acetate, CO_2 and H_2 is unfavorable thermodynamically unless the reaction is coupled to a H_2 sink to maintain low partial pressures of H_2; this may be accomplished through the

use of H_2-consuming methanogens (to reduce CO_2 to CH_4) or H_2-consuming sulfate reducing bacteria (to reduce sulfate to sulfide). These phenomena have been documented (58-60) and the reaction scheme is shown in Table 1. It has been suggested that similar syntrophic mechanisms may contribute to bioconversion of various aromatic compounds, including benzoate (59). The consortium of bacteria acts synergistically and the overall reaction becomes thermodynamically more favorable when reaction products (such as H_2) from initial metabolic steps are continuously removed and maintained at low concentrations. Many investigations to date involving anaerobic bioconversion of aromatic compounds have relied on mixed culture interactions with several different bacterial groups participating in the overall bioconversion to CH_4 and CO_2. As mentioned previously, Ferry and Wolfe (56) demonstrated the anaerobic conversion of benzoate to CH_4 and CO_2 by a microbial consortium and they identified acetate as a key intermediate. Benzoate degradation was dependent on the presence of the methane producing bacteria for removal of compounds produced by the benzoate degrader. Methane producing bacteria are known to metabolize only a restricted number of substrates; these include acetate and various one-carbon compounds such as formate, methanol, methylamine, and H_2 plus CO_2 (61). Methanogens are not capable of metabolizing any other compounds of greater "complexity" than acetate; thus, several different microbial groups are necessary for the bioconversion of complex substrates, such as aromatic compounds, to CH_4. Some investigations of aromatic bioconversion under methanogenic conditions demonstrated intermediates such as cyclohexane carboxylate 2-hydroxycyclohexane carboxylate, heptanoate, valerate, propionate, butyrate, and acetate (36,55,62). These results suggest that ring cleavage yields aliphatic acids which are further metabolized to more simple organic acids before methane evolution and this data supports the proposed reductive pathways for bioconversion of simple aromatic compounds to CH_4. The proposed pathway for the anaerobic bioconversion of benzoate and ferulic acid indicates reduction of the double bonds of the aromatic nucleus followed by ring cleavage to yield aliphatic acids (36,49,56), as shown in Figure 4.

Other groups of aromatic compounds have been anaerobically metabolized to CH_4. Healy

Table 1. Equations and free-energy changes for the anaerobic oxidation of butyrate and propionate with subsequent production of CH_4; note the dramatic favorable change in free-energy. Taken from Boone and Bryant (60).

	$\Delta G^{o'}$ (kJ/reaction)
$CH_3CH_2CH_2COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+$	(+48.1)
$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + HCO_3 + H^+ + 3H_2$	(+76.1)
$2CH_3CH_2CH_2COO^- + HCO_3 + H_2O \rightarrow 4CH_3COO^- + H^+ + CH_4$	(-39.4)
$4CH_3CH_2COO^- + 12H_2 \rightarrow 4CH_3COO^- + HCO_3 + H^+ + 3CH_4$	(-102.4)

and Young (63,64) examined a range of lignin-derived mono-aromatic compounds and reported mixed culture biodegradation of vanillic and ferulic acids, benzoate, catechol, phenol, and syringic acid. Chemielowski and others (36,37,63) have demonstrated that phenol and other monoaromatic compounds are degraded to CH_4 using acclimated digestor sludge. Balba and Evans (35) reported that benzoate enrichments also utilized other substituted monoaromatics such as phloroglucinol and cinnamate, and Healy and Young (63) reported bioconversion of phenol and catechol to CH_4 and CO_2. Kaiser and Hanselmann (65) reported the metabolism of syringic acid, gallic acid, and other monoaromatic substrates using microbial inocula from lake sediments; they observed a homofermentative metabolism of gallic acid to acetate by a coccoid isolate and demonstrated further metabolism to CH_4 if coupled with methanogens. These results indicate that aromatic compounds with differing number, type, and position of functional groups may affect their overall biodegradative potential. In the instance of gallic acid, for example, a consortium of microbes was not obligatory for bioconversion of the parent compound but was obligatory for complete conversion to CH_4 and CO_2. Mountfort and Bryant (53) characterized *Syntrophus buswellii* from anaerobic digester sludge which fermented benzoate and hydrocinnamate to acetate, CO_2 and H_2 but only when cocultured with a H_2-oxidizing bacterium such as a methanogen. Finally, Ehrlich (66) demonstrated methane-production from creosote contaminated aquifier samples, indicating that natural microbial consortia became adapted to coal-tar runoff and possibly degraded complex aromatics from creosote waste. In fact, a nitrate-respiring pseudomonad was isolated capable of phenol degradation (67). From these and other studies it is evident that many different monoaromatic compounds are degraded under methanogenic as well as nonmethanogenic conditions.

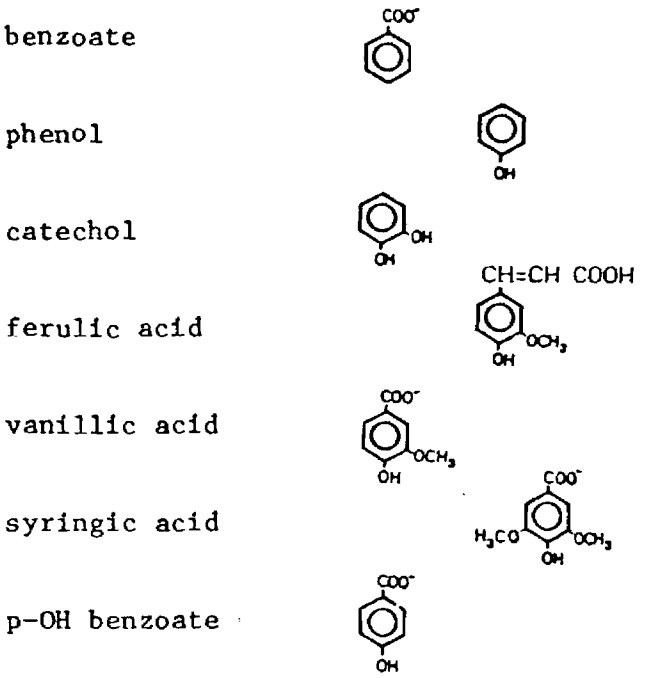
In addition to the study of Kaiser and Hanselmann, it has been shown by others that pure cultures of diverse bacteria are also capable of anaerobic bioconversion of aromatic compounds. *Coprococcus* and *Streptococcus* species, isolated from bovine rumen microflora (68,69), are capable of phloroglucinol degradation to acetate and CO_2. Phloroglucinol, or 1,3,5-trihydroxybenzene, forms part of certain plant flavonoids and is also degraded by the

anaerobic photosynthetic bacterium *Rhodopseudomonas* (70). *Pseudomonas cepacia* metabolizes ferulic acid anaerobically to 3-methoxy-4-hydroxystyrene and 3-methoxy-4-hydroxy-phenyl-propionic acid. Further, *Pelobacter acidigallic*, a marine sediment isolate, ferments trihydroxy-benzenes such as gallic acid, pyrogallol, trihydroxy-benzoate and phloroglucinol to acetate and CO_2 under strict anaerobic conditions (71). Cocultures of *Pelobacter* and acetotrophic methanogens yielded aromatic ring cleavage and complete conversion of those substrates to CH_4 and CO_2.

As mentioned previously, Widdel (52) demonstrated bioconversion of benzoate and phenyl-propionate by several new isolates of sulfate-reducing bacteria. A list of substituted aromatic compounds reported to be metabolized under anaerobic conditions is presented in Figure 5. One marine isolate, *Desulfonema magnum*, was capable of complete mineralization of benzoate to CO_2 in the presence of sulfate (72). Cyclohexane carboxylate, a proposed intermediate in the reductive ring cleavage pathway, was not metabolized by *Desulfonema*.

Polycyclic aromatic hydrocarbons (PAH's) comprised of two to four fused benzene rings are likely constituents of certain coals and are known to be rather recalcitrant with respect to microbial bioconversion. Their persistance in nature is probably due to their low solubility in water and their resonance energy (73) or aromatic stability. In fact, the chemical behavior and stability (reactivity) of an aromatic ring is largely dependent upon the distribution of electron density (45). Comparison of a homocyclic aromatic nucleus (benzene) and a heterocyclic aromatic nucleus (pyrimidine) reveals a significantly different degree of stability, indicating that electron density in heterocyclic aromatics is lower than in benzene and thus more susceptible to nucleophilic attack and subsequent ring cleavage. In order to improve the biodegradative potential of complex PAH's, pretreatment methods to destabilize the aromatic nucleus may be a plausible alternative to enhance bioconversion. Miller et al. (74) have assessed the effects of photolytic pretreatment on the fate of benzopyrene in microbial test systems. They reported that the presence of H_2O_2 enhanced the photolysis rate and subsequent mineralization of benzopyrene in biological test systems and may provide a means for successful detoxification of PAH.

Figure 5. Examples of substituted monoaromatic compounds metabolized under anaerobic conditions.



Very recently, Mihelcic and Luthy (75) examined the microbial degradation of the polycyclic aromatic hydrocarbons naphthalene and acenaphthene. Results from their studies indicated that anaerobic biodegradation occurred only under conditions of denitrification. These investigators also observed 1-naphthol bioconversion under conditions of anaerobiosis in the presence and absence of nitrate, but control experiments also exhibited conversion of 1-naphthol with time, suggesting abiotic degradation in their test system. Bioconversion of naphthalene and acenaphthene was observed after an initial lag period of several weeks and results were reported only for low concentrations (0.4-7 mg/L) of the PAH's (75). Additional studies by these investigators further demonstrated nitrate-dependent bioconversion of naphthalene and acenaphthene in soil-water systems. It was also shown that PAH sorbtion was correlated with the organic content of the test system and these investigators postulated that microbial bioconversion of specific organics may be influenced by the availability of the substrate in the aqueous phase (76).

Finally, attention must be focused on studies demonstrating the biological conversion, either by aerobic or anaerobic means, of coal or coal-related compounds. Very few studies have been reported concerning bioconversion of the natural substrate, coal. Cohen and Gabriele (77) in 1982 reported that basidiomycete fungi of the genera *Polyporus* and *Poria* converted a North Dakota lignite coal to an unknown liquid product. This was one of the first demonstrations of bioconversion of the natural substrate. More recently, Ward (78) reported the isolation of several different fungi, including members of *Aspergillus, Candida, Mucor, Paecilomyces* and *Penicillium*, from a natural exposed lignite coal seam. These fungi grew on lignite coal as the sole source of organic carbon and energy and, in some cases, a viscous liquid deposit resulted, indicative of coal liquefaction. Ward (79) further demonstrated that *Penicillium* and *Candida* digested solid lignite coal to a water-soluble digestion product of unknown composition. UV and infrared analyses indicated that a complex solution of organic compounds was produced.

The above investigations represent the current state of knowledge concerning aerobic coal bioconversion. One further report, a patent by Johnson (80), indicates possible anaerobic

bioconversion of coal. The author presents evidence suggesting enhanced methane production from activated sewage sludge when amended with lignite, subbituminous, or bituminous coal. However, no documentation of coal conversion was presented, only enhanced gas production (as CH_4) when amended with coal. To date, no other reports are available concerning anaerobic bioconversion of coal.

It is the intention of the project team to utilize the information presented in this text for the development of microbial cultures capable of the anaerobic bioconversion of coal, coal-related compounds, and selected model compounds (representative of coal constituents) to fuel related products such as CH_4.

II. Selection of Model Compounds for Biodegradation Experiments.

Information acquired from an initial assessment of low-rank coal biodegradation suggested that polycyclic aromatic organic compounds, containing various oxygenated and aliphatic functional groups, were representative of organic molecules found as constituents of low-rank coal. The most likely aromatic compounds amenable to biodegradation under anaerobic conditions are probably oxygenated monoaromatic compounds such as phenol and various oxygenated derivatives of phenol. These compounds, however, are probably not predominant constituents of coal because they are not routinely found during chemical degradation and characterization of coal. Further, studies have been performed and were presented in the previous section which demonstrate bioconversion of mono-aromatic organic compounds. Polycondensation reactions, forming polycyclic aromatic organic compounds, are thought to occur during coalification of lignin; therefore, simple monoaromatic derivatives are unlikely to persist. Thus, as "model compounds" for initial biodegradation studies, we have chosen polycyclic aromatic organic compounds of low complexity and containing oxygenated and/or methylated functional groups. These compounds seem to be the most likely candidates representative of constituents of low-rank coal which are amenable to anaerobic bioconversion to methanogenic precursors. Initial studies will be performed primarily using hydroxyl and carboxyl containing derivatives of naphthalene and phenanthrene.

Specifically, 2-naphthol, 1-naphthoic acid, and 9-phenanthrol will be investigated. Additional studies included biodegradation of a sulfur containing aromatic compound, dibenzothiophene.

Other prevalent constituents of low-rank coal include various hydrocarbons and aliphatic chains, such as polymethylene groups, which may or may not be attached to aromatic rings and may interconnect aromatic clusters. However, these fully reduced compounds are not likely amenable to anaerobic oxidation and thus will not be studied in this project.

III. Culture Medium and Anaerobic Procedures.

Anaerobic techniques and methods for the preparation of culture medium for initial biodegradation studies are considered to be "state-of-the-art" and are described by Balch et al. (61). Briefly, the medium components are added to distilled water and gently heated to a boil under an O_2-free gas mixture composed of 80% N_2 plus 20% CO_2. All gases used for anaerobic cultivation are scrubbed free of trace amounts of O_2 by passing the gas stream through a column of reduced copper turnings heated to 250° C. Reducing agents (cysteine ·HCl and Na_2S) are added after boiling to lower the reducing potential of the medium to a level sufficient for growth of methanogenic bacteria (-300 mV). Resazurin was also added to culture media as a color indicator of redox conditions. After cooling, the flask containing the reduced, anaerobic medium was sealed and transferred inside an O_2-free anaerobic chamber. Trace amounts of O_2 that may have been introduced into the chamber were removed by the presence of palladium-coated ceramic pellets which catalyze the conversion of O_2 and H_2 to form H_2O. The anaerobic medium was dispensed into specialized culture tubes (Bellco Glass, Inc.; Vineland, N.J.) designed for strict anaerobic cultivation. Tubes were sealed with butyl rubber stoppers (butyl rubber is more effective against gas diffusion), removed from the anaerobic chamber, and crimped with aluminum seals to secure the rubber stoppers. Utilizing a specially designed gassing manifold connected to a vacuum pump, the gas phase within the sealed tubes was exchanged with an O_2-scrubbed gas mixture composed of 80% N_2 plus 20% CO_2 at 2 atmospheres pressure. Media were sterilized by autoclaving at 121° C, 15 psi for 20

minutes. A more detailed description of anaerobic cultivation procedures used in this study has been presented (61).

Anaerobic stock solutions of the model compounds were prepared by adding appropriate aliquots of the compounds to O_2-free absolute ethanol, prepared by N_2 sparging, to yield final concentrations of 2.5 and 25 mM. Addition of 20 μl of either the 2.5 or 25 mM stock solution to 5.0 ml of culture medium yielded final concentrations of the model compounds of 10 or 100 μM, respectively. These low final concentrations of model compounds were chosen for initial biodegradation studies because of possible toxic (inhibitory) effects of the compounds (at higher concentrations) to relevant microbial components.

The specific model compounds at the desired concentrations were added anaerobically to replicate culture tubes containing the test medium to be described. Medium supplemented with a specific model compound was then inoculated with a bacterial consortium obtained from appropriate natural anaerobic habitats. Specific control samples containing no model compound or a heat-sterilized inocula were also included. Replicate samples of the inoculated medium plus model compound were incubated at 37^{o} C in the dark; at specified time intervals (initial; one month incubation; two month's incubation, etc.), experimental samples were removed for gas chromatographic analysis to determine the extent of biodegradation of the model compound. Preliminary extraction experiments demonstrated that it was not feasible to remove small sample aliquots from individual culture tubes for assessment of biodegradation because of problems associated with adsorption of model compounds to both organic and inorganic surfaces within the tube. In order to accurately quantitative bioconversion (loss of substrate), the entire contents of each individual culture tube (test sample) was extracted and analyzed. Replicate tubes were thus prepared, inoculated and incubated for initial biodegradation studies; samples were then sacrificed at given time points and frozen for later analysis.

The test system for bioconversion studies with benzene derived coal extract of subbituminous coal was essentially the same as the test system with "model compounds". The main exception was in the method of addition of the coal extract. The benzene-soluble

fraction of the coal extract was relatively insoluble in the aqueous (polar) solvent of the biodegradation test system because of the inherent insolubility of benzene in water. Thus, the benzene-derived coal extract was concentrated and diluted into acetone (1:6) to achieve the same final concentration of constituents as in the original benzene-coal extract. This benzene/acetone mixture was added to appropriate experimental media for anaerobic bioconversion studies. Anaerobic digestor sludge was chosen as the inocula for these studies due to the diverse nature of organic constituents found in this complex environment and the diverse microflora also present. As in previous bioconversion experiments, strict anaerobic conditions were maintained at all times.

A. Media. The composition of the standard culture medium used in most initial biodegradation experiments is presented in Table 2. The medium was specifically formulated from current literature (61) and supports the nutritional needs of most methane-producing bacteria described to date as well as other metabolically diverse bacteria which may also be important constituents of the mixed microbial population necessary for the bioconversion of complex organic compounds to CH_4. The medium consists of various minerals, vitamins and trace elements required by or stimulatory to most organisms and is pH controlled by use of a bicarbonate/CO_2 buffer. The final pH of the medium under an atmosphere of 80% N_2 plus 20% CO_2 was 7.0.

IV. Bacterial Inocula for Use in Biodegradation Studies.

A number of samples from various anaerobic habitats have been collected and used as a source of bacterial inocula for initial biodegradation studies. To date, three different types of samples have been used in biodegradation experiments, including (i) active anaerobic sludge from a local municipal sewage treatment facility; (ii) anaerobic sediment from a local freshwater eutrophic swamp and pond; and (iii) sediment from a stream bed which receives leachate from a lignite coal deposit. The first two samples listed were 'anaerobic', owing to their apparent high organic content and diverse microflora (contained methanogens). The

Table 2. Composition of Medium Used in Biodegradation Studies

	per liter[a]
K_2HPO_4	0.3 g
Mineral Solution[b]	50 ml
Trace Metals Solution [c]	10 ml
Trace Vitamin Solution [d]	10 ml
$Fe(NH_4)_2(SO_4)_2 \cdot 7H_2O$	0.002 g
Resazurin	0.001 g
$NiCl_2 \cdot 6H_2O$	0.0005 g
Na_2SeO_4	0.002 g
L-Cysteine HCl	0.5 g
$Na_2S \cdot 9H_2O$	0.5 g

a) Ingredients are added to distilled water to give a final volume of 1 liter. Cysteine and Na_2S are added after boiling the medium under an 80% N_2-20% CO_2 gas mixture, the final gas phase of tubed medium being an 80% N_2-20% CO_2 gas mixture at two atmospheres of pressure.

b) Contains in grams per liter of distilled water: KH_2PO_4, 6; NaCL, 12; $MgSO_4 \cdot 7H_2O$, 2.6; $CaCl_2 \cdot 2H_2O$, 0.16.

c) Contains, in grams per liter of distilled water (pH to 7.0 with KOH): nitrilotriacetic acid, 1.5; $MgSO_4 \cdot 7H_2O$, 3.0; $MnSO_4 \cdot 2H_2O$, 0.5; NaCl, 1.0; $FeSO_4 \cdot 7H_2O$, 0.1; $CoSO_4$ or $CoCl_2$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; $ZnSO_4$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; $AlK(SO_4)_2$, 0.01; H_3BO_3, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.01. Dissolve nitrilotriacetic acid with KOH to pH 6.5; then proceed to add minerals.

d) Contains in milligrams per liter of distilled water: biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; thiamine hydrochloride, 5; riboflavin, 5; nicotinic acid, 5; d,l-calcium pantothenate, 5; vitamin B_{12}, 0.1; p-aminobenzoic acid, 5; lipoic acid, 5.

e) Modified from Balch et al., (61).

stream bed sediment sample appeared sandy and granular; although it is unlikely that 'anaerobic' conditions prevailed with this sediment, it is possible that facultatively anaerobic bacteria could be present which degrade complex organic compounds.

All samples used in initial biodegradation experiments were collected under anaerobic conditions by completely filling the collection vial and sealing the vial tightly to prevent oxygen invasion. Transfer of inocula to biodegradation medium was accomplished using either syringe and needle transfer procedures or by transfer inside an O_2-free Freter type anaerobic chamber. Care was taken to prepare a homogeneous suspension (prepared under O_2-free atmosphere) of the original anaerobic sediment to ensure that each experimental tube received a representative inocula.

V. Analytical Methods

A. Separation and Quantitation of Model Compounds.

The method of choice for separation and quantitation of "model compounds" used in initial biodegradation studies was determined to be gas chromatography, based on sensitivity of detection and reproducibility. The following is a summary of the experimental protocol for analysis of model compounds.

1. Methodology

Test compounds: 2-naphthol, 99% (Aldrich); 1-naphthoic acid, 98% (Aldrich); 9-phenanthrol, technical grade (Aldrich), dibenzothiophene (Aldrich).

Surrogate compound: fluorene

Internal standard: hexamethyl benzene (HMB)

GC conditions:

Gas chromatograph: Hewlett Packard 5980 A

Column: DB-5 (5% phenylmethyl silicone; 15 m long, 0.52 mm i.d., J&W Scientific)

Carrier gas: N_2 (8 ml/min)

Temperature program: 130° C (3 min), 15° C/min to 260° C

Attenuation and range: 3

A chromatogram of the test compounds plus surrogate compound and internal standard is presented in Figure 6. The concentrations of the various compounds are: surrogate and internal standard, 10 mg/L; 2-naphthol and 9-phenanthrol, 200 M; 1-naphthoic acid, 500 μM. Results indicate that two minor problems exist: 1-naphthoic acid shows tailing, and 9-phenanthrol seems to be resolved into two peaks. GC procedures for separation and quantitation of the internal standard (hexamethyl benzene), surrogate compound (fluorene), and 2-naphthol were acceptable.

Quantitation of 1-naphthoic acid was determined to be more reproducible and at lower detection limits using a gas chromatography column different from that used for 2-naphthol. Problems associated with peak tailing due to the polar characteristics of naphthoic acid were resolved by employing a chromatography column of intermediate polarity. Thus, gas chromatography conditions for greater resolution and reproducible quantitation of 1-naphthoic acid are as follows:

Gas Chromatograph: Hewlett Packard 5890A

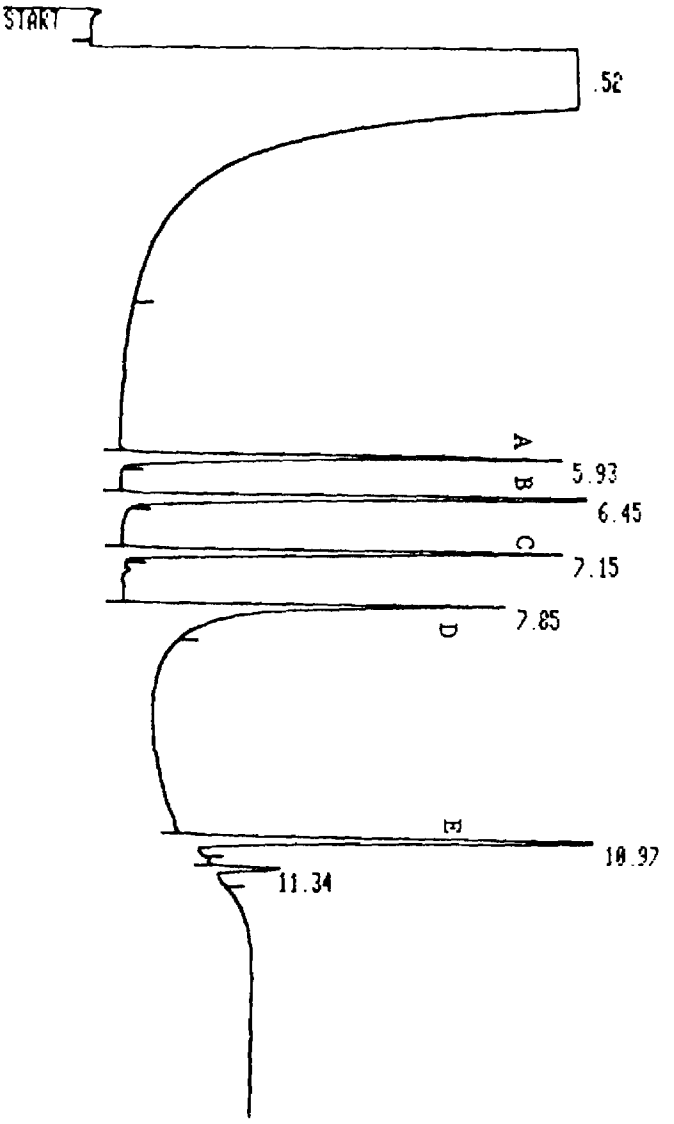
Column: HP 5% methyl silicone (fused silica);

10 m long, 0.53 mm ID

Carrier Gas: N_2 (5 ml/min)

Temperature Program: 130 C (3 min), 15 C/min to 250 C

Quantitation of the model compound, 9-phenanthrol, using the gas chromatography conditions employed previously (5% phenylmethyl silicone column) was not acceptable due to the problem of resolution of this model compound into 2 separate peaks. Initial results of gas chromatography-mass spectrometry of 9-phenanthrol indicates that the "two peak" GC resolution of this compound represents isomers of 9-phenanthrol. It appears that upon incubation in our biodegradation 'test system', the concentration ratio of the two isomers changes toward greater production of the 'second' peak. At this time we have no explanation for this phenomenon. Bioconversion potential of 9-phenanthrol will be based on quantitation of both isomers.



RUN # 403
WORKFILE ID: C2
WORKFILE NAME:

AREA%

RT	AREA	TYPE	AR/HT	AREA%
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5.93	236080	PB	0.075	0.140
6.45	433910	PB	0.062	0.257
7.15	204840	PB	0.065	0.121
7.85	314290	PB	0.118	0.186
10.97	323280	PB	0.071	0.192
11.34	28768	PB	0.061	0.017

TOTAL AREA= 1.6883E+08
MUL FACTOR= 1.0000E+00

Figure 6. Chromatogram of model compounds, surrogate compound, and internal standard. A, internal standard; B, 2-naphthol; C, surrogate; D, 1-naphthoic acid; E, 9-phenanthrol.

Quantitation and separation of dibenzothiophene was achieved using the same methodology as listed above for 2-naphthol and 9-phenanthrol.

2. Method assurance. The surrogate compound (fluorene) and internal standard (HMB) were used for method assurance during extraction procedures and GC analysis, respectively. The results of this mode of analysis were acceptable since the recovery of the surrogate compound was within an acceptable and reproducible range (±5%); refer to Table 3.

3. Instrumentation accuracy and detection limits. Table 4 summarizes the results of the accuracy of the gas chromatography/FID response by duplicate injections at several concentrations of 9-phenanthrol. At the approximate detection limit of 30 μM 9-phenanthrol, the deviation was 5.2% and decreased to 1.9% at 600 μM 9-phenanthrol. Less than 5% deviation was within an acceptable range of instrumentation accuracy.

The detection limits of 2-naphthol, 1-naphthoic acid and 9-phenanthrol were 10, 300, and 30 μM, respectively. The use of very low concentrations of "model compounds" in the test system needs special consideration because of detection limits. For example, if 6 μM phenanthrol in 5 ml of test medium is employed, then extraction (assuming 100% recovery) into a final organic solvent volume of 1 ml produced 30 μM phenanthrol, which is close to detection limits. Thus, further concentration of the extracted model compound would be necessary. Figures 7 through 11 illustrate standard calibration curves for the model compounds tested thus far. All calibration curves showed high correlation (>0.99). Note that some of the calibration curves are linear over a restricted range of concentrations for specific model compounds. The calibration curve for dibenzothiophene was linear over a range of concentrations from 50 - 200 μM.

B. Preparation and Analysis of Coal Extracts

Extracts of raw coal samples were prepared as described below and were used as "test compounds" for anaerobic biodegradation experiments. The coal sample used in

Table 3. Recovery of surrogate (fluorene, 2 mg/L) from simulated biodegradation system using extraction procedures (Figure 12) in presence and absence of model compound 9-phenanthrol. OFW (organic free water), supernatant and sediment fractions were obtained, spiked with appropriate compounds, and extracted.

	% recovery of surrogate		
9-phenanthrol present (μM)	OFW	Supernatant	Sediment
0	68.4 72.4 (71.9±2.8) 73.8	74.4 75.6 (75.4±0.9) 76.1	76.3 77.2 (79.9±4.3) 80.2 85.8
10	70.2 75.1 (72.7±2.4) 72.8	78.0 79.5 (78.7±0.8) 78.7	83.2 80.0 (82.2±1.9) 83.3
100	82.7 82.1 (81.2±2.1) 78.8	83.5 81.3 (82.2±1.2) 81.7	84.1 82.0 (83.4±1.2) 84.1

Table 4. The accuracy of GC/FID response by duplicate injections of three concentrations of 9-phenanthrol. Data corrected with internal standard.

9-Phenanathrol (μM)	FID Response (area)	Average Area	% Deviation
30	34976 38824	36900	5.2
80	106747 100893	103820	2.8
600	987084 1025043	1006064	1.9

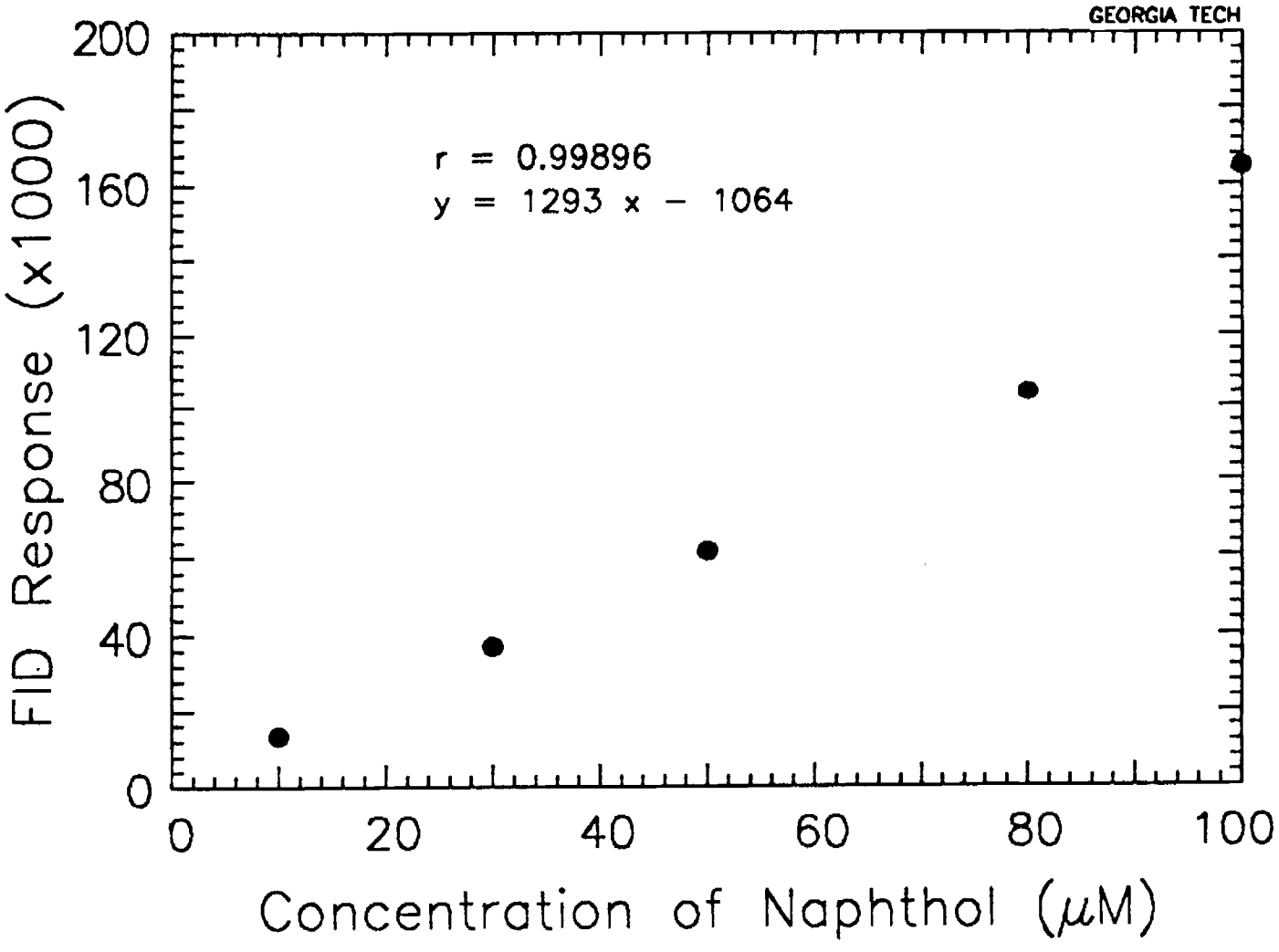

Figure 7. Standard calibration curve for 2-naphthol. Results are the average of duplicate determination.

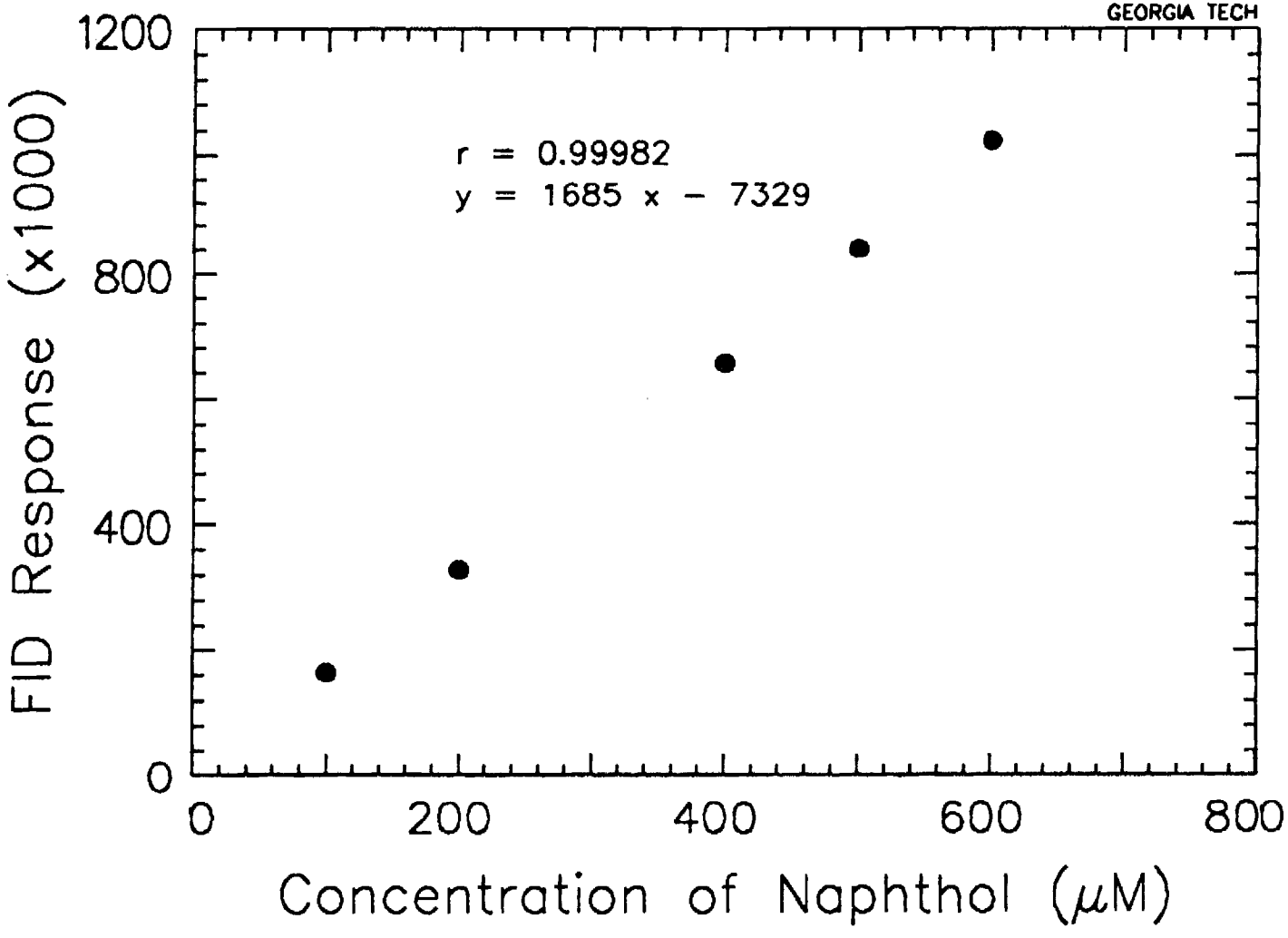

Figure 8. Standard calibration curve for 2-naphthol. Results are the average of duplicate determinations.

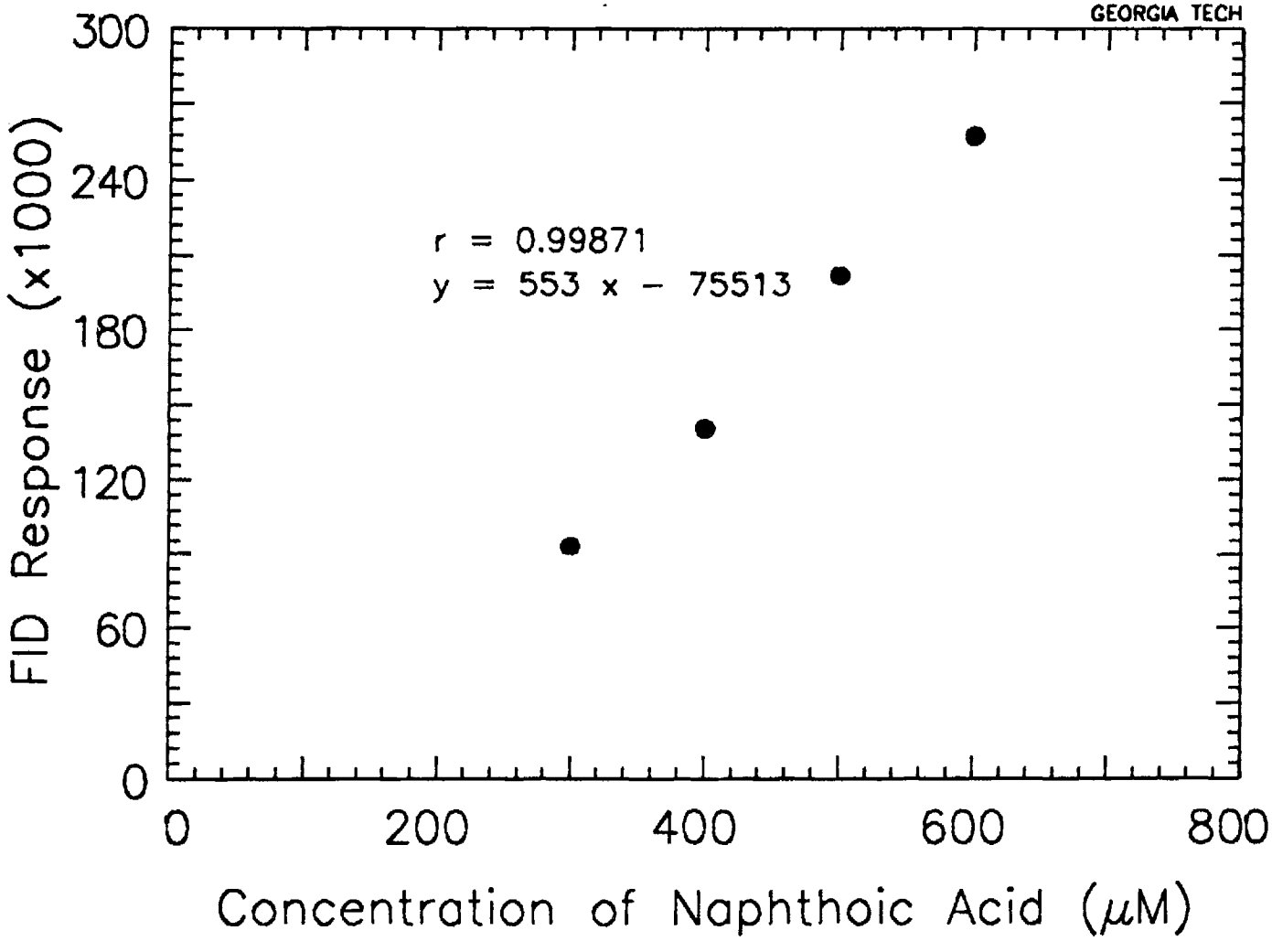

Figure 9. Standard calibration curve for 1-naphthoic acid. Results are the average of duplicate determinations.

Figure 10. Standard calibration curve for 9-phenanthrol. Results are the average of duplicate determinations.

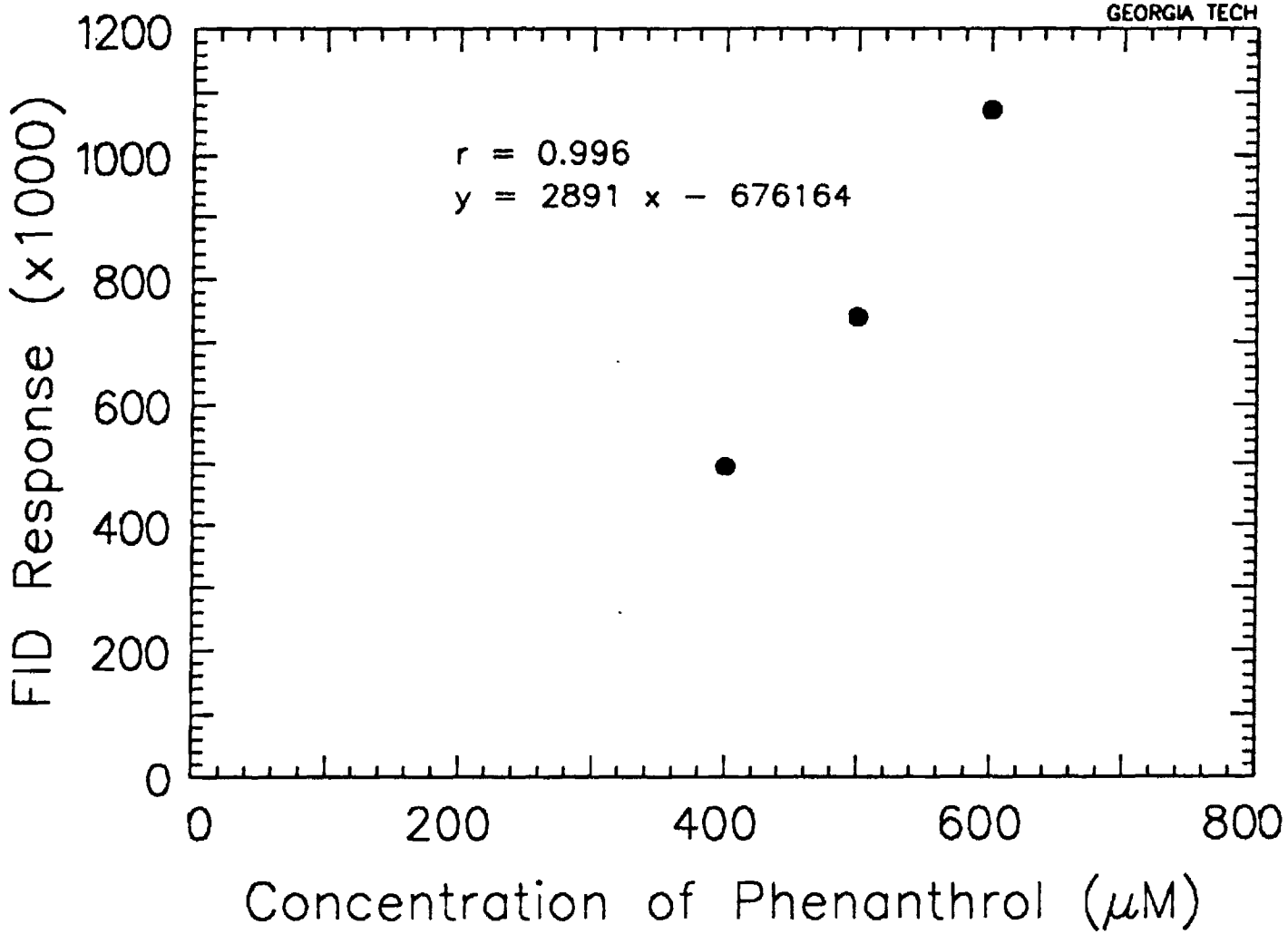

Figure 11. Standard calibration curve for 9-phenanthrol. Results are the average of duplicate determinations.

biodegradation studies was designated as "subbituminous coal" and was received from the Grand Forks Project Office. Due to the extensive time involved in the preparation of the coal extract and the analytical methods for separation and quantitation of the various components of the extracted coal, only subbitumious coal was tested for potential anaerobic bioconversion technology.

Extraction of coal was carried out in a Soxhlet extraction apparatus using benzene as the solvent. Twenty grams of subbituminous coal was crusted to a fine powder using a mortar and pestle and added to 200 ml of HPLC grade benzene. The resulting slurry was subjected to the Soxhlet extraction and extraction of the coal powder was repeated with 3 additional volumes of benzene (800 ml total). The combined benzene-extracts were concentrated to 20 ml under a stream of N_2 and stored in the dark at 2°C prior to analysis and subsequent use in biodegradation experiments. The yield of benzene-soluble constituents was 21.8%. Use of acetone and methylene chloride as solvents was also attempted but only a low recovery of extractable coal was obtained with these solvents. Thus, the benzene-extractable fraction was believed to contain a significant amount of extractable organics from coal.

The benzene-extract of subbituminous coal was divided into three identical parts for further studies. One part was stored for future use in pretreatment (oxidation) studies, another for use in biodegradation experiments, and the final aliquot for initial characterization by gas chromatography and for efficiency of extraction from a test system. Characterization of the benzene derived coal-extract was performed by gas chromatography/flame ionization detection since this method of analysis is extremely sensitive. An aliquot of the coal-extract was concentrated under N_2 to dryness and redissolved in an equal volume of methelene chloride. An aliquot of the methylene-chloride soluble fraction was spiked with surrogate (fluorene) and internal standard (hexamethylbenzene) and injected into the GC for analysis. GC conditions are listed below:

Gas chromatograph: Hewlett Packard 5980A

Column: J & W Scientific DB-5 (5% phenylmethyl silicone);

15 m x 0.52 mm i.d.

Carrier gas: N_2 at 8 ml/min.

Temperature program: 120°C (3 min.), 15°C/min. to 280°C

A standard gas chromatogram of the subbituminous coal extract is presented in Figure 12 as reference. Disregarding the peaks representative of the internal standard and surrogate compound, 6 components are easily resolved above background noise. No attempt has been made to identify any of these resolved components; however, these peaks are assumed to be representative, predominant organic constituents of subbituminous coal and will be used as the basis of "model compounds" for bioconversion studies.

In order to examine the efficiency of extraction of the predominant constituents of the benzene-derived coal extract from a simulated test system, a preliminary study was performed before initiation of biodegradation experiments. An aliquot (100 μl) of the benzene-derived coal extract was diluted with acetone to a final volume of 700 μl and spiked into an aqueous medium (5 ml) simulating the biodegradation test medium. It was necessary to first dissolve the benzene extract into a more polar solvent (such as acetone) before addition to the aqueous solution in order to provide dissolution of the coal extract. Following mixing and short term incubation (30 min - 1 hour), the entire 5 ml contents of the test system was extracted with methylene chloride as described previously for extraction of model compounds. The percentage recovery of the surrogate compound and the six predominant components identified from the standard coal extract are presented in Table 5. The extraction efficiency of at least four of the six predominant constituents was sufficiently high to warrant further investigation in bioconversion studies.

VI. Extraction Method Verification and QA/QC.

The first step for extraction method verification was conducted with organic free water

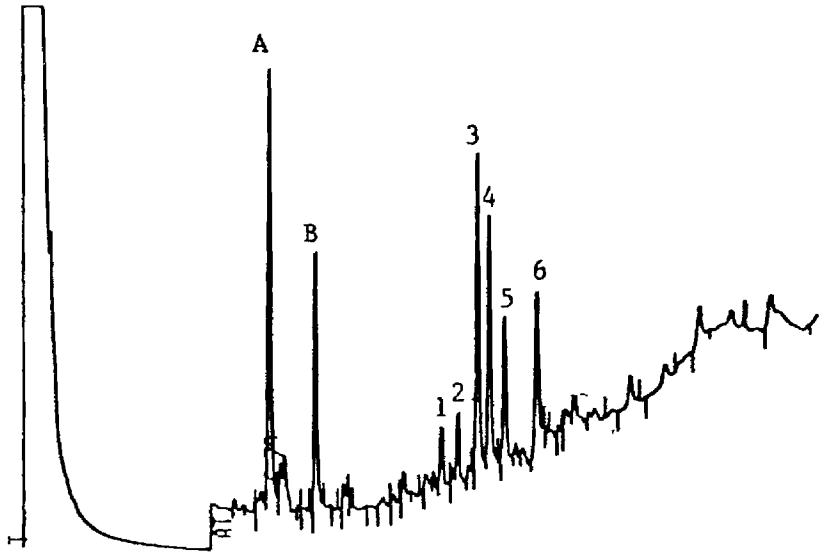

Figure 12. Chromatogram of benzene-derived extract from a sample of low-rank coal. Major constituents are labeled as compounds 1-6. A, internal standard (hexamethyl benzene); B, surrogate (fluorene).

Table 5. Recovery of surrogate (fluorene) and major constituents (peaks 1-6) of benzene-derived extract from low-rank coal in a simulated biodegradation experiment.

		% Recovery (peak number)					
Sample	Surrogate	1	2	3	4	5	6
A	83	99	88	90	77	43	66
B	81	87	77	72	54	13	25
C	88	103	93	94	80	20	31

(OFW) spiked with two concentration levels of the test compound. The procedure is as follows: two concentration levels of 2-naphthol and 9-phenanthrol (10 and 100 μM) were spiked in OFW. Blank samples of organic free water were also prepared, and all samples were performed in triplicate. Samples were spiked with surrogate compound at 2 ppm and the extraction procedures of Figure 13 were followed. Table 6 shows the recovery of the model compounds plus the surrogate compound. The average initial surrogate recovery was 102.8 $\pm$ 18.8%; further experiments resulted in more reproducible surrogate recovery (75.9 $\pm$ 2.9%). The recovery of 2-naphthol was 90.7 $\pm$ 9% and 77.7 $\pm$ 4.8% at 10 and 100 μM concentration levels. The 10 μM concentration level of 9-phenanthrol was not quantifiable and a relatively low recovery (49.4 $\pm$ 9.4%) was found at 100 μM 9-phenanthrol. The recovery of surrogate and 2-naphthol were within an acceptable range. The problem of low recovery of 9-phenanthrol was due to the problem of two peaks resolved during chromatography, as illustrated in Figure 6. Phenanthrol dissolved in organic solvent resolves into one major and one minor peak, while phenanthrol dissolved in water and extracted resolves into two peaks of almost identical intensity. The second peak represents an isomer of 9-phenanthrol, as determined by GC-mass spectrometry.

Further experiments were performed to examine extraction and recovery of model compounds and surrogate from an experimental test system. Two different procedures were initially tested to examine recovery of model compounds. In the first procedure, test medium was inoculated with sterilized anaerobic sediment and then the aqueous phase (supernatant) was separated from the sediment phase by centrifugation. This experiment was performed to establish if the model compound was bound to organic or inorganic surfaces or if it was free in solution. Also, problems arise (emulsions) during extraction of a "slurry" with organic solvents. After separation of the phases, model compounds (at two different concentrations, 10 μM and 100 μM) and surrogate were added to each of the two phases. Extraction of the surrogate and model compounds was then performed as indicated in Figure 13, beginning with CH_2Cl_2 extraction. Gas chromatographic analysis of the surrogate, 2-naphthol and 9-phenanthrol at 10 μM and 100 μM levels in the supernatant phase and the sediment phase is

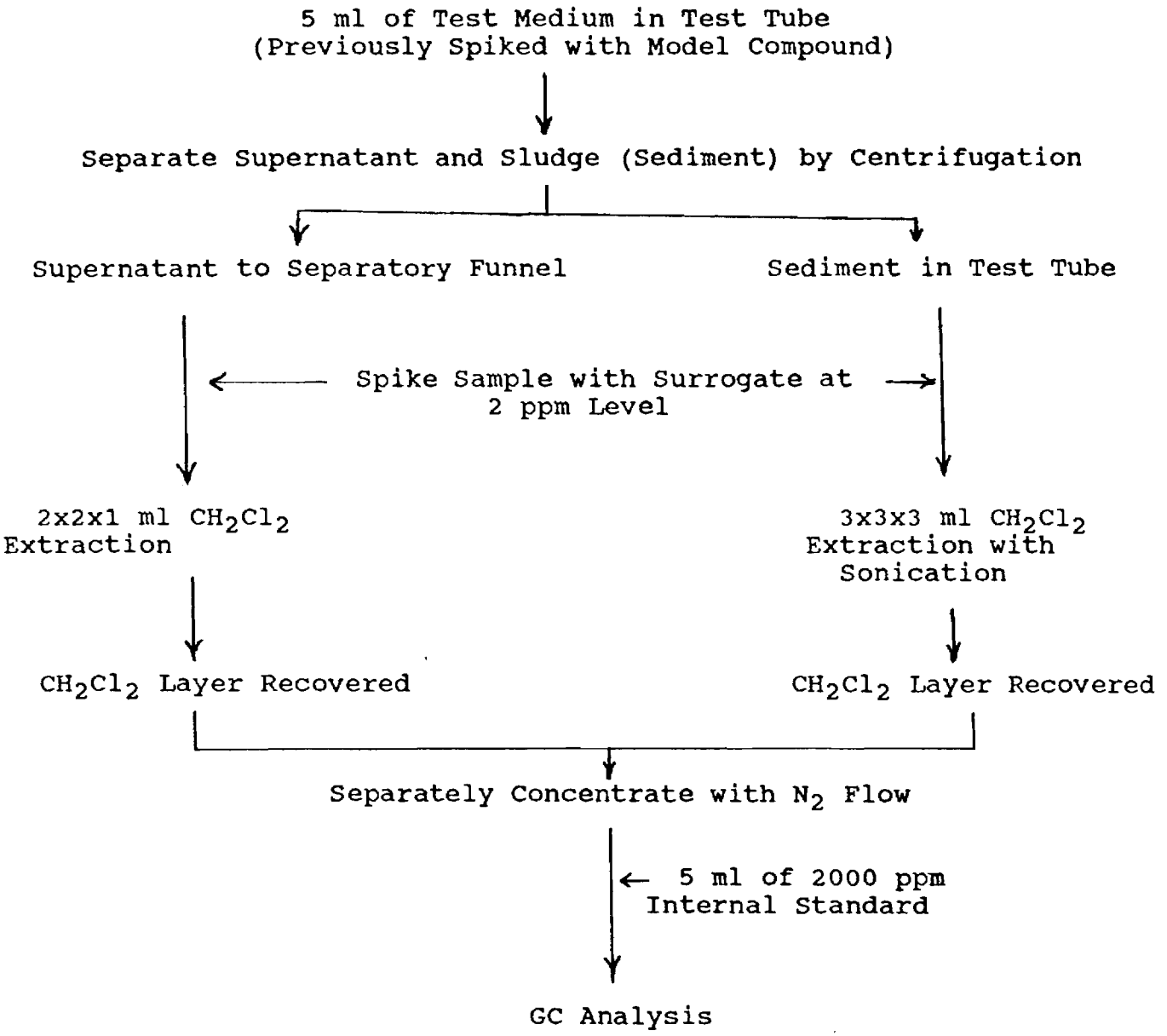

Figure 13. Finalized Flow Diagram of Extraction Methods of Model Compounds From Test System

Table 6. Recovery of test compounds 2-naphthol and 9-phenanthrol from organic free water (OFW) plus surrogate at two concentrations of model compounds.

Concentration (μM)	% Recovery					
	Surrogate[a]		2-Naphthol		9-Phenanthrol	
0	84.0 86.4 94.3	88.2±5.4	—		—	
10	87.7 102.0 98.7	96.1±7.5	96.0 80.3 95.8	90.7±9	NQ[b]	
100	115.4 120.8 135.7	124.0±10.5	79.5 73.8 79.8	77.7±4.8	58.1 50.5 39.5	49.4±9.4

[a]Surrogate compound added at 2ppm per 5ml of OFW
[b]Not quantifiable (below detection limit)

presented in Table 7. Results indicate that the surrogate was recovered at reproducible levels from both the supernatant fluid and from the sediment. The mean surrogate recovery ranged from 75.4 ± 0.9% to 83.4 ± 1.2%, which is within an acceptable range. The recovery of 2-naphthol was efficient (85.9 ± 0.4% to 94.4 ± 3.4%) and was in close agreement from both the supernatant and sediment phases; the % recovery did not appear to vary when tested at low and high substrate concentrations. However, recovery of 9-phenanthrol was more variable and the percentage recovery was lower when compared to the recovery of either the surrogate or 2-naphthol. The percentage recovery of 9-phenanthrol from the supernatant liquid ranged from 63.6 ± 4.2% at the 10 μM concentration to 75.1 ± 5.4% at the 100 μM level. The mean % recovery of 9-phenanthrol from the sediment phase was less, and ranged from 51.0% to 60.2%. As previously mentioned, the model compound 9-phenanthrol was resolved into two peaks by gas chromatography and represents isomers of 9-phenanthrol.

Results were more dramatic when the test system plus inoculum was spiked with model compounds before separation into the supernatant phase and sediment phase. In this second procedure, surrogate was added to both the supernatant liquid and sediment phase after separation by centrifugation; however, model compounds were added to the test system before separating the supernatant liquid from the sediment material. This procedure is analogous to the experimental protocol that will be followed to assess initial biodegradation experiments. Results of this experiment are presented in Table 8.

An important finding in this procedure is indicated by the absence of detectable levels of 9-phenanthrol in the supernatant liquid at both 10 μM and 100 μM concentrations. Apparently, 9-phenanthrol resides in the sediment phase and is not associated with the supernatant liquid. These results indicate that 9-phenanthrol is absorbed to organic and/or inorganic constituents of the "sediment" which are introduced by the inoculum. The mean percentage recovery of 9-phenanthrol in the sediment phase ranged from 56.9 ± 4.2% at 100 μM 9-phenanthrol to 59.9 ± 8.3% at 10 μM 9-phenanthrol and these results correspond well to the percentage recovery of "sediment phase" phenanthrol obtained from the initial procedure (Table 6) in which model compounds were added to the already separated phases. In similar experiments,

Table 7. Percentage recovery of Surrogate (fluorene), and model compounds (2-naphthol, 9-phenanthrol) from a simulated biodegradation test system. All compounds were added to the test system after separation into supernatant fraction and sediment fraction.

		% Recovery[a]		
	Conc (μM)	Surrogate	2-Naphthol	9-Phenanthrol
Recovered from supernatant fraction	0	75.4 ± 0.9	--	--
	10	78.7 ± 0.8	94.4 ± 3.4	63.6 ± 4.2
	100	82.2 ± 1.2	85.9 ± 0.4	75.1 ± 5.4
Recovered from sediment fraction	0	79.9 ± 4.3	--	--
	10	82.2 ± 1.9	89.8 ± 2.6	51.0
	100	83.4 ± 1.2	86.0 ± 6.4	60.2 ± 6.0

[a] values are means of triplicate determinations

Table 8. Percentage recovery of surrogate (fluorene) and model compounds (2-naphthol, 9-phenanthrol) from a simulated biodegradation test system. Model compounds were added to the test system before separation into supernatant and sediment fractions.

	% Recovery[b]		
Sample	Supernatant	Sediment	Total
2-Naphthol (10 μM)	80.0 ± 5.9	19.6 ± 2.2	99.6 ± 3.8
(100 μM)	77.2 ± 2.8	25.5 ± 2.8	102.7 ± 4.7
9-Phenanthrol (10 μM)	ND	59.9 ± 8.3	59.9 ± 8.3
(100 μM)	ND	56.9 ± 4.2	56.9 ± 4.2
Surrogate[a] (Fluorene, 2mg/L) with 10 μM 2-Naphthol plus 10 μM 9-Phenanthrol	114.2 ± 15.5	116.4 ± 6.8	---
Surrogate[a] (Fluorene, 2mg/L) with 100 μM 2-Naphthol and 100 μM 9-Phenanthrol	119.6 ± 8.3	133.6 ± 6.8	---

ND = Not Detected

[a] Surrogate was added to both supernatant and sediment fractions after separation of fractions, containing both model compounds at either 10 μM or 100 μM concentration.

[b] Values are means of triplicate determinations.

the mean percentage recovery of 2-naphthol from the supernatant liquid phase ranged from 77.2 ± 2.8% to 80.0 ± 5.9% and the percentage recovery in the sediment phase ranged from 19.6 ± 2.2% to 25.5 ± 2.8%. Thus, the majority of 2-naphthol resides in the aqueous phase rather than bound to organic and/or inorganic constituents of the inoculum. These results confirm our earlier speculation that the model compounds may "bind" or absorb to various components (glassware, organics, inorganics) of the test system; thus sampling may not be accurate by merely withdrawing a sample "aliquot" from a test system with time in order to follow substrate loss. Instead, we will continue to "sacrifice" the entire contents of a test system (test tube) at specific time points, separate the supernatant liquid from the sediment phase, and extract both phases in order to obtain a representative value for the concentration of the model compound in the reaction tube. This protocol will eliminate any possible sampling error due to absorption of the model compound of interest to non-aqueous constituents.

The model compound dibenzothiophene was also tested in biodegradation experiments and the percent recovery of dibenzothiophene from a simulated biodegradation test system is presented in Table 9. Extraction was performed on the entire contents of the individual test systems at dibenzothiophene concentrations of 20 and 200 μM. Surrogate recovery ranged from 81-88% recovery and averaged 85.7% ± 4.0. Dibenzothiophene recovery was also efficient (mean recovery was 84.3% ± 3.5 at 20 μM and 83.7% ± 4.5 at 200 μM).

Table 9. Recovery of dibenzothiophene and surrogate compound (phenanthrene) from a simulated biodegradation test system.

DBT Concentration		% Recovery Surrogate	% Recovery DBT
20 μM	(A)	88	88
	(B)	88	84
	(C)	81	81
20 μM	(A)	88	88
	(B)	88	84
	(C)	81	79

VII. Analysis of Anaerobic Biodegradation Experiments.

A. Anaerobic Bioconversion of Monoaromatic Compounds.

Initial experiments were performed to assess the potential for anaerobic bioconversion of "model", low-rank coal constituents. The following section (VII-B) focuses on bioconversion of "model" aromatic constituents which were selected as representative coal constituents of a more complex nature (2 and 3 fused aromatic ring substituents). This section focuses on some initial experiments designed to evaluate the anaerobic bioconversion of more simple (monoaromatic) aromatic compounds which may be representative of some low-rank coal constituents and to test the biopotential of inocula used in subsequent studies. Published literature (37,56,63,64) and the studies presented here confirm the role of microbes in anaerobic bioconversion of mono-aromatic constituents. Previous studies in our lab as well as additional studies with inocula from anaerobic habitats used in this study have established the anaerobic bioconversion of substituted aromatics, including phenol, p-cresol, and catechol (Tables 10-11). In most cases, partial or significant lag phases were observed before initiation of bioconversion of most aromatic constituents when anaerobic inocula was used which had no prior exposure to the aromatic substrate. Further, inhibition of bioconversion was noted at high levels of the substrates tested. As an example in case, phenol bioconversion mediated by anaerobic inocula obtained from a municipal anaerobic digestor was inhibited by high initial concentrations of phenol (1000 mg/L); however, when the inocula was initially exposed to lower concentrations (500 mg/L) of phenol, bioconversion proceded with a short lag period followed by acclimation to higher doses of phenol (800 mg/L, 1200 mg/L). These results confirm the presence of a population of bacteria capable of bioconversion of and acclimation to higher levels of the aromatic constituent. These results also provide evidence for the existance of microbes in "naturally occurring" anaerobic environments with vast biodegradation potential. As shown in Table 12, a number of investigations have been performed which exhibit the extensive metabolic capabilities of bacteria from anaerobic environments, including biodegradation of aromatic compounds under anaerobic conditions.

Table 10. Anaerobic bioconversion of phenol to CH_4; inocula was anaerobic sewage sludge 10% (w/v).

Incubation Time	Phenol (total μmol)	CH_4 (total μmol)
0	266	0
10	241	62
20	82	181
30	14	540
40	280*	705
50	147	944
60	38	1230

All values are corrected for controls; initial liquid culture volume was 50 ml with 80% N_2/20% CO_2 culture atmosphere.

* sample was reamended with phenol (500 mg/L)

Table 11. Summary of results depicting anaerobic bioconversion of catechol and p-cresol with anaerobic sludge (10% w/v) as inocula.

Incubation Time (days)	catechol (μmol)	CH_4 (μmol)
0	25	0
45	15	22
90	N.D.	51
	p-cresol(μmol)	CH_4(μmol)
0	23	0
45	17	16
90	5	46

All values are corrected for controls; initial liquid culture volume was 50 ml with 80% N_2/20% CO_2 culture atmosphere.

* N.D. = none detected

Table 12. Microbial mediated anaerobic bioconversion of selected aromatic constituents.

Compound	Inocula	Reference
2 amino-benzoate	*Pseudomonas* sp.	82
benzoic acid	sewage sludge enrichment culture	41 54,56,83
catechol	sewage sludge	63
o, m, p-cresol	sewage sludge	84,85
ferulic acid	enrichment culture	64
indole	enrichment culture	86
lignin	enrichment culture	87
phenol	sewage sludge	35,63,88

B. Anaerobic Bioconversion of "Model" Coal Constituents.

In order to assess anaerobic bioconversion of more complex aromatic constituents, biodegradation experiments were performed with the model compounds 2-naphthol, 1-naphthoic acid, 9-phenanthrol, and dibenzothiophene. Analyses have been performed for experiments in which sludge from an anaerobic sewage digester and anaerobic "swamp" sediment were used as the source of bacterial inocula.

Experiments were performed using individual model compounds at final concentrations of both 10 μM and 100 μM. In most cases, a supplemental experiment was performed with the addition of glucose (10 mM final concentration) to examine whether addition of an accessory growth substrate (glucose) would have any effect on biodegradation of the added model compound.

Results of initial biodegradation experiments using anaerobic swamp sediment at both 10 μM and 100 μM concentrations of 2-naphthol are presented in Tables 13-14. In experiments with 100 μM 2-naphthol, the supernatant liquid and sediment material were extracted separately. Greater than 95% of the recovered 2-naphthol resided in the supernatant liquid, as indicated by the T_0 (initial) samples. Samples obtained and analyzed at times T_1 (one month incubation) and T_2 (after two month's incubation) indicated that no loss or bioconversion of the model compound had occurred. In addition, experiments which were supplemented with glucose (10 mM final conc.) exhibited no detectable bioconversion after an incubation period of one month (T_1 plus glucose). Recovery of the surrogate compound in these experiments was within an acceptable range (as compared to preliminary experiments described previously; Tables 6-8).

Similar results were obtained for experiments inoculated with anaerobic "swamp" sediment but supplemented with 2-naphthol at a final concentration of 10 μM (Table 14). In this case, extraction of 2-naphthol from the test system was performed without prior separation of the liquid and sediment material since greater than 95% of the 2-naphthol present was demonstrated to reside in the liquid phase in experiments with 100 μM of 2-naphthol and in

Table 13. Results of initial biodegradation experiment inoculated with anaerobic "swamp" sediment and containing 100 μM 2-naphthol.

Incubation Time	Supernatant			Sediment			Total Corrected 2-Naphthol[c]
	% Surrogate Recovery	2-Naphthol (μM) Actual	Corrected[a]	% Surrogate Recovery	2-Naphthol (μM) Actual	Corrected[b]	
Initial (T_o)	98.3	459.5	368.4	88.6	14.0	12.9	381.3
T_o	92.6	438.5	373.2	88.2	—	—	373.2
30 Days (T_1)	90.3	492.0	429.3	84.8	12.0	11.6	440.9
T_1	89.9	512.5	449.2	92.7	16.0	14.1	463.3
60 Days (T_2)	83.2	518.0	490.6	90.2	15.5	14.0	504.6
T_2	91.3	454.0	391.8	92.6	—	—	391.8
T_o Plus Glucose	94.9	531.5	441.3	81.1	11.5	11.6	452.9
	83.6	484.5	456.7	90.3	12.5	11.3	468.0
T_1 Plus Glucose	83.4	525.0	496.0	90.6	22.0	19.8	515.8
	89.7	506.0	444.5	88.9	22.0	20.2	464.7

[a] Corrected supernatant 2-naphthol based on 78.8% surrogate recovery from preliminary experiments.

[b] Corrected sediment 2-naphthol based on 81.6% surrogate recovery from preliminary experiments.

[c] Predicted recovery of 2-naphthol equals 500 μM (based on concentration of samples by 5-fold).

Table 14. Results of initial biodegradation experiment inoculated with anaerobic "swamp" sediment and containing 10 μ M 2-naphthol.

Incubation Time	% Surrogate Recovery	2-Naphthol (μM) Actual	2-Naphthol (μM) Corrected[b]
Initial:			
(T_o)	88.1	46.9	42.8
T_o	94.8	54.9	46.5
30 Days:			
(T_1)	82.4	49.0	47.8
T_1	87.2	58.4	53.8
60 Days:			
(T_2)	93.6	57.5	49.3
T_2	81.3	52.8	52.2
T_o Plus Glucose	92.0	54.4	47.5
	91.6	56.0	49.1
T_1 Plus Glucose	87.8	57.4	52.2
	90.1	63.3	56.4

[a] Predicted recovery of 2-naphthol equals 50 μ M (based on concentration of recovered samples by 5-fold).

[b] Corrected value based on surrogate recovery of 80.3%, as calculated from preliminary experiments of recovery of extracted compounds.

preliminary experiments described previously (Tables 7-8). Addition of glucose (10 mM final conc.) to some experimental tubes (T_0 + glucose, T_1 + glucose) seemed to have no effect on bioconversion of 2-naphthol after an incubation period of one month.

Analysis of another model compound, 9-phenanthrol, in the initial biodegradation experiments inoculated with anaerobic "swamp" sediment, is presented in Table 15. Methylene chloride extractions from T_0, T_1 and T_2 samples, corresponding to initial, one month, and two months incubation times, were performed without separation of liquid and sediment since it was previously shown that 9-phenanthrol primarily resides in the sediment phase. Results indicated a slight decrease in 9-phenanthrol concentration after an incubation period of 2 months. Experiments supplemented with glucose enhanced 9-phenanthrol bioconversion compared to experimental tubes with no glucose addition after an incubation time of one month.

Biodegradation experiments performed with 9-phenanthrol as primary substrate and inoculated with 10% v/v anaerobic sludge were also somewhat promising in terms of bioconversion potential. After a period of incubation of 150 days at 30°C, the concentration of 9-phenanthrol was dramatically lower (92%) in one experimental sample compared to the initial level of 9-phenanthrol (Table 16). Only a very slight reduction in 9-phenanthrol was observed after a 30 day incubation period. Sterile controls also exhibited an apparent reduction in 9-phenanthrol concentration (16-45%) after incubation for 150 days but not to the extent of 9-phenanthrol reduction in experimental samples. The significant decrease in 9-phenanthrol concentration in sterile controls may be attributed to chemical transformation reactions, biological transformation due to contaminant microbes, or possibly due to a decreased efficiency of extraction of 9-phenanthrol with increased incubation time. This type of phenomenon has been observed with nitrogen-containing aromatic constituents such as trinitrotoluene (81); trinitrotoluene was shown to bind to a humus fraction of organic-rich composting piles and the retention of the aromatic constituent increased with increased time of incubation. Thus, it is possible that this type of phenomenon accounts for the apparent reduction in aromatic concentration in biodegradation test systems.

Table 15. Results of initial biodegradation experiment inoculated with anaerobic 'swamp' sediment and containing 100 μM 9-phenanthrol.

Incubation Time		% Surrogate Recovery	Corrected[(a)] 9-phenanthrol (μM)[(b)]
Initial:	T_o	76.3	359.0 > 363.9 ± 18.2
	T_o	92.9	368.7
	T_o + glucose	90.0	378.8 > 368.9 ± 18.5
	T_o + glucose	95.8	359.0
30 days:	T_1	91.0	296.0 > 352.9 ± 17.6
	T_1	92.8	409.8
	T_1 + glucose	80.2	223.0 > 277.8 ± 13.9
	T_1 + glucose	79.0	332.6
60 days:	T_2	53.5	--
	T_2	101.2	188.7

(a) Corrected 9-phenanthrol concentration based on mean surrogate reocvery of 80.3%

(b) Predicted recovery of 9-phenanthrol, assuming no bioconversion, equals 500 μM, based on concentration of samples for analysis.

Table 16. Results of anaerobic biodegradation experiment inoculated with anaerobic digestor sludge and containing 100 μM 9-phenanthrol.

	Sample	% Surrogate Recovery	Corrected 9-phenanthrol (μM)
T_o	A	60.1	86.2
	B	72.9	103.4
T_{30}	A	73.2	75.1
	B	74.8	92.4
T_{150}	A	88.9	7.7
	B	79.9	84.0
T_o	A sterile	72.6	84.9
	B sterile	76.1	101.2
T_{150}	A sterile	70.8	44.8
	B sterile	59.8	16.3

Results of other biodegradation experiments, using anaerobic sewage sludge as a source of bacterial inocula, are presented in Table 17. Biodegradation experiments with 2-naphthol and 2-naphthol supplemented with glucose (10 mM final concentration) have been performed. After an incubation period of 2 months, results are somewhat inconclusive regarding bioconversion of 2-naphthol. There does appear to be an indication of 2-naphthol bioconversion since a lower concentration of 2-naphthol is evident in samples after an incubation period of one month (T_1) and in one of two samples analyzed after an incubation of two months (T_2) when compared to initial (T_0) samples. It should be emphasized that each sample analyzed at a particular time point represents an individual test system and is not a subsample of a continuously incubated sample. Thus, each data point must be interpreted as an individual test system.

In this test system, the % recovery of the surrogate compound was comparable to data obtained in the early phases of this project (Table 6-8) and a greater proportion of the 2-naphthol was associated with the supernatant liquid compared to the sediment. There does appear to be a trend toward increased accumulation of 2-naphthol in the sediment phase with time and less 2-naphthol in the liquid phase. The cumulative data for 2-naphthol concentration indicates that an 11 to 16% decrease in 2-naphthol occurred from T initial to T_2 and T_1, respectively.

Further experiments were performed to assess the anaerobic bioconversion of a potentially relevent sulfur containing aromatic constituent of low-rank coals, dibenzothiophene (DBT). As indicated in a previous section, recovery of DBT from a simulated test system at concentrations of 20 and 200 μM were 84.3 ($\pm$ 3.5) % and 83.7 ($\pm$ 4.5) %, respectively. Bioconversion experiments were performed using anaerobic sludge as inocula and the results are presented in Table 18. Cultures inoculated with anaerobic sludge exhibited a significant reduction in dibenzothiophene concentration from the initial incubation time to a final sampling time of 3 monthes. In experiments with an initial DBT concentration of 50 μM, samples exhibited a significant reduction of DBT to levels of approximately 4 μM after 3 months incubation, suggesting significant bioconversion. A similar trend was observed

Table 17. Results of initial biodegradation experiment inoculated with anaerobic sewage sludge and containing 100 μM 2-naphthol.

Incubation Time	Supernatant % Surrogate Recovery	Supernatant 2-Naphthol (μM) Actual	Supernatant 2-Naphthol (μM) Corrected[a]	Sediment % Surrogate Recovery	Sediment 2-Naphthol (μM) Actual	Sediment 2-Naphthol (μM) Corrected[b]	Total Corrected 2-Naphthol[c]
Initial (T_o)	81.8	270.0	260.1	99.5	115.2	94.5	354.6
T_o	80.0	268.1	264.1	78.4	120.1	125.0	389.1
30 Days (T_1)	80.3	204.5	200.7	97.5	128.2	107.3	308.0
T_1	83.4	217.6	205.6	100.4	134.1	109.0	314.6
60 Days (T_2)	87.7	214.1	192.4	100.3	135.5	110.2	302.6
T_2	78.2	226.5	228.2	96.9	156.7	132.0	360.2
T_o Plus Glucose	85.4	263.1	242.8	79.6	143.6	147.2	390.0
	87.1	229.0	207.2	76.3	125.7	134.4	341.6
T_1 Plus Glucose	79.9	172.1	169.7	95.3	187.2	160.3	330.0
	83.6	211.2	199.1	79.0	147.2	152.0	351.1

[a] Corrected supernatant 2-naphthol concentration based on 78.8% surrogate recovery from preliminary experiments.

[b] Corrected sediment 2-naphthol concentration based on 81.6% surrogate recovery from preliminary experiments.

[c] Predicted recovery of 2-naphthol equals 500μM (based on concentration by 5-fold).

Table 18. Results of anaerobic biodegradation experiment inoculated with anaerobic sludge and containing 50-200 μM dibenzothiophene.

Compound	DBT Concentration (μM)			
	T_o	T_1	T_2	T_{final}
Experimental:				
· 50 μM DBT (initial)	56,39	23,21	32,31	3.5,4.0
· Surrogate recovery (%)	86,91	96,87	95,94	86,141
· 200 μM DBT (initial)	220	107,81	103,160	32,38
. Surrogate recovery (%)	87,91	98,96	77,86	110,88
Control:				
· 50 μM DBT	41	--	24	1.3
· 200 μM DBT	188	--	89	69

for samples amended with a higher initial level (200 μM) of dibenzothiophene and in additional experiments inoculated with anaerobic sediment. In these experiments, however, sterile controls also exhibited a significant reduction in the parent compound, indicating that "bioconversion" reactions may not have been the only mechanism in which dibenzothiophene concentration was lowered. Additional studies need to be performed in order to accurately assess this phenomenon. At this time, it cannot be conclusively stated that dibenzothiophene was "bioconverted" under anaerobic conditions. It is possible that DBT was either transformed abiologically, transformed by contaminating bacterial populations or heat resistant bacteria, or the lowered final concentration of DBT recovered was due to a lower extraction efficiency following prolonged incubation as previously stated.

C. Anaerobic Biodegradation Experiments with Coal-Extracts and Oxidized Coal-Extracts

Final biodegradation experiments focused on the assessment of low-rank coal bioconversion under strictly anaerobic conditions. Because of the limitations of using intact coal particles as substrate, biodegradation experiments were performed using a benzene-extract of a low-rank coal sample. As previously stated, six predominant peaks (components) were detected after extraction and analysis of the benzene derived coal extract by the gas chromatographic procedures previously described. These six components were tentatively "identified" by GC retention times and their concentrations were followed during subsequent anaerobic bioconversion experiments. The retention times of the six constituents was similar to the retention times for several of the two and three-ring "model" compounds tested earlier in this study, indicating that compounds of similar molecular weight/chemical characteristics were present in the benzene-coal extract.

Anaerobic biodegradation experiments were performed as described previously for "model compounds" with inocula from an anaerobic municipal digestor and from anaerobic sediment. For both inocula tested, results indicated little evidence of bioconversion of the major constituents of the benzene-derived coal extract. For peak number one, a reduction of approximately 50% was noted in the final analyzed sample, which represented an incubation

time of twelve weeks (Table 19). None of the other five remaining components were reduced in concentration to any appreciable extent. Further, no detectable conversion was noted for any of the six constituents in control experiments. The percent recovery of the surrogate compound in the coal-extract bioconversion experiments was greater than 90% for most samples, indicating that extraction procedures were efficient and acceptable.

In an effort to enhance bioconversion of the major constituents of the benzene-derived coal extract, coal-pretreatment studies were initiated. Samples of the benzene-coal extract from low rank coal were pre-treated with H_2O_2 before subsequent use in biodegradation experiments. Experimental details of H_2O_2 pretreatment are presented elsewhere. A comparison of the gas chromatographic profiles of oxidized and non-oxidized coal extracts was similar, although not identical. As expected, the concentrations of the major constituents of the original benzene-derived coal extract were reduced after being subjected to oxidation at different H_2O_2 levels. The results presented in Table 20 show the % recovery of the six major constituents of "oxidized" benzene coal-extract relative to non-oxidized coal extract. It is evident that for all three oxidation levels tested, the % recovery of the six major constituents was reduced significantly, indicating some chemical oxidation of the major compounds to perhaps 'related' organic constituents. The identity of the compounds resulting from the peroxide treatment was not determined; from the gas chromatographic elution profile, it did not appear that new or different constituents were present at a significantly high level. Biodegradation experiments were performed with oxidation level 1 coal-extract to assess the effects of pretreatment (oxidation) of the coal-extract on anaerobic bioconversion. Bioconversion experiments were performed as previously described for model compounds; inocula was obtained from an anaerobic sludge digestor and gas chromatographic profiles of the four major constituents of the partially "oxidized" coal-extract were followed with time as an index of bioconversion potential. An anaerobic bioconversion experiment with level 1 oxidation of benzene-derived coal extract is presented in Table 21. In some samples, a reduction in the concentrations of components number one, two and four were noted. Most control samples exhibited little or no significant reduction in the concentration of the major

Table 19. Results of biodegradation experiment inoculated with 10% anaerobic sludge and amended with low-rank coal extract.

Samples		% Recovery of Major Components (Peak No.)						
		Surrogate	1	2	3	4	5	6
Experimental								
T_o	A	95	177	77	77	43	80	85
	B	124	170	64	61	27	64	96
T_f	A	108	90	83	72	38	40	76
	B	104	77	60	92	64	118	85
Control								
T_o	A,B	98,76	158,171	59	57,35	38,13	75,10	70,24
T_f	A,B	89,70	179	71	39,31	30,22	59,23	40,52

Peaks 1-6 represent major constituents of benzene-derived low-rank coal extract (refer to Figure 12)

Table 20. Percent recovery of major components of benzene-derived coal extract after oxidation with H_2O_2.

	% Recovery (Peak No.)							
	Surrogate	1	2	3	4	5	6	7
Oxidation Level 1	101	39	48	28	13	17	15	72
Oxidation Level 2	102	32	39	35	28	21	13	12
Oxidation Level 3	92	43	40	37	35	31	19	26
Unoxidized	92	99	99	90	92	94	94	127

constituents. Final time point samples (three months incubation at 30°C) did not exhibit a significant reduction in the concentration of any of the four major constituents of the "oxidized" coal extract compared to initial levels. However, each sample analyzed in the overall experiment represented an individual experiment and not a subsample of a continuing incubated sample. Thus, it is difficult to ascertain if significant anaerobic bioconversion of the oxidized coal extract constituents occurred under these conditions with anaerobic digestor sludge as inocula, but there was evidence for reduction of specific constituents in some samples.

Table 21. Results of biodegradation experiment inoculated with anaerobic sludge and amended with H_2O_2 oxidized, benzene-extract of low-rank coal.

	% Recovery (Peak No.)				
	Surrogate	1	2	3	4
Experimental					
T_o	98,106	100,109	100,118	100,137	100,115
T_2	124	39	60	82	26
T_{final}	84,93	73,91	83,105	87,116	73,94
Control					
T_{final}	79,72	77,88	86,101	105,106	43,96

VIII. Preliminary Toxicity Studies.

Inocula from anaerobic sewage sludge was added to test medium in the presence and absence of model compounds and supplemented with glucose; methane production was followed with time, over a time period of 65 hours, to determine if addition of the model compound had any toxic effect on the microbial consortia responsible for the production of CH_4 from glucose. The results presented in Table 22 and Figure 14 indicate that 2-naphthol, 1-naphthoic acid, and 9-phenanthrol exhibited little or no toxic effect on CH_4 production from glucose at either 10 μM or 100 μM concentrations. Compared to control samples, CH_4 production occurred at virtually the same rate during the first 42 hours of incubation. From 42 hours to 65 hours, there was an indication of a slight reduction in CH_4 production in the presence of all model compounds tested; the greatest inhibition occurred in experiments with the highest concentration (100 μM) of 2-naphthol and 1-naphthoic acid.

Additional toxicity experiments were performed with a pure culture of a methanogenic bacterium, *Methanospirillum hungatei*, cultivated with H_2 plus CO_2 as the source of carbon and energy; the model compounds 2-naphthol, 1-naphthoic acid, and 9-phenanthrol, corresponding to 10 μM and 100 μM final concentrations, were added to the pure culture. Preliminary results suggest that 2-naphthol and 1-naphthoic acid, at both concentrations tested, had little or no effect on growth and CH_4 production by *Methanospirillum hungatei*; however, 9-phenanthrol, at 10 μM and 100 μM levels, does appear to inhibit growth and CH_4 production significantly. Additional studies were performed to determine the minimal concentration of 9-phenanthrol that inhibited growth of *M. hungatei*. Results presented in Table 23 indicated that a concentration of 10 μM or less of 9-phenanthrol did not inhibit growth of *M. hungatei*; 25 μM 9-phenanthrol resulted in a 29% inhibition while concentrations greater than 50 μM resulted in complete inhibition of growth. Addition of a sterilized sediment inoculum, similar to the inoculum level used in biodegradation experiments, to *M. hungatei* inoculated medium plus various concentrations of 9-phenanthrol resulted in a reduction of inhibition. No inhibition was observed in experiments supplemented with sediment and 100 μM 9-phenanthrol; complete inhibition still occurred at 250 μM

Table 22. Effect of model compounds 2-naphthol, 1-naphthoic acid, and 9-phenanthrol on CH_4 production from glucose. All cultures inoculated with anaerobic digestor sediment. Results are means of duplicate experiments.

	μ moles CH_4 produced						
		1-Naphthoic Acid		2-Naphthol		9-Phenanthrol	
Time (hours)	Control	10 μM	100 μM	10 μM	100 μM	10 μM	100 μM
0	0.67	0.50	0.49	0.49	0.45	0.46	0.47
18	8.13	8.50	9.38	8.94	8.81	9.63	8.00
42	28.1	29.9	28.9	28.7	29.1	28.8	30.1
65	57.6	56.7	43.9	53.5	44.1	46.6	52.7

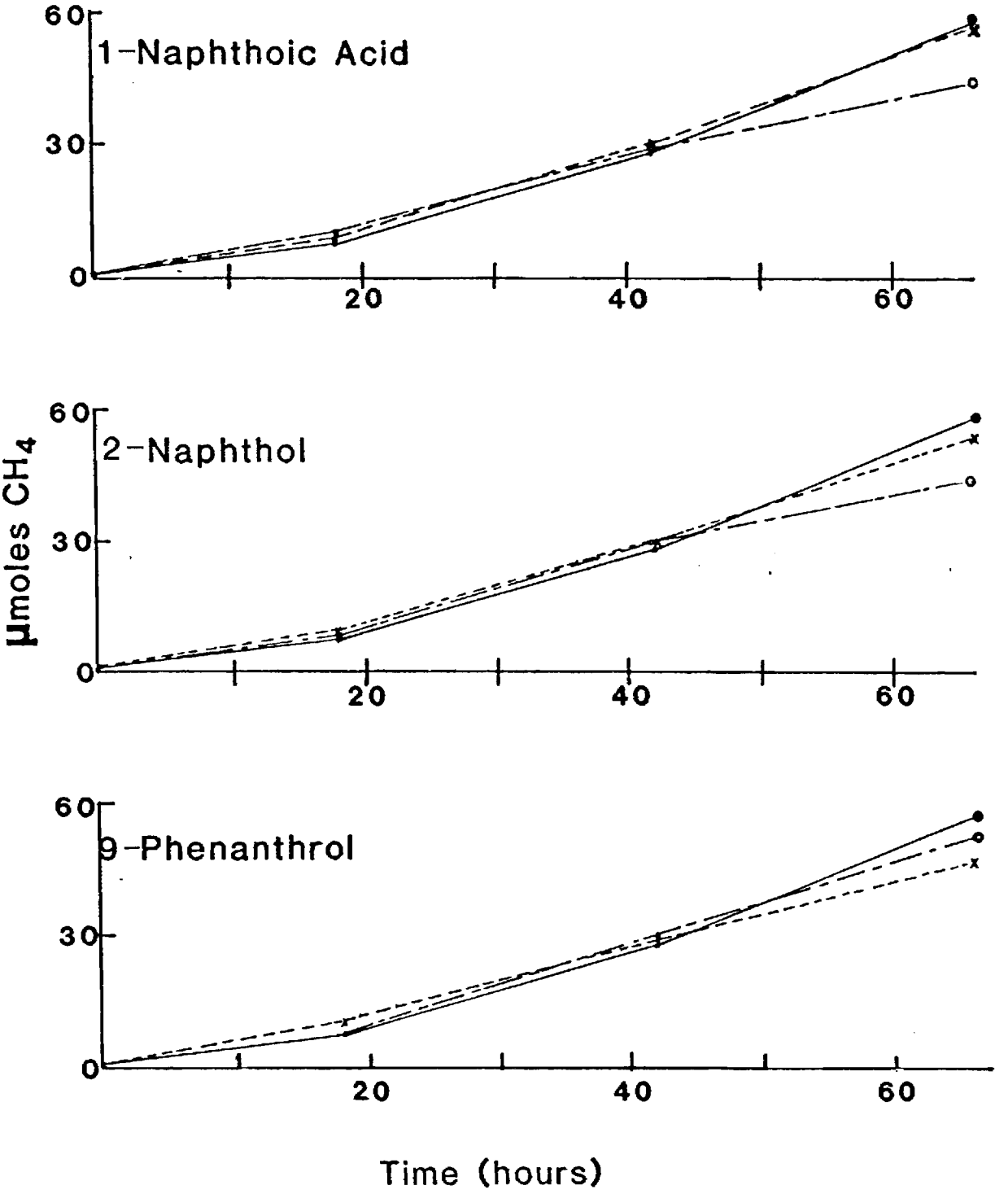

Figure 14. Effect of model compounds 1-naphthoic acid, 2-naphthol, and 9-phenanthrol on CH_4 production from glucose. Samples inoculated with anaerobic sewage sludge. (●, control; x, 10μM concentration; ○, 100μM concentration)

Table 23. Effect of 9-phenanthrol addition on growth of the methanogen *Methanospirillum hungatei*.

	Growth[a](O.D. 600 nm)				
	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours
9-phenanthrol (μM)					
0	0.02	0.16	0.37	0.48	0.55
1	0.02	0.09	0.29	0.40	0.54
10	0.03	0.10	0.27	0.41	0.53
25	0.03	0.08	0.19	0.29	0.39
50	0.04	0.05	0.05	0.05	0.05
75	0.04	0.04	0.03	0.03	0.03
100	0.05	0.05	0.04	0.04	0.04

[a] Values are the average of triplicate determinations.

9-phenanthrol, however.

Thus, toxicity of the model compounds may be a function of whether the model compound is 'available' to the particular organism in question or not. 9-phenanthrol was previously shown to be associated with 'sediment' (organic and/or inorganic material added as inoculum in experiments inoculated with anaerobic sediment) in several experiments (Table 8) and thus may not exert the same toxic effect in a "mixed" culture system as compared to a pure culture (more controlled) test system. These studies are important to determine which microbial processes may potentially be affected by specific (toxic) model compounds.

IX. Conclusions

The objectives of the research contract entitled "Biological Degradation of Low-Rank Coal" were divided into three primary tasks:

Task 1: Assessment of Low-Rank Coal Biodegradation

Task 2: Biodegradation of Model Compounds

Task 3: Biodegradation of Coal

The present study was undertaken to examine the potential for bioconversion of low-rank coal and coal-related constituents under anaerobic conditions and to explore the feasibility for bioconversion to fuel-related products. The conclusions of the completed project are described below.

Task 1: Assessment of Low-Rank Coal Bioconversion.

A literature review of coal chemistry and microbial processess associated with biotransformation of coal and coal-related constituents was performed. Only a very limited number of investigations have been reported related to biotransformation of coals, coal slurries or coal extracts, either under aerobic conditions or under strictly anaerobic conditions. In fact, the reported investigations were restricted to microbial transformation of selected lignite (probably oxidized) coals by a group of fungi under aerobic conditions. No studies have been reported which involve coal biotransformation under anaerobic conditions. However, a much greater amount of information is available related to microbial bioconversion of simple and complex aromatic constituents which may comprise low-rank coals, including monoaromatic and polycyclic aromatic organic compounds with differing functional groups. Most studies have described bioconversion of aromatic constituents under aerobic conditions, and specific microorganisms have been isolated and identified which are capable of aerobic biotransformation of monoaromatics as well as polycyclic aromatic hydrocarbons. More recently, investigations have been performed which verify the anaerobic

biotransformation of certain mono-aromatic organic compounds, particularly those associated with the structure of lignin. Thus, it appears that microbes exist in natural habitats which are capable of anaerobic bioconversion of less complex aromatic compounds which may be representative constituents of low-rank coal structure. Little evidence is available which supports the notion that significant bioconversion occurs for the more complex polycyclic aromatic hydrocarbons, which indeed may be the predominant constituents of coal structure.

Task 2: Biodegradation of Model Compounds.

Following the assessment of coal chemistry and literature related to microbially-mediated coal and coal constituent bioconversion, "model compounds" were selected for use in initial bioconversion studies. The "model compounds" chosen for the present study included monoaromatics (phenol, cresol) as well as more complex aromatic constituents of the polycyclic aromatic hydrocarbon family. Specifically, 1 and 2-naphthol, 9-phenanthrol, and dibenzothiophene were chosen as "model" constituents which may be representative polycyclic aromatic hydrocarbons found in low-rank coals. Experimental procedures were developed for extraction, separation and quantitation of the various complex aromatic constituents in order to be able to accurately assess bioconversion potential. An experimental test system was designed and employed to follow bioconversion under strictly anaerobic conditions and using various naturally occurring anaerobic inocula. Extraction efficiencies and quantitation of the "model compounds" were within acceptable limits for an accurate assessment of bioconversion potential. Results from these bioconversion experiments indicated that the more simple aromatic constituents were metabolized anaerobically to CH_4 using natural microbial consortia after an initial lag phase. Cultures were subsequently acclimated to increased concentration of the monoaromatic constituents after a period of initial adaptation. More complex aromatic compounds (naphthols, phenanthrol, dibenzothiophene) were not readily amenable to bioconversion under anaerobic conditions. In some instances, a reduction in the initial level of the complex aromatic constituent was noted after prolonged incubation but this phenomenon was inconsistent with differing inocula and with replicate samples. Thus, it is

uncertain or unlikely that significant anaerobic bioconversion of more complex aromatic constituents occurs under experimental conditions used in this study.

Preliminary studies were also performed to assess the potential toxic effects of some "model" aromatic constituents on specific diverse microbial cultures and undefined microbial consortia. For most experiments and for most compounds tested, little or no toxic effects were observed on growth of several known test microbes at the concentrations of the test compounds used in biodegradation experiments. However, some toxicity was noted with certain compounds at higher concentrations.

Task 3: Biodegradation of Coal.

Final experiments were performed to assess the potential for anaerobic bioconversion of constituents of a natural coal sample. Because of the limitations in using intact coal particles, a benzene-extract of a low-rank coal sample was employed in these anaerobic bioconversion experiments. Extraction efficiencies and gas chromatographic analyses were optimized for assessment of coal-extract bioconversion and results identified six major constituents as potential substrates. Subsequent anaerobic bioconversion experiments indicated little or no significant biotransformation of any of the six major constituents from the benzene-derived extract. Hydrogen peroxide treatment of the coal-extract was performed to enhance bioconversion potential via generation of oxidized constituents of the coal-extract. Experimental results indicated a modest enhancement of bioconversion for one of the four major constituents. In summary, it appears that complex extractable constituents from coal are relatively recalcitrant to anaerobic bioconversion by natural anaerobic inocula.

References

1. J.W. Larsen. 1981. Chemistry and Physics of Coal Utilization-1980. p. 1-27. American Institute of Physics Proceedings, No. 70.

2. B.M. Benjamin et al. 1978. Fuel 57:269.

3. W.A. Kirby, J.R. Lakey and R.J. Sarjant. 1954. Fuel 33:480.

4. D.W. Van Krevelen. 1961. Coal. Elsevier Publ. Co.

5. K.E. Chung, I.B. Goldberg and J.J. Ratto. 1985. EPRI report, Feb. 1985, Project 2147-4 (AP-3889).

6. R. Narayan. 1986. EPRI report, Feb. 1986, Project 2383-3 (AP-4441).

7. P.H. Gray and H.G. Thorton. 1928. Zentralbl. Bakteriol. II 73:74.

8. E.J. McKenna and R.E. Kallio. 9:183.

9. D.T. Gibson. 1972. Crit. Rev. Microbiol. 1:199.

10. J.J. Perry. 1979. Microbiol. Rev. 43:59.

11. D. Claus and N. Walker. 1964. J. Gen. Microbiol. 36:107.

12. G.M. Klecka and D.T. Gibson. 1981. Appl. Environ. Microbiol. 41:1159.

13. R.E. Cripps and R.J. Watkinson. 1978. Biodegradation of Hydrocarbons -1. Appl. Sci. Publs., London.

14. R.L. Raymond, W.V. Jamison and J.O. Hudson. 1967. Appl. Microbiol. 15:857.

15. R.S. Wodzinski and J.E. Coyle. 1974. Appl. Microbiol. 27:1081.

16. D.T. Gibson, V. Mahadevan, D.M. Jerina, H. Yagi and H.J. Yeh. 1975. Science 189:295.

17. B. Haccius and O. Helfrich. 1958. Arch. Microbiol. 28:394.

18. P.K. Ayengar, O. Hayaishi, M. Nakjima and I. Tomida. 1959. Biochim. Biophys. Acta. 33:111.

19. D.T. Gibson, J.R. Koch and R.E. Kallio. 1968. Biochemistry 7:2653.

20. D. Claus and N. Walker. 1964. J. Gen. Microbiol. 36:107.

21. C. Colla, A. Fiecchi and V. Treccani. 1959. Ann. Microbiol. Enzymol. 9:87.

22. S. Dagley. 1975. Essays Biochem. 11:81.

23. P.A. Williams. 1976. Biochem. Soc. Trans. 4:452.

24. P.H. Gray and H.G. Thornton. 1928. Zentralbl. Bakteriol. II. 73:74.

25. M. Blumer and N. Rudram. 1970. J. Inst. Petrol. 56:99.

26. W.W. Youngblood and M. Blumer. 1975. Geochim. Cosmochim. Acta 39:1303.

27. M. Blumer and W.W. Youngblood. 1975. Science 188:53.

28. R.A. Hites, R.E. LaFlamme and J.W. Farrington. 1977. Science 198:829.

29. R.J. Gordon. 1976. Envir. Sci. Technol. 10:370.

30. J. Borneff, F. Selenka, H. Knute and A. Maximos. 1968. Environ. Res. 2:22.

31. C.E. Cerniglia, 1984. p. 99-128, In: R.M. Atlas (ed.), Petroleum Microbiology, Macmillan, New York.

32. M. Blumer and N. Rudrum. 1970. J. Inst. Petrol. 56:99.

33. W. Giger and M. Blumer. 1974. Anal. Chem. 46:1663.

34. D.T. Gibson and V. Subramanian. 1984. Microbial Degradation of Organic Compounds. Marcel Dekker, New York.

35. M.T. Balba and W.C. Evans. 1977. Biochem. Soc. Trans. 5:302.

36. W.C. Evans. 1977. Nature 270:17.

37. L.Y. Young. 1984. Microbial Degradation of Organic Compounds. Marcel-Dekker, New York.

38. D. Tarvin and A.M. Buswell. 1934. J. Am. Chem. Soc. 56:1751.

39. F.M. Clark and L.R. Fina. 1952. Arch. Biochem. Biophys. 36:26.

40. H.A. Barker. 1956. Bacteriol Fermentations. John Wiley and Sons, New York.

41. L.R. Fina and A.M. Fiskin. 1960. Arch. Biochem. Biophys. 91:163.

42. T. Oshima. 1965. Z. Allg. Mikrobiol. 5:386.

43. B.F. Taylor and M.J. Heeb. 1972. Arch. Mikrobiol. 83:165.

44. P.L. Dutton and W.C. Evans. 1969. Biochem. J. 113:525.

45. D.F. Berry, A.J. Francis and J.M. Bollag. 1987. Microbiol. Revs. 51:43-59.

46. R.J. Williams and W.C. Evans. 1973. Biochem. Soc. Trans. 1:186-187.

47. R.J. Williams and W.C. Evans. 1975. Biochem. J. 148:1-10.

48. M. Guya and G. Hegeman. 1969. J. Bacteriol. 99:906.

49. J.B. Healy, Jr., L.Y. Young and M. Reinhard. 1980. Appl. Environ. Microbiol. 39:436.

50. T.M. Vogel and D. Grbic-Galic. 1986. Appl. Environ. Microbiol. 52:200-202.

51. E.R. Stadtman, T.C. Stadtman, I. Pastan and L.D. Smith. 1972. J. Bacteriol. 110:758-760.

52. F. Widdel. 1980. PhD. Thesis, Univ. of Gottingen, FRG.

53. D.O. Mountfort and M.P. Bryant. 1982. Arch. Microbiol. 133:249.

54. P.M. Nottingham and R.E. Hungate. 1969. J. Bacteriol. 98:1170.

55. L.R. Fina, R.L. Bridges, T.H. Coblentz and F.F. Roberts. 1978. Arch. Microbiol. 118:1631.

56. J.G. Ferry and R.S. Wolfe. 1976. Arch. Microbiol. 107:33.

57. D. Grbic-Galic and L.Y. Young. 1982. Abst. Ann. Mtg. Amer. Soc. Microbiol. 020:199.

58. M.P. Bryant, E.A. Wolin, M.J. Wolin and R.S. Wolfe. 1967. Arch. Microbiol. 59:20.

59. M.J. McInerney, M.P. Bryant and N. Pfennig. 1979. Arch. Microbiol. 122:129.

60. D. Boone and M.P. Bryant. 1980. Appl. Environ. Microbiol. 40:626.

61. W.E. Balch, G.E. Fox, L.J. Magrum, C.R. Woese and R.S. Wolfe. 1979. Microbial Rev. 43:260.

62. E.R. Shlomi, A. Lankhorst and R.A. Prins. 1978. Microbiol. Ecol. 4:249.

63. J.B. Healy, Jr., and L.Y. Young. 1978. Appl. Environ. Microbiol. 35:216.

64. J.B. Healy, Jr., and L.Y. Young. 1979. Appl. Environ. Microbiol. 38:84.

65. J.P. Kaiser and K.W. Hanselmann. 1982. Experientia 38:167.

66. G.G. Ehrlich, E.M. Godsy, D.F. Goerlitz and M.F. Hult. 1983. Dev. Ind. Microbiol. 24:235-245.

67. G.G. Ehrlich, D.F. Goerlitz, E.M. Godsy, and M.F. Hult, 1982. Ground Water. 20:703-710.

68. G.C. Tsai and G.A. Jones. 1975. Can. J. Microbiol. 133:195.

69. G.C. Tsai, D.M. Gates, W.M. Ingeldew and G.A. Jones. 1976. Can. J. Microbiol. 22:159.

70. P.J. Whittle, D.O. Lunt and W.C. Evans. 1976. Biochem. Soc. Trans. 4:490.

71. B. Schink and N. Pfennig. 1982. Arch. Microbiol. 133:195.

72. F. Widdel, G.W. Kohring and F. Mayer. 1983. Arch. Microbiol. 134:286-294.

73. H.B. Klevens. 1954. J. Phys. Colloid Chem. 54:283-298.

74. R.M. Miller, G.M. Singer, J.D. Rosen, and R. Bartha. 1988. Appl. Environ. Microbiol. 54:1724-1730.

75. J.R. Mihelcic and R.G. Luthy. 1988. Appl. Environ. Microbiol. 54:1182-1187.

76. J.R. Mihelcic and R.G. Luthy. 1988. Appl. Environ. Microbiol. 54:1188-1198.

77. M.S. Cohen and P.D. Gabriele. 1982. Appl. Environ. Microbiol. 44:23.

78. B. Ward. 1985. System. Appl. Microbiol. 6:236.

79. H.B. Ward. 1985. Bioenergy 84 Proceedings, Vol. 3, Elsevier Publ., London.

80. G.E. Johnson. 1972. U.S. Patent No. 3,640,846. Production of Methane by Bacterial Action.

81. D.L. Kaplan and A.M. Kaplan. 1982. Appl. Environ. Microbiol. 44:757-760.

82. K. Braun and D.T. Gibson. 1984. Appl. Environ. Microbiol. 48:102.

83. D.R. Shelton and J.M. Tiedje. 1984. Appl. Environ. Microbiol. 47:850.

84. P.M. Fedorak and S.E. Hrudey. 1984. Water Res. 18:361.

85. S.A. Boyd, D.R. Shelton, D. Berry, and J.M. Tiedje. 1983. Appl. Environ. Microbiol. 46:50.

86. Y.T. Wang, M.T. Suidan and J.T. Pfeffer. 1984. Appl. Environ. Microbiol. 48:1058.

87. R. Benner and R.E. Hodson. 1985. Appl. Environ. Microbiol. 50:971.

88. G. Bakker. 1977. FEMS Letters 1:103-108.