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**ANAEROBIC BENZENE DEGRADATION IN CULTURE AND
HYDROCARBON DEGRADATION IN THE SUBSURFACE ENVIRONMENT**

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ABSTRACT OF DISSERTATION

Anaerobic Benzene Degradation in Culture and Hydrocarbon Degradation in the Subsurface Environment

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Understanding of microorganisms and pathways involved in anaerobic benzene degradation is limited. Stable isotope probing of DNA was used to identify key members of a previously characterized, sulfate-reducing benzene degrading consortium. DNA extracts of cultures incubated with [$^{13}\text{C}_6$]- or [$^{12}\text{C}_6$]benzene were separated into ^{13}C - and ^{12}C -labeled fractions by CsCl density gradient centrifugation. Sequencing and Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis of the 16S rRNA gene identified TRF 270 (bp), a *Desulfobacterium* like phylotype, which was first to derive the bulk of the ^{13}C label for DNA synthesis, and is thus likely involved in activation of benzene degradation.

To understand the pathway of anaerobic benzene metabolism, degradation and inhibition tests were used. Based on these tests toluene was eliminated, and benzoate was identified as a possible intermediate. Metabolites detected in cultures amended with [$^{13}\text{C}_6$]benzene or [$^{13}\text{C}_6$]phenol indicate that in this consortium there are 2 different pathways of benzoate formation, one forms universally labeled ([^{13}C -UL]benzoate), and the other forms ring labeled benzoate. Pathway that forms [^{13}C -UL]benzoate is dominant

during benzene degradation in which the benzene ring is carboxylated by a carbon derived from another benzene ring. This pathway is different from the proposed pathway of benzene degradation via phenol, as the labeling pattern of ^{13}C -labeled benzoate formed from $[^{13}\text{C}_6]\text{benzene}$ or $[^{13}\text{C}_6]\text{phenol}$ is not identical. In conclusion, a novel pathway that activates one benzene ring through its reaction with products of another benzene ring likely exists in this consortium.

Groundwater impacted by a manufacturing gas plant site was used for detection and quantification of metabolic intermediates of polycyclic aromatic hydrocarbons and gene analogues encoding alpha subunit of benzylsuccinate synthase (*bssA*), as evidence for natural attenuation. Highest concentrations of metabolic intermediates of anaerobic naphthalene and 2-methylnaphthalene degradation were detected in an impacted monitoring well (MW)-24, near the source. Quantitative analysis of 16S rRNA gene indicated that bacterial population was enriched in the impacted wells, while *bssA* gene containing bacterial community was enriched in MW-24. Detection of not one, but two different indicators specific to the presence and activity of microorganisms provides strong evidence for *in situ* anaerobic microbial processes.

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DEDICATION

The work in this thesis is dedicated to the spirit of science that is kindled in our hearts when we are first amazed by the wonders of this world, and we choose to understand it through observation and experimentation, in the effort to find answers to all that happens to us and surrounds our being.

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CHAPTER 1

Literature review

1.1 Petroleum hydrocarbons in the environment

1.1.1 Inputs

Petroleum fuels and other petroleum products are an important component of our modern life. Over 84 million barrels of oil are used worldwide per day (176). Crude oil and refined petroleum products can be released into the environment through accidental spills and leaks, during extraction, transport and consumption, in addition to the input of oil through natural seeps.

The National Research Council (NRC) estimates that the releases of oil from extraction and transportation of petroleum represent less than 10 % of the input due to human activities to the North American marine waters, while chronic releases during consumption of petroleum including urban runoff, polluted rivers, and discharges from commercial and recreational marine vessels, contribute up to 85% of the anthropogenic load (134). According to the NRC the annual worldwide estimates of petroleum input into the sea exceeds 1.3 million metric tons (134). The United States Environmental Protection Agency (USEPA) runs the Underground Storage Tank (UST) program, which provides broad based statistics for petroleum releases into the environment. The UST program reported that in 1994 there were more than 300,000 UST sites, with more than a million underground storage tanks. There were 119,000 confirmed instances of release of gasoline or similar petroleum bulk fuels to the groundwater or soil at these UST sites (1). These releases are significant since the potential hazard of a leaking UST is that the petroleum or hazardous waste can contaminate the groundwater supplies that serve as

drinking water sources for half of all the Americans. According to the latest EPA report there are 623,319 active tanks in the U.S. under the UST program (178).

1.1.2 Fate and Effects

In the subsurface and surface environments the composition of petroleum can be altered by chemical, physical and biological processes such as oxidation, photolysis, evaporation, dissolution, and biodegradation, collectively called weathering (186). These processes also affect the distribution and redistribution of spilled oils in the environment (191).

The effect of petroleum release is not directly related to the volume, but instead is a complex function of the rate of petroleum release, the nature of petroleum (its chemical components) and the local ecosystem exposed (134). The components of crude oil (saturated hydrocarbons, aromatic hydrocarbons, and resins and asphaltenes (175)), when released into the environment can adversely affect its health and the quality of its natural resources. Environments such as marshes, mudflats and subtidal areas are sensitive to contaminant impacts (129). Human populations are also at risk and are more likely to be exposed to transport fractions of petroleum products and additives (1). Groundwater contamination by petroleum products can affect public water supply, as groundwater aquifers are an important source of fresh water in the U.S.

1.1.3 Bioremediation of impacted environments

Bioremediation techniques exploit inherent capabilities of microorganisms to degrade (transform and/or mineralize) environmental contaminants. Although conventional methods of remediation, such as physical removal, are often the first response option, they rarely complete the clean-up of oil spills. Bioremediation has

emerged as a promising technology, particular as a secondary treatment option for oil cleanup. It has several potential advantages over conventional methods such as being cost effective, less intrusive to the contaminated site, and more environmentally benign in terms of its products (177). Among all the remediation technologies for treating impacted groundwater, bioremediation appears to be an economical and efficient treatment option (63).

1.2 Biodegradation of monoaromatic hydrocarbons and PAHs

Biodegradation of hydrocarbons has been the focus of many studies, as it is not only relevant for decontamination of impacted sites, but also for the oil weathering processes. Aerobic biodegradation of hydrocarbons has not been included in this review. Please refer to reviews (6, 7, 30, 107, 170, 183) for details.

Many hydrocarbons previously considered to be recalcitrant under anaerobic conditions are now known to undergo anaerobic biodegradation. These include the monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and xylenes (BTEX) and their alkyl substituents (propylbenzene, 1,3,4-trimethylbenzene), aromatic monoterpene (*p*-cymene), polycyclic aromatic hydrocarbons (PAHs) (naphthalene, phenanthrene, 2-methylnaphthalene, acenaphthene, biphenyl, fluorene, fluoranthene), *n*-alkanes (hexane, heptane, octane, dodecane, hexadecane), isoprenoid alkane (pristane, 2,6,10,14-tetramethylpentadecane), alkynes (acetylene) and several alkenes. Please refer to reviews (20, 32, 64, 123, 148, 172, 192) for details.

The following sections briefly cover pure cultures, enrichments, and activation mechanisms of BTEX compounds and some PAHs. (Information about anaerobic benzene degradation has been covered in more detail, as it is relevant to the experimental

chapters.) Anaerobic degradation of BTEX compounds is well documented. It is known that some PAHs such as naphthalene, phenanthrene, biphenyl, acenaphthene, and fluorene can be degraded anaerobically, but until now it is not yet clear if more condensed PAHs can support growth or are transformed through co-metabolism (64, 123).

1.2.1 Benzene

Understanding of the anaerobic pathways of benzene degradation is primarily based on a small number of enrichment cultures and a few recently isolated pure cultures.

Pure cultures

The only known bacterial isolates capable of anaerobic benzene degradation are *Dechloromonas* strains RCB and JJ (41), and *Azoarcus* DN11 and AN9 (97). These strains are reported to be capable of benzene mineralization with nitrate as an electron acceptor. *Dechloromonas* strain RCB is also capable of mineralizing benzene with nitrate, chlorate, perchlorate and oxygen as terminal electron acceptors. In addition, it can mineralize toluene and ethylbenzene with nitrate as an electron acceptor (34). Additional characterization of strain DN11 has shown that it is also capable of degrading toluene, *m*-xylene and benzoate, while *o*- and *p*-xylene were degraded in the presence of toluene. Strain DN11 cannot degrade phenol, which is proposed as one of the metabolic intermediates of anaerobic benzene degradation (28, 76, 180, 185). 16S rRNA gene specific qPCR analysis of DN11 in bioaugmented benzene contaminated groundwater microcosms showed that the abundance of strain DN11 decreased after benzene was degraded (96).

Enrichment cultures

Only a few stable enrichment cultures capable of benzene mineralization under anaerobic conditions are available. These include 1 methanogenic, 2 sulfate-reducing, 1 nitrate-reducing and 1 iron-reducing enrichment.

Methanogenic enrichment

This enrichment culture was established with soil and groundwater from an oil refinery in Oklahoma (181). Using phylogenetic analysis based on the 16S rRNA gene it was shown that the two dominant bacterial phylotypes in this enrichment grouped with *Desulfosporosinus* sp., or with *Desulfobacterium aniline*, and with the phylotype SB-21 (described in sulfate-reducing enrichment (A)). The *archaeal* phylotypes in this enrichment grouped with the acetoclastic methanogens or with methanogens capable of using H₂ and CO₂, and formate. qPCR analysis with 16S rRNA gene specific primers showed strong co-relation between gene copies of *Desulfobacterium* sp. clone OR-M2 and benzene degradation activity (48).

Sulfate-reducing enrichments

(A) This enrichment was established with sediment from a deep-sea hydrocarbon seep in Guaymas Basin, Mexico (used for experiments in this thesis). Phelps et al. (146) described this enrichment originally as having 12 different phylotypes, including γ , δ , and ϵ Proteobacteria, Cytophageles, low G+C content Gram-positives and a deeply rooted clone. Using stable isotope probing, we recently identified that the 16S rRNA gene phylotype SB-21 is the dominant member of this consortium *likely* to initiate anaerobic benzene degradation (140). It is a sulfate-reducing phylotype, which was first identified in this consortium in 1998 (146). Our recent study shows that this phylotype can derive

bulk of the carbon from [$^{13}\text{C}_6$]benzene within 8 days of amendment (140). (refer A1 and A2 in the appendix)

(B) This enrichment culture was established from a Mediterranean lagoon (133). It is dominated by oval-shaped cells, related to clade Deltaproteobacteria that includes sulfate-reducing bacteria capable of degradation of naphthalene and other aromatics, and is also related to 16S rRNA gene clones (93 % and 91% similarity to SB-21 and SB-30 respectively) from the sulfate-reducing benzene degrading consortium described in (A) earlier. This oval shaped phylotype accounts for 85 % of the cells by DAPI analysis and is considered as the ‘candidate species’ for anaerobic benzene degradation.

Nitrate-reducing enrichment

This enrichment was established with soil and groundwater from a gas station in Toronto (181). Based on 16S rRNA gene sequence analysis the dominant phylotype in this enrichment was closely related to *Sterolibacterium denitrificans*, and was phylogenetically similar to *Azoarcus* sp. and benzene degrading *Dechloromonas* sp. JJ.

Iron-reducing enrichment

This enrichment was established with sediment from a former coal-gasification plant in Gliwice, Poland. DNA stable isotope probing was used to identify *Clostridia* as primarily responsible for oxidation of benzene in this enrichment (103).

Communities associated with *in situ* benzene degradation

Characterization of communities associated with *in situ* benzene degradation and benzene degrading enrichments indicates the presence of diverse microorganisms (36, 85, 111, 187). Rooney-Varga et al. (160) observed elevated levels of microorganisms from

the family *Geobacteraceae* in a Fe(III)-reduction zone also associated with benzene degradation.

Pathways of anaerobic benzene degradation

Mineralization of benzene has now been documented in several studies, primarily based on enrichment cultures or microcosm experiments under nitrate-reducing (26, 34, 41, 97), sulfate-reducing (98, 113, 145), methanogenic (76, 98), and Fe(III)-reducing (3, 91, 98, 160) conditions and in undefined anaerobic conditions (54).

Kazumi et al. (98) demonstrated mineralization of benzene under diverse environmental conditions using ^{14}C label, while Phelps et al. (147) demonstrated loss of benzene in a BTEX mixture in sulfate-reducing enrichments established from the NY/NJ harbor.

The initial step in benzene activation is considered to be hydroxylation (28, 76, 180, 188), methylation (180) or carboxylation (28, 102, 149, 180) (which may be a series of steps). In this thesis the hydroxylation of benzene to phenol is referred to as the ‘phenol pathway’, while methylation of benzene to toluene is referred to as the ‘toluene pathway’ of anaerobic benzene degradation. The proposed pathways of benzene degradation are shown in Figure 1.1. These pathways were elucidated based on the detection of ^{13}C , ^{14}C or ^2H labeled intermediates after incubation with isotope-labeled substrates. Studies with isotope labeled substrates have been summarized in Table 1.1. To date, to our knowledge, such studies have been based only on enrichment cultures or microcosms. Benzoate (28, 102, 149, 180), phenol (28, 180), and toluene (180) have been detected as labeled intermediates, while only unlabeled cyclohexanone (76) has been detected.

Vogel and Grbic-Galic (185) suggested that water is the source of the OH group of phenol. They observed that when cultures were grown with unlabeled benzene and 9% ^{18}O -labeled water, 8% ^{18}O -labeled phenol was formed in a methanogenic enrichment culture. Chakarborty and Coates (33) suggested that free hydroxyl radical was the donor of OH group based on the study with a benzene degrading pure culture *Dechloromonas* strain RCB. They observed that benzene degradation was reduced to 0 %-30 % in the presence of a free radical scavenger, as compared to 55% degradation in controls. Chakarborty and Coates also observed sequential formation of phenol and benzoate when cultures were amended with unlabeled benzene and nitrate as the electron acceptor. Recent studies by Kunapuli et al. (102) have shown that phenol can be formed abiotically during sampling or sample analysis, when reduced media is exposed to air (102). Similar observations were made in our study. This indicates that phenol detected in previous studies of anaerobic benzene degradation may be an artifact.

Methylation of benzene to toluene has been observed in only one study so far (180). Coates et al. (42), proposed methylation of benzene to toluene as an activation mechanism because addition of toluene inhibited benzene degradation in cultures of *Dechloromonas* strain RCB, although no direct evidence was presented. Coates et al. also reported that benzene degradation was stimulated in the presence of vitamin B₁₂ and inhibited in presence of propyl iodide (an inhibitor of cobalamin mediated methylation reactions). Methylation of benzene has been observed in human bone marrow (42). S-adenosyl-methionine or methyl-tetrahydrofolate are considered to be the methyl group donors during the methylation of benzene ring, since such a reaction is energetically favorable (42).

The source of the carboxyl group of benzoate has also been investigated. Two separate studies based on enrichment cultures have noted contradictory results when ^{13}C -labeled bicarbonate was used along with unlabeled benzene. The carboxyl carbon of benzoate detected in these studies either had the ^{13}C -label (102) or was unlabeled (149). Phelps et al. (149) tested ^{13}C -labeled acetate as the carboxyl group donor in a sulfate-reducing benzene degrading enrichment with negative results. These results indicate that in some benzene degrading enrichment cultures the carboxyl carbon of benzoate formed from benzene is derived from bicarbonate, while in others it is not. Detection of universally labeled benzoate in [$^{13}\text{C}_6$]benzene amended cultures (28) indicates that the carboxyl carbon of benzoate originated from ^{13}C labeled benzene. As a consequence of these different and at times contradictory results, as yet no consensus has been reached on a universal mechanism for benzene activation.

1.2.2 Toluene

Of all the BTEX compounds toluene is the most easily degradable under anaerobic conditions (106, 147). Several pure cultures capable of degrading toluene anaerobically are now available. These include denitrifying *Thauera aromatica* strains T1 (60, 171) and K172 (2), *Thauera* sp. DNT-1 (168), *Azoarcus tolulyticus* strain Tol4 (201), and ToN1 (153), *Azoarcus* spp. (67), *Dechloromonas aromatica* strains RCB and JJ (34, 41), iron-reducing *Geobacter metallireducens* GS-15 (112, 114), and *Geobacter grbiciae* strains TACP-2T and TACP-5 (40), sulfate reducing *Desulfobacula toluolica* Tol2 (152), oXyS1 (82) and PRTOL1 (18). In addition, toluene degradation has also been seen under manganese reducing (105, 106), methanogenic, and fermentative conditions (53, 106). Toluene degradation has also been shown to occur with humic acids and the

model anthraquinone-2,6-disulfonate (AQDS) (31) as electron acceptors. Toluene can be assimilated as a carbon source by anoxygenic phototrophs (196).

Work by Evans et al. has greatly contributed to the progress made in understanding anaerobic toluene degradation. Evans et al. (61) documented mineralization of toluene under denitrifying conditions, and isolated denitrifying strain T1 (59, 60) (later named as *Thauera aromatica* strain T1), which produced metabolites benzylsuccinic acid and benzyl fumaric acid when grown with toluene (58). Formation of similar metabolites (2-methylbenzyl-succinic acid and 2-methylbenzyl fumaric acid) was observed during *o*-xylene degradation (58). Although earlier thought of as dead end products of toluene metabolism, work by Coschigano and Young (46), and Coschigano et al. (45) using mutants of strain T1 helped identify the regulatory mechanism of toluene degradation that leads to formation of fumarate addition product, benzylsuccinate, as a result of toluene activation.

So far only one mechanism of anaerobic toluene activation has been repeatedly observed in various pure cultures. This reaction involves the glycyl radical enzyme benzylsuccinate synthase that catalyzes the addition of fumarate to the methyl group of toluene to form benzylsuccinate (15, 16, 19, 44, 46, 84, 95, 109, 172), which subsequently leads to the formation of benzoyl-CoA, a central intermediate of anaerobic degradation of aromatic compounds (81).

1.2.3 Ethylbenzene

Four nitrate reducing pure cultures capable of ethylbenzene degradation have been isolated. Strain EbN1, PbN1 (153) (formerly *Thauera*, now *Azoarcus*) and *Azoarcus* strain EB1 (8), all can oxidize ethylbenzene, but have shown a limited ability to

oxidize other aromatic hydrocarbons under anaerobic conditions. *Dechloromonas aromatica* RCB (34) is capable of degrading ethylbenzene as well as toluene and benzene. Strain EbS7 is the only known sulfate-reducing pure culture and has limited substrate range (99). The degradation of ethylbenzene occurs through two different pathways. In the nitrate-reducing cultures (8, 151) the methylene group of the side chain undergoes a dehydrogenation reaction followed by hydroxylation to form 1-phenylethanol, which is subsequently converted to acetophenone, and then to the central intermediate of anaerobic degradation, benzoyl-CoA. The OH group for the initial hydroxylation reaction is derived from water (8), and the reaction is catalyzed by ethylbenzene dehydrogenase, a member of the dimethyl sulfoxide reductase family of molybdoprotein-containing enzymes. An alternative pathway for anaerobic ethylbenzene degradation was seen in sulfate-reducing strain EbS7. This pathway involves reactions similar to toluene degradation, i.e., fumarate addition to the methyl group forming 1-phenylethyl-succinate (99). For further details please refer to reviews (32, 64, 172).

1.2.4 Xylenes

Degradation of xylene isomers (*o*-, *m*- and *p*-xylene) has been studied under nitrate-reducing (61, 80, 101, 198), iron-reducing (22, 91), sulfate-reducing (55) and methanogenic conditions (53). No isolate able to mineralize *p*-xylene under anaerobic conditions is yet available. Several pure cultures capable of degrading *o*- and *m*-xylene (86, 153) with nitrate as an electron acceptor have been isolated. Sulfate reducing strains oXyS1 and mXyS1 can use *o*- and *m*-xylene, respectively as a sole source of carbon and energy (82), and are members of the *Desulfobacteriaceae* family. Strain oXyS1 is closely related to *Desulfobacterium cetonicum*, and *Desulfosarcina variabilis*, while

mXyS1 is distantly related to *Desulfococcus multivorans*. These isolates are also able to grow on toluene, ethyltoluene, isopropyltoluene and crude oil. Strain OX39, a sulfate reducer belongs to the genus *Desulfotomaculum* and can degrade both *o*- and *m*-xylene (130). In one of the first studies Evans et al. (58) identified metabolites of *o*-xylene degradation, 2-methylbenzyl-succinic acid and 2-methylbenzyl fumaric acid in denitrifying strain T1. Beller and Spormann (15) and Beller et al. (18) demonstrated that toluene degrading denitrifying strain T and sulfate-reducing strain PRTOL1 could transform xylene isomer(s) to isomer(s) of benzylsuccinate, indicating a fumarate addition reaction during degradation. Based on the detection of methylbenzylsuccinates in the culture supernatants, and as products of in vitro reactions of enzyme extracts with different substrates, the initial steps in degradation of *o*-, *m*- and *p*- xylene are considered to be fumarate addition reactions as in anaerobic toluene degradation pathways (17, 100, 130).

1.2.5 Naphthalene

Mineralization of naphthalene has been demonstrated under both nitrate- and sulfate-reducing conditions (9, 23, 39, 200), while degradation has also been reported under methanogenic conditions (35). Very few pure cultures degrading naphthalene have been isolated so far. Sulfate-reducing strains NaphS2 (69), NapS3 and NapS6 (132), are closely related to each other and belong to the *Desulfobacteriaceae* family of *Deltaproteobacteria*. These are also closely related to *m*-xylene degrading strain mXyS1 (82) and benzene degrading sulfate-reducing 16S rRNA gene clones SB-30 and SB-21 as well (146). *Pseudomonas*- and *Vibrio*- like strains are also reported to partially mineralize naphthalene under nitrate-reducing conditions (159).

Proposed pathways of anaerobic naphthalene degradation include different initial reactions as well as different downstream metabolic steps (figure 1.2). Zhang and Young (200) first proposed carboxylation as the initial step in activation of naphthalene (figure 1.2 C). They observed that the carboxyl group of 2-naphthoic acid (2-NA) detected in a sulfidogenic consortium grown with unlabeled naphthalene and ^{13}C -labeled bicarbonate, was ^{13}C -labeled. Zhang, et al. (199) proposed that subsequent to carboxylation, naphthalene undergoes sequential ring reduction steps, in which the unsubstituted ring of naphthalene is reduced first, followed by the substituted ring (Figure 1.2C). These conclusions were based on detection of ^2H -labeled 2-NA, 5,6,7,8-tetrahydro-2-NA, hexahydro-2-NA, octahydro-2-NA and decahydro-2-NA in sulfate-reducing enrichment cultures grown with deuterated naphthalene (199). Carboxylation of naphthalene, followed by ring reduction was also observed by Meckenstock et al. (124) and Annweiler et al. (5) in sulfate-reducing enrichment cultures when ^{13}C or ^2H label was used. Zhang et al. (199) suggested that water acts as the proton donor for ring reduction of naphthalene since ^2H from $^2\text{H}_2\text{O}$ was incorporated in 5,6,7,8-tetrahydro-2-NA detected in a sulfate-reducing enrichment cultures grown with unlabeled naphthalene.

Bedessem et al. (9) proposed hydroxylation of the naphthalene ring as an activation mechanism based on detection of naphthalenol in an enrichment culture under sulfate-reducing conditions (Figure 1.2A), grown with unlabeled naphthalene.

Safinowski and Meckenstock (162) detected deuterated naphthyl-2-methylsuccinic acid and deuterated naphthyl-2-methylene-succinic acid, in a sulfate-reducing enrichment culture grown with deuterated naphthalene. These metabolites were earlier identified as intermediates of anaerobic 2-methylnaphthalene degradation (4). Based on

identification of similar metabolites in naphthalene and 2-methylnaphthalene grown sulfidogenic enrichment cultures, Safinowski and Meckenstock (162) proposed that the initial reaction in naphthalene degradation is methylation to 2-methylnaphthalene (Figure 1.2B), which is further activated by a fumarate addition reaction (Figure 1.2D) mediated by benzylsuccinate synthase-like enzymes (162). They suggested that carboxyl group of 2-naphthoic acid, which is formed after initial carboxylation of naphthalene, may be converted to a methyl group by a CO-dehydrogenase enzyme associated with acetate oxidation.

Recent protein expression studies with sulfate-reducing naphthalene degrading pure cultures NapS2, NapS3 and NapS6 do not support the hypothesis of methylation of naphthalene to methylnaphthalene (132). Musat et al. observed that (132) benzylsuccinate synthase like proteins were produced only when sulfate-reducing pure cultures were grown with 2-methylnaphthalene, but not when grown with naphthalene. Benzylsuccinate synthase catalyzes toluene degradation in all known isolates (84, 192). Similar enzymes also catalyze the fumarate addition reaction to several aromatic substrates including *o*-, *m*- and *p*-xylenes, *o*-, *m*-, and *p*-cresols, ethylbenzene, 2-, 3- and 4-fluorotoluenes, 1-methyl-1-cyclohexane, *n*-hexane and hexadecane (17, 29, 79, 99, 184). Expression of such an enzyme in 2-methylnaphthalene grown cells along with its absence in naphthalene grown cells suggests that 2-methylnaphthalene, but not naphthalene undergoes fumarate addition reaction. Thus, naphthalene may not undergo methylation to 2-methylnaphthalene, as suggested by Safinowski and Meckenstock (162).

1.2.6 Phenanthrene

Although no pure cultures degrading phenanthrene under anaerobic conditions have been isolated so far, phenanthrene mineralization has been observed in enrichments under both sulfate- and nitrate-reducing conditions (39, 49, 200). Phenanthrene degradation has also been observed under methanogenic conditions (35) and in marine sediments under anaerobic conditions (174). Zhang and Young (200) suggested that the first step in phenanthrene degradation is carboxylation, since ^{13}C -labeled phenanthrene carboxylic acid was detected in sulfate-reducing enrichment cultures incubated with unlabeled phenanthrene and [^{13}C]bicarbonate. Similar observations were made recently using another sulfate-reducing enrichment by Davidova et al. (49). Denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene indicated that the enrichment had low diversity, yielding only two OTUs. Clone Phe4A was 91 % identical to *Desulfobacterium cetonicum*, while clone Phe4C was 99% identical to sulfate-reducing bacterium *Desulfococcus oleovorans* Hxd3, which has been described as an alkane degrader.

1.2.7 2-methylnaphthalene

Mineralization of 2-methylnaphthalene has been observed in sulfate-reducing sediments (43). Studies by Annweiler et al. (4, 5) using a sulfate-reducing enrichment culture demonstrated that 2-methylnaphthalene activation involves a fumarate addition reaction as in toluene degradation (Figure 1.2D). Succinyl adducts, naphthyl-2-methylsuccinic acid and naphthyl-2-methylene-succinic acid were detected in cultures amended with unlabeled 2-methylnaphthalene. Similar results were obtained by Safinowski et al. (161) with another sulfate-reducing enrichment culture named N47. The downstream metabolism of 2-methylnaphthalene is proposed to be identical to naphthalene degradation

involving ring reduction steps. In the ring reduction pathway of 2-methylnaphthalene and naphthalene, 2-naphthoic acid is considered to be a common intermediate (5). Sullivan et al. (173) proposed that an alternative pathway of 2-methylnaphthalene degradation exists, which involves carboxylation of 2-methylnaphthalene on the unmethylated ring followed by sequential ring reductions (Figure 1.2E).

1.3 Biomarkers of anaerobic hydrocarbon degradation

Natural attenuation is the reduction in toxicity, mass and mobility of the contaminants at the impacted site, without human intervention, owing to both physical (dilution, sorption, precipitation) and biological processes (biodegradation) (166). To establish *in situ* remediation occurring at impacted sites through natural attenuation, three steps are suggested by the U.S. Environmental Protection Agency (179): (i) groundwater chemistry data that demonstrate a clear and meaningful trend of decrease of contaminant mass and/or concentration over time; (ii) hydrogeologic and geochemical data that demonstrate indirectly the types of natural attenuation processes active at the site; and (iii) data that provide direct evidence for degradation taking place in actually contaminated site media (microcosms) or *in situ* degradation (aquifer). Assessment of *in situ* anaerobic biodegradation of hydrocarbons at impacted sites can be achieved by (i) study of signature metabolites of hydrocarbons in the field samples (metabolic biomarkers), (ii) compound specific isotope analysis (CSIA) of the target compounds, and (iii) study of functional genes involved in anaerobic biodegradation of target compounds (molecular biomarkers). The following sections include a brief review of metabolic and molecular biomarkers used in different studies. Readers are referred to reviews (87, 125, 163) for CSIA.

1.3.1 Benzylsuccinate synthase gene as a molecular biomarker

Benzylsuccinate synthase (Bss) is a member of the glycyl radical family of enzymes (167). In anaerobic toluene degradation Bss catalyzes the activation of toluene by a fumarate addition reaction to the methyl group generating benzylsuccinate (84), which is then converted to benzoyl-CoA, the central intermediate of degradation of monoaromatic hydrocarbons. Bss is a heterohexamer with three different subunits, 94 KDa (α), 12 KDa (β), and 10 KDa (γ) (109). Exposure to oxygen inactivates the enzyme producing an α' fragment of 90 KDa. The genes coding for the three subunits and the activating enzyme form a single operon (109). *BssA* gene encodes for the alpha subunit of benzylsuccinate synthase. The gene product is 860 amino acids long (109).

Bss catalyzes toluene degradation in all known isolates (84, 192). Bss-like enzymes also catalyze the fumarate addition reaction to several other aromatic substrates including *o*-, *m*- and *p*-xylenes, *o*-, *m*-, and *p*-cresols, ethylbenzene, 2-, 3- and 4-fluorotoluenes, 1-methyl-1cyclohexane, *n*-hexane and hexadecane (17, 29, 79, 99, 184). Bss like enzymes are also considered to be active in 2-methylnaphthalene (4, 5) and naphthalene degradation (162). Since Bss-like enzymes catalyze hydrocarbon degradation in phylogenetically diverse microorganisms only under anaerobic conditions, the presence of *bssA* gene in anoxic environments can be used as a specific indicator for the presence of microorganisms that have the potential to degrade hydrocarbons under anaerobic conditions. A 16S rRNA gene based assay of bacteria capable of hydrocarbon degradation is not feasible, as the diversity of microorganisms associated with anaerobic degradation of hydrocarbons is vast (192). In contrast, the conserved enzyme, Bss, in the catabolic pathway can be used as a biomarker (194).

Beller et al. (13) first developed a real-time PCR or quantitative PCR (qPCR) assay for anaerobic toluene degrading bacteria in aquifer sediment, based on the abundance of *bssA* gene. The primers and probes for the Taq man® assay were based on *bssA* gene sequences from 4 denitrifying isolates. The assay was used to assess the effect of ethanol on BTEX degradation in microcosms established with aquifer sediment. A 100-1000 fold increase in *bssA* gene copies was seen in the microcosms with most rapid toluene loss. The results were confirmed with a slot blot analysis, cloning and sequencing of the putative *bssA* gene.

Winderl et al. (194) developed degenerate PCR primer sets for the *bssA* gene based on 7 toluene degrading isolates. These primers were able to amplify *bssA* gene from toluene degrading isolates other than denitrifiers. One of the primer sets when used to amplify genomic DNA samples from three distinct tar-oil contaminated sites, indicated that the *bssA* gene based communities in these sites were distinct. From one site, *bssA* genes closely related to *Geobacter* spp. were isolated, while at another sulfate-reduction dominated site, previously unidentified sequences were found. Subsequently a qPCR assay for a specific cluster of the *bssA* genes identified at these sites was used for the study of spatial distribution of *bssA* genes in the aquifer column (193). With high-resolution sampling, Winderl et al. identified a zone of high sulfidogenic activity beneath the BTEX plume. This zone was also associated with increased absolute and relative abundance of specific *bssA* gene copies indicating the presence of a highly specialized toluene degrading community beneath the plume core.

Recently Beller et al. (14) developed a qPCR primer set for *bssA* assay based on sequences from two sulfate-reducing isolates and a methanogenic toluene degrading

consortium. This *bssA* qPCR assay was applied along with identification of metabolic biomarkers and CSIA to assess natural attenuation of BToX in the subsurface (also see 1.3.3).

1.3.2 Metabolic intermediates of anaerobic hydrocarbon degradation as biomarkers

Metabolic intermediates of anaerobic degradation of target compounds can serve as indicators of *in situ* biodegradation if these fulfill a set of prerequisites (77): (i) unequivocal and unique biochemical relationship to the parent hydrocarbon, (ii) release from the microbial cells to the extracellular medium, (iii) no commercial or industrial production, or uses, and (iv) generation as intermediate of mineralization rather than a product of co-metabolism. Young and Phelps (195) suggested that a metabolic intermediate could be used as a biomarker if it is biodegradable rather than stable, as this ensures that the intermediate is formed during active biodegradation of the target compound, and is not a result of processes in the past. In addition, it was also suggested that the metabolic biomarker normally should be absent in unimpacted environments, and should be water soluble for ease of sampling.

Various metabolic intermediates of toluene, ethylbenzene, xylenes, naphthalene and 2-methylnaphthalene have been detected in groundwater at impacted sites. Table 1.2 summarizes metabolites of alkylbenzenes detected in subsurface environments in various studies. It was noted that detection of metabolic intermediates (of toluene) works well at higher substrate concentration, as they are produced in fractional amounts as compared to the substrates (122). Beller (11) evaluated various intermediates of alkylbenzenes as metabolic indicators based on their specificity, stability and production as intermediates as against dead end products. In this study the author concluded that benzylsuccinates or

E-phenyllitaconates were superior to benzoates as metabolic biomarkers, due to their specificity, lack of commercial use and uniqueness to anaerobic metabolism.

Very few studies have focused on identification of metabolic biomarkers of anaerobic naphthalene and 2-methyl naphthalene degradation in the subsurface (72, 144). Metabolites of PAHs that have been detected in subsurface environments are listed in table 1.3. At a maintenance yard impacted site, Phelps et al. (144) detected metabolites of anaerobic naphthalene degradation (2-NA, tetrahydro-2-NA, hexahydro-2-NA) along with methyl-naphthoic acid (intermediate of anaerobic 2-methylnaphthalene degradation) in the groundwater, indicating anaerobic metabolism of naphthalene and 2-methylnaphthalene in the subsurface environment. In addition, the concentration of 2-NA could be co-related to various zones of the impacted aquifer.

In a tar oil impacted site Ohlenbusch et al. (139) detected relatively high concentrations of 1-NA, 2-NA, and 2-MNA as compared to hydroxynaphthoic acids (the latter are metabolites of aerobic naphthalene degradation) in the groundwater. These results indicate that anaerobic conditions prevailed in the impacted subsurface and that, contaminants were undergoing anaerobic biodegradation.

Gieg et al. (72) detected methyl-NA, dimethyl-NA, 2-NA, 1,2,3,4-tetrahydro-2-NA, and 5,6,7,8-tetrahydro-2-NA in different petroleum impacted aquifers along with several alkylsuccinic acids (the latter are metabolites of anaerobic alkane degradation). Griebler et al. (77) used signature metabolite detection along with CSIA at a former gasworks site. In this study, metabolic intermediates of naphthalene and methylnaphthalene, such as 1-NA, 2-NA, 1,2,3,4-tetrahydro-2NA, 5,6,7,8-tetrahydro-2-

NA, and naphthyl-2-methyl-succinic acid were detected indicating *in situ* biodegradation in the impacted groundwater (also see 1.3.3)

Thus, the presence of multiple metabolites of anaerobic hydrocarbon degradation in impacted environments provides an indication that these sites have developed anoxic conditions, and that hydrocarbons present at these sites are undergoing *in situ* anaerobic biodegradation. Therefore, these metabolites can be used as indicators of natural attenuation of hydrocarbons under anaerobic conditions, and can be used for qualitative assessment of impacted sites and evaluating different remediation strategies.

1.3.3 Evaluation of multiple biomarkers of anaerobic hydrocarbon degradation

Multiple biomarkers of anaerobic hydrocarbon degradation can be used for independent assessment of impacted sites for evidence of *in situ* biodegradation.

Beller et al. (14) studied the effect of ethanol on degradation of BToX in the subsurface in a controlled release study. A combination of quantitative analysis of the *bssA* gene and detection of specific metabolites of alkylbenzenes was used along with CSIA. No single method was able to provide a definitive assessment of *in situ* biodegradation processes, but the result of all three biomarkers provided evidence of TX degradation in both treatments. Benzylsuccinates were detected exclusively, in the BToX with ethanol treatment, while carbon isotope fractionation was evident in the BToX only treatment. A 200-fold increase in the *bssA* gene copies was seen in BToX only treatment, but higher absolute *bssA* gene numbers were observed in BToX with ethanol treatment.

Griebler et al. (77) evaluated a former gas works impacted site in Germany. Metabolites of degradation of *o*- and *p*-xylene, ethylbenzene, 2-methylnaphthalene and naphthalene were detected at the site indicating anaerobic degradation of hydrocarbons *in*

situ. Selected BTEX compounds, along with 1-methylnaphthalene, 2-methylnaphthalene and naphthalene showed $^{13}\text{C}/^{12}\text{C}$ isotope fractionation, but correlation between isotope fractionation and concentration was not observed for all of the compounds (also see 1.3.2).

At an impacted subarctic aquifer site McLelvie et al. (122) observed carbon and hydrogen fractionation in selected BTEX compounds at both low and high concentration of substrates. In contrast, intermediates of alkylbenzenes could only be detected at high substrate concentrations.

These studies indicate that use of multiple biomarkers provides more reliable data as evidence for natural attenuation at contaminated sites.

1.4 Stable Isotope Probing: A Tool for Assessing Active Microbial communities

In this thesis various techniques of molecular biology were used for analysis of experimental samples, including routine laboratory methods like polymerase chain reaction, cloning and DNA sequencing, in addition to fingerprinting techniques like terminal restriction fragment length polymorphism analysis, and restriction fragment length polymorphism. A review of all these methods is beyond the scope of this chapter, and the discussion here is limited to DNA stable-isotope probing, a recently developed technique for analysis of active members in a microbial community.

Traditional methods of bacterial community analysis are biased due to selective isolation of culturable organisms, or have failed to link the culture independent detection methods with specific microbial function (75). Stable isotope probing (SIP) was developed to identify organisms involved in the metabolism of specific substrates, with minimal disturbance to the environmental samples (137). Stable isotope probing involves

labeling of cellular components, such as DNA, RNA, or phospholipid fatty acids, with a stable isotope of carbon (or nitrogen in case of nucleic acids) to elucidate the phylogenetic relationships between various organisms in a given environment and to derive functional information about the biota in it. Thus, SIP provides a link between function and identity of the uncultivated microorganisms (138). For a detailed reviews of SIP please refer to (51, 62, 66, 119, 135, 137, 138, 150, 155, 189, 190). Stable isotope labeling of phospholipid fatty acids, DNA and RNA was first demonstrated by Boschker et al. (21), Radajewski et al. (154) and Manefield et al. (120), respectively. Recently protein-SIP was also used to identify labeled proteins using specific substrates in laboratory microbial cultures (92, 93).

1.4.1 Density gradient centrifugation

DNA- and RNA-SIP primarily involve amendment of ^{13}C or ^{15}N labeled substrate to an environmental sample from which subsamples are taken over a predefined time interval to extract the nucleic acids. The labeled DNA or RNA is separated from the unlabeled fraction by using cesium chloride (154) or cesium trifluoroacetate (120) density gradient centrifugation, respectively. Density gradient centrifugation forms the basis of DNA and RNA based SIP. It was developed as a method to study the physical characteristics of macromolecules (127), and was later applied to study DNA replication in *E.coli* (126). Variations of the original method of DNA separation using CsCl gradients and recovery of DNA from the gradients have been discussed by Neufeld et al. (137).

It is estimated that 20 % incorporation of ^{13}C -labeled substrate into the DNA (154) or RNA (121) will be sufficient to resolve the labeled nucleic acids from unlabeled

ones, and maintain a confidence, a link between identity of and function of the active microorganism. It is also suggested that isotope fractionation will not have a confounding effect on RNA-based stable isotope probing studies (121). Technical considerations of ^{15}N DNA-SIP and RNA-SIP have been discussed by Cadisch et al. (27, 121).

1.4.2 Analysis of the samples

Separate DNA/RNA fractions can be characterized by measuring the buoyant density of each fraction and the heavy isotope content of the labeled nucleic acids in the fractions can be quantified by isotope ratio mass spectrometry (IRMS) analysis (120). Purity of the ^{13}C labeled fraction can be increased by a second round of density centrifugation (121). In addition to these analyses, it is important to show that a particular RNA (or DNA) species is enriched in the high-density fractions, without a concomitant increase in the low-density fractions (121).

The separate nucleic acid fractions in a SIP study can be characterized by various fingerprinting methods such as terminal-restriction fragment length polymorphism (TRFLP), or DGGE, along with cloning and sequencing of the genes of interest, often 16S rRNA genes. In addition, quantitative measurements of total nucleic acids and the specific template, e.g., 16S rRNA gene or 16S rRNA, in the samples can be done by using fluorometry and quantitative PCR analysis, respectively (116). The molecular characterization of the nucleic acids is primarily of interest as it can be used to identify microorganisms active under diverse environmental conditions, and results of such analysis can also be used for guiding isolation attempts (94, 97). On the other hand metagenomic analysis of SIP samples provides access to the entire genome of active

bacteria and can also be used for increasing the frequency of detection of genes of interest (165).

1.4.3 Applications of SIP

Microbial ecologists have applied stable isotope probing to address a diverse array of questions about the environment, which have previously been difficult to tackle. These include identifying microorganisms that can (i) assimilate simple carbon substrates, (bicarbonate (65), methane (21, 110, 131, 154), methanol (73, 118, 136, 141, 156), acetate (21, 38, 74, 88, 141, 164), propionate (37, 117)); (ii) degrade toxic compounds (benzene (92, 97, 103, 111, 140), pyrene (169), phenol (50, 143), naphthalene (94, 143), 2, 4, dichlorophenoxyacetic acid (47)) or (iii) degrade complex substrates such as cellulose (56). SIP studies have also elucidated the flow of carbon compounds through the soil microbial community (50), and trophic interactions between microorganisms in an ecosystem (118, 138), including study of micropredators in soil microbial food webs (115). SIP also has been applied to study microorganisms relevant to wastewater treatment technologies, such as study of glycogen accumulating organisms during enhanced phosphorus removal (128), and palmitate degrading microorganisms in methanogenic sludges (83). SIP can also be used as a substrate independent approach to assess the effects of environmental factors such as moisture content on growth of microorganisms (by using ^{18}O -labeled H_2O) (138), as well as to study unique environmental processes such as nitrogen fixation by free-living diazotrophs (by using $^{15}\text{N}_2$) (24). Along with laboratory based SIP studies, field based stable isotope assays have also been developed to reduce the artifacts caused by physical and nutritional disturbances of environmental samples during laboratory incubations (143).

SIP can also be used to study the plant microbe interactions in the rhizosphere. The labeling of bacterial nucleic acids in the rhizosphere might be inefficient due to the dilution of the label when it is transferred from the plants to the microbial community and can make it difficult to track the flow of ^{13}C label. (78). This can set lower limits on density of rhizosphere bacteria that can be detected by SIP (157).

1.4.4 DNA vs RNA-SIP

DNA-SIP is unique as it provides the ability to isolate the entire genome of microorganisms involved in the metabolism of substrates, thus providing an advantage over other methods such as fluorescent *in situ* hybridization-microautoradiography (FISH-MAR) and phospholipid fatty acid analysis (PLFA) (108, 142), that attempt to link metabolic activity with taxonomic identity (154). SIP also provides access to genes involved in the specific functions, which allows the use of several molecular biology techniques for identification of new metabolic pathways (154). However, DNA-SIP requires that the organism are actively growing and replicating *in situ* during the pulse of heavy isotope, which may not always be the case (121). 16S rRNA analysis can be used alternatively as the 16S rRNA molecule has a higher copy number, a higher turnover rate and is produced independently of replication as compared to DNA (121). Thus, RNA-SIP may be more sensitive than DNA-SIP, as it should be possible to reduce the amount of substrate or the length of incubation that is required to obtain a link between the metabolic function and taxonomic identity.

1.4.5 Considerations for DNA- and RNA-SIP experiments

Both DNA and RNA-SIP experiments are dependent on the availability of the labeled substrate (preferably uniformly labeled), or the ability to synthesize it,

satisfactory labeling of the nucleic acids with the heavy isotope, and efficient separation of the labeled and unlabeled nucleic acids, as well as the sensitivity of the separation method and the molecular methods used for analysis of such samples. The time frame chosen for the sampling, as well as the substrate concentrations are crucial for the results of the experiments to be relevant to the environmental conditions. Use of higher-weight isotopes may also retard the growth of microorganisms (197) and affect the distribution and quantity of cellular components (182).

Long incubation with labeled substrates can lead to cross-feeding of the microorganism, due to the uptake of labeled intermediates of degradation (156). Gallagher et al. (68) suggested use of carrier DNA to decrease the incubation time needed to get detectable target DNA from samples for SIP studies. Carrier DNA can be used to visualize bands in CsCl density gradients using low biomass of sample DNA. The transfer of the ^{13}C or ^{15}N intermediates between different microorganisms, on the other hand, can be made use of to study the flow of carbon or nitrogen in the environment (138).

High G + C content of organisms can generate confounding results, as it can interfere in the separation of labeled and unlabeled nucleic acids in density gradients. Addition of bis-benzimide to the density gradients in such a case can be used to overcome the effect of natural variation in the buoyant density of DNA due to high G+C content (25).

1.4.6 Advances in stable isotope analysis

Since the first use of stable isotopes in PLFA analysis and subsequently in DNA and RNA analysis, the field of stable isotope probing has acquired new techniques with

improved sensitivity. SIP has been coupled with fluorescent *in situ* hybridization and microautoradiography (FISH-MAR) to identify the likely critical players in an environment before identifying the active population based on an individual cell analysis (73, 74). SIP coupled with single cell Raman-FISH microscopy was recently applied to identify aerobic naphthalene-degrading microorganisms in a groundwater aquifer (89). This technique allows single cell structure–function analysis, and can be used at only 10% incorporation of ^{13}C label (90). Nano-secondary ion mass spectrometry (nanoSIMS) can analyze stable or radioisotope content of single cells at resolutions higher than Raman microscopy. It exceeds the sensitivity of microautoradiography, and can detect stable isotopes with a precision of 1% (104). Protein-SIP, on the other hand, can detect ^{13}C incorporation as low as $\pm 1\text{-}2\%$ and it can be used as a combined indicator of specific metabolic activity as well as obtaining phylogenetic information (93).

1.5 Conclusions

To develop better applications based on microbial activities to remediate subsurface sites contaminated due to anthropogenic activities, it is first necessary to understand the fundamentals of anaerobic degradation of different compounds. Work presented in this thesis is a combination of projects –some aimed at answering basic questions using aerobic laboratory cultures, while others demonstrate the application of developments made through laboratory studies for environmental site clean up. Techniques spanning different disciplines were applied to identify active microorganism, their metabolic processes, and genetic make up, for both laboratory cultures and field samples.

Table 1.1 Summary of isotope labeling studies of metabolites of anaerobic benzene degradation ^a									
Reference	Vogel, (185)	Grbic-Galic (76)	Weiner (188)	Caldwell (28)		Phelps (149)	Ulrich (180)		Kunapuli (102)
Inoculum	consortium	enrichment	Sediment	enrichment w/sediment		enrichment	enrichment		enrichment
Label used	[¹⁸ O]H ₂ O	[¹⁴ C ₆]benzene	[¹⁴ C ₆]benzene	[¹³ C ₆]benzene		[² D ₆]benzene	[¹³ C ₆]benzene		[¹³ C ₆]benzene
e- acceptor	CO ₂	CO ₂	CO ₂	SO ₄ ²⁻	CO ₂ /Fe(III)	SO ₄ ²⁻	NO ₃ ⁻	CO ₂	Fe(III)
Controls	Without [¹⁸ O]H ₂ O	sterile and media controls	NA	sterile controls		sterile controls	media controls		sterile and media controls
Phenol	+	+	+	+	+	-	-	+	in cultures and controls
4-HB	NA	NA	NA	-	-	NA	-	-	NA
Toluene	NA	NA	NA	NA	NA	NA	ring	ring	NA
Benzoate	NA	NA	NA	UL	unlabeled	+	ring	ring	UL and ring
Benzylsuccinate	NA	NA	NA	NA		NA	-	-	NA
Cyclohexanone	NA	^b	NA	-	-	NA	NA	NA	NA

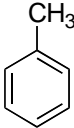
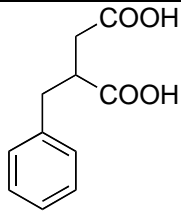
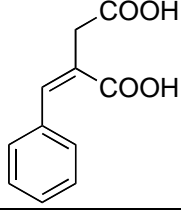
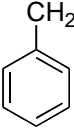
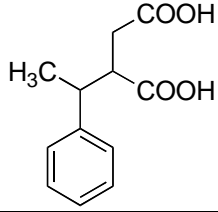
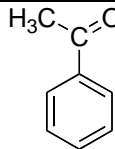
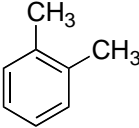
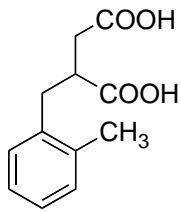
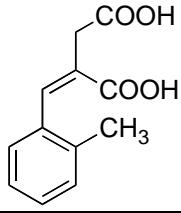
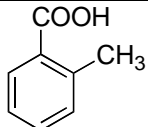
^a Only studies documenting detection of metabolites from labeled substrate are included in the table

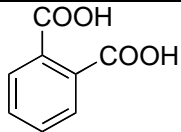
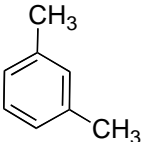
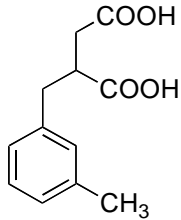
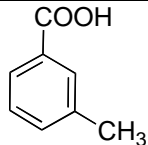
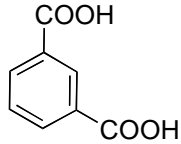
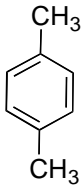
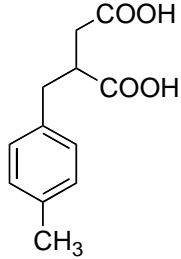
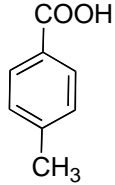
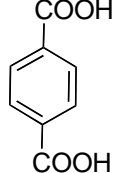
^b cyclohexanone detected in this study was not labeled

4-HB: 4-hydroxybenzoate

UL: universally labeled, ring: ring labeled

Table 1.2. Metabolic intermediates of anaerobic alkylbenzene degradation that have been detected at hydrocarbon impacted sites (10, 12, 14, 57, 71, 72, 77, 158, 195)

Substrate	Intermediate	
Toluene 	benzylsuccinic acid	
	phenylitaconic acid	
Ethylbenzene 	1-phenylethylsuccinic acid ^a	
	acetophenone ^{b,c}	
<i>o</i> -xylene 	2-methylbenzylsuccinic acid	
	2-methylphenylitaconic acid	
	<i>o</i> -toluic acid ^e	

	phthalic acid	
<i>m</i> -xylene 	3-methylbenzylsuccinic acid	
	<i>m</i> -toluic acid ^e	
	isophthalic acid	
<i>p</i> -xylene 	4-methylbenzylsuccinic acid	
	<i>p</i> -toluic acid	
	terephthalic acid	

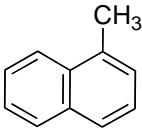
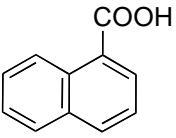
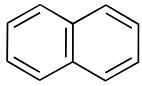
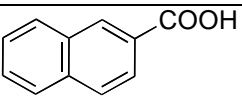
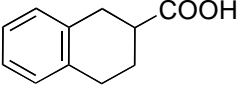
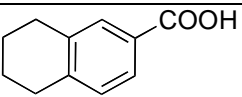
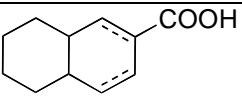
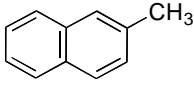
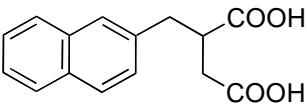
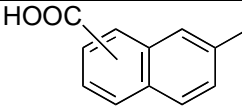
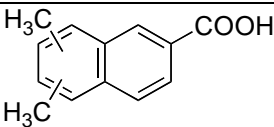
a: product of fumarate addition of ethylbenzene

b: product of hydroxylation of ethylbenzene

c: used in industries

e: can be produced aerobically (70)

Table 1.3. PAHs and their metabolic intermediates detected in subsurface environments
(77, 122, 144, 195)

Substrate	Metabolic intermediates	
1-methylnaphthalene 	1-naphthoic acid ^a	
naphthalene or 2-methylnaphthalene 	2-naphthoic acid ^b	
	1,2,3,4-tetrahydro-2-naphthoic acid	
	5,6,7,8-tetrahydro-2-naphthoic acid	
	hexahydro-2-naphthoic acid	
2-methylnaphthalene 	naphthyl-2-methylsuccinic acid ^c	
	methylnaphthoic acid ^c	
Substrate not verified in lab studies	dimethylnaphthoic acid	

a: putative metabolite of 1-methylnaphthalene (77)

b: can be produced aerobically (52)

c: is also proposed as a metabolite of naphthalene(162)

Figure 1.1. Proposed pathways of anaerobic benzene degradation (Adapted from (64)).

A) hydroxylation to phenol (28, 76, 180, 188), B) methylation to toluene (28, 102, 149, 180), and C) carboxylation to benzoate (28, 102, 149, 180). Square brackets indicate transient intermediate.

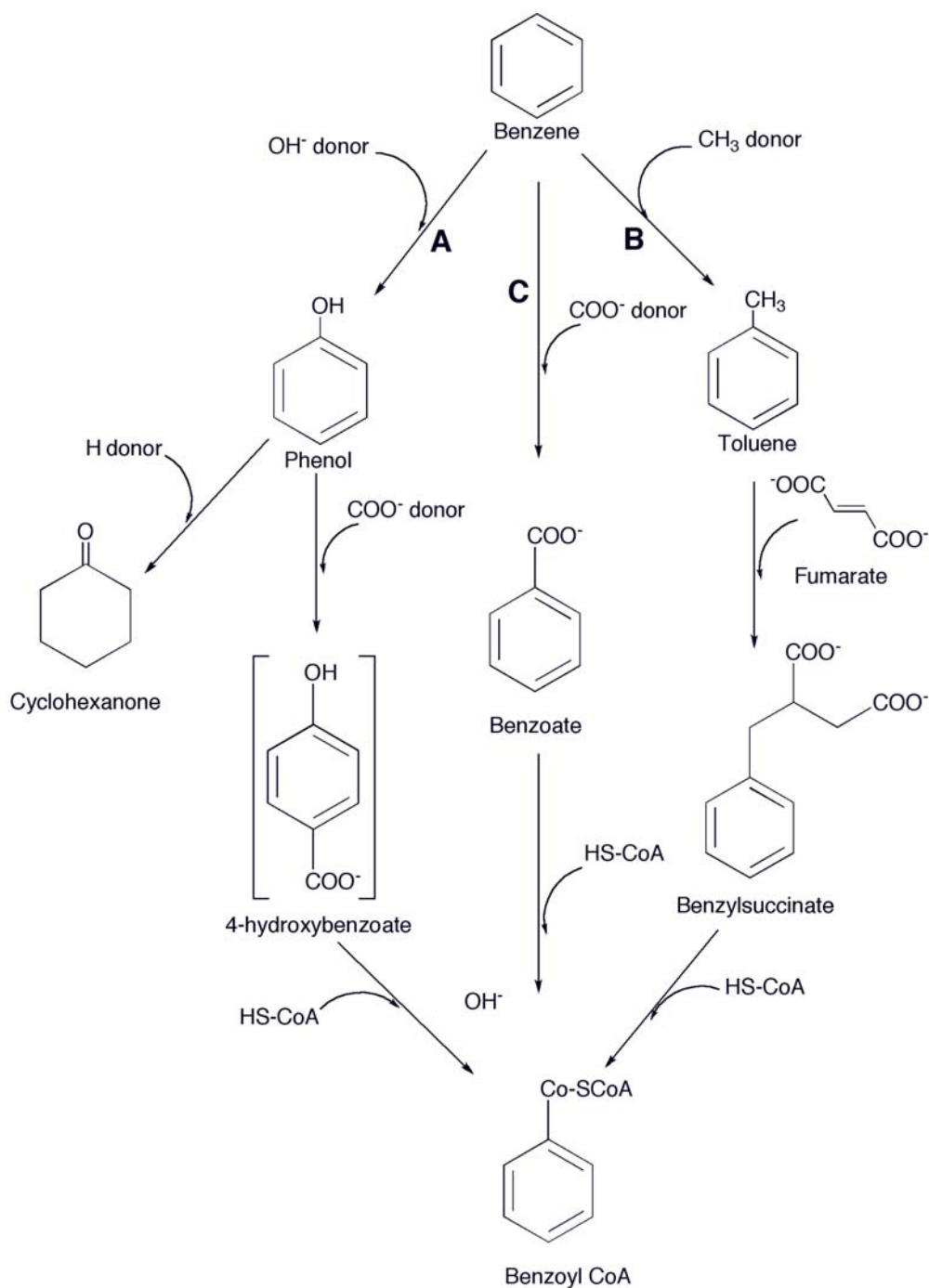
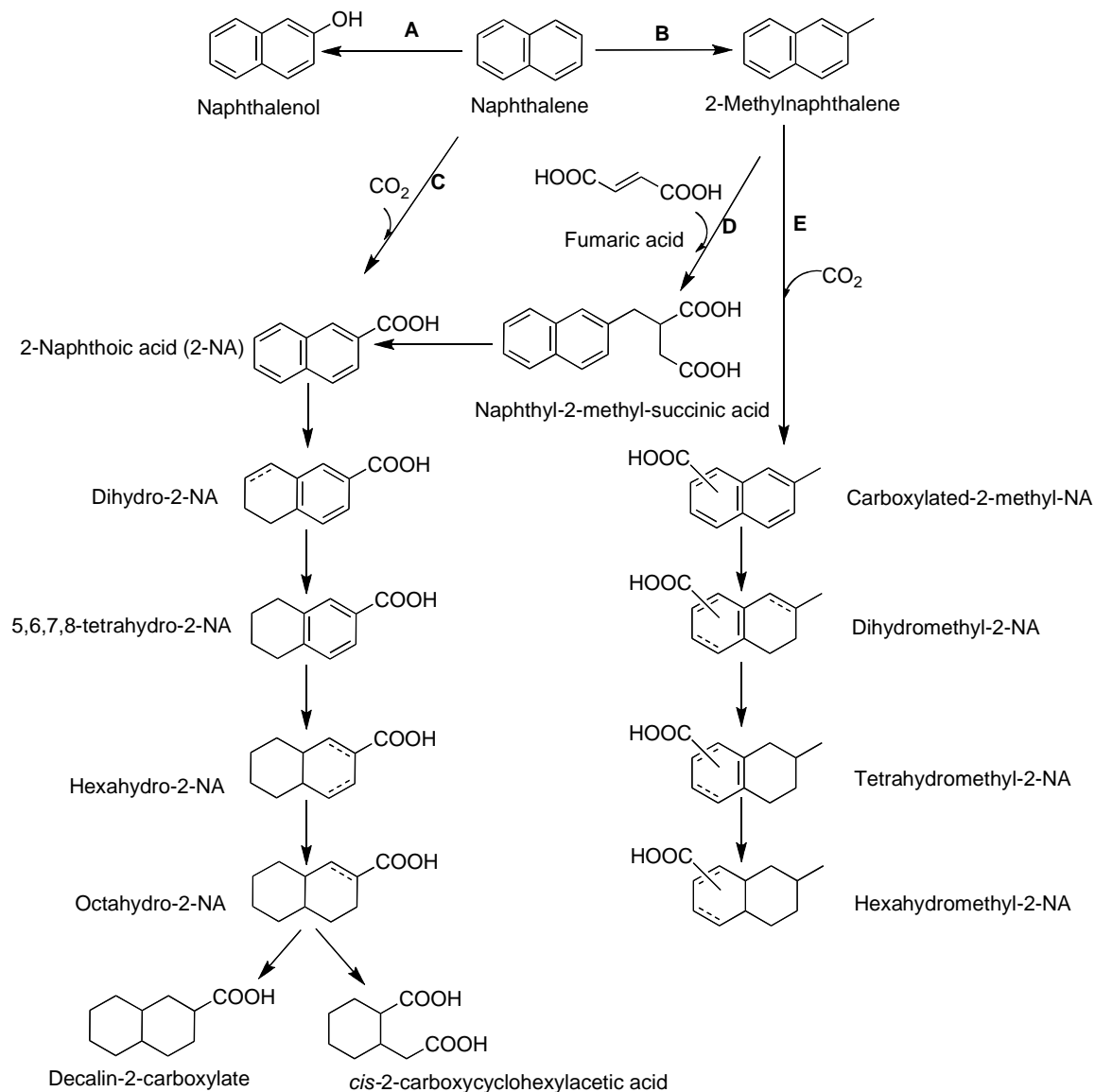


Figure 1.2. Proposed pathways of naphthalene and 2-methylnaphthalene degradation. A) Hydroxylation of naphthalene to naphthalenol (9) B) Methylation of naphthalene to 2-methylnaphthalene (162), C) Carboxylation of naphthalene to 2-naphthoic acid followed by ring-reduction (199, 200), D) Fumarate addition of to 2-methylanaphthalene E) Carboxylation of 2-methyl naphthalene (173).



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CHAPTER 2

Identification of Critical Members in a Sulfidogenic Benzene-Degrading Consortium by DNA Stable Isotope Probing

ABSTRACT

Benzene is a known carcinogen, and one of the most prevalent organic contaminants in groundwater. Therefore, determining which specific microorganism(s) may be responsible for anaerobic benzene degradation would be valuable for optimizing bioremediation strategies. A previously described consortium that is able to mineralize benzene with sulfate as the terminal electron acceptor was used in this study and DNA-stable isotope probing was used to identify the member of this consortium that is dominant during the anaerobic benzene degradation processes. This consortium was originally established with sediment from the Guaymas Basin, Mexico. Subcultures of the consortium were amended with 134 μM uniformly labeled [^{12}C]- or [^{13}C]benzene and newly synthesized ^{13}C -labeled DNA was separated by cesium chloride density gradient centrifugation, using archaeal DNA as carrier. PCR amplification of 16S rRNA genes and terminal restriction fragment length polymorphism analysis indicated that a 270 bp peak incorporated the majority of the ^{13}C label during the incubation, ultimately accounting for 46-59%, of the area in the active community profile. Clonal library analysis was used to identify the 16S rRNA gene associated with the 270 bp terminal restriction fragment, which closely matched clone SB-21 (Genbank accession number: AF029045), previously described from the consortium. The results demonstrate the importance of SB-21 in benzene degradation by this consortium, as it can derive majority of the carbon from benzene during its degradation under sulfidogenic conditions.

2.1 INTRODUCTION

Benzene is a carcinogen and is one of the mono-aromatic compounds in gasoline (2003 average concentration was 0.94 vol% (32)) along with toluene, ethylbenzene, and xylenes, collectively known as BTEX. These compounds can potentially contaminate groundwater supplies. In 1986 an estimated 189,000 non-farm, underground, motor fuel storage tanks in the U.S. (35 % of the total) were judged to be leaking (34). In 2007 there were 474,127 confirmed releases from underground storage tanks (33). Since subsurface environments can rapidly turn anoxic after a contamination event, determining the microorganisms that can degrade benzene under anaerobic conditions would help to understand the process and to determine methods for subsurface bioremediation. Although anaerobic degradation of benzene has been studied extensively for the last two decades, there is a very limited understanding about the mechanisms of degradation and the organisms that are involved in this process. For reviews, refer to (6, 23). Earlier, *Dechloromonas* strains, RCB and JJ, were reported that could mineralize benzene with nitrate as an electron acceptor (5). Subsequently, another study employing RNA-based stable isotope probing (SIP) in combination with denaturing gradient gel electrophoresis (DGGE) has demonstrated that two strains of *Azoarcus*, DN11 and AN9, isolated from gasoline contaminated groundwater, can mineralize benzene under denitrifying conditions (12). To our knowledge, unlike these nitrate-reducing conditions, no pure culture capable of benzene degradation under iron- or sulfate-reducing conditions has been identified to date. The current understanding of anaerobic benzene degradation is thus based mostly on the study of enrichment cultures, with very little insight into the

roles that different microorganism execute in the mineralization of benzene under anaerobic conditions.

In this report, DNA-based SIP and terminal restriction fragment length polymorphism analysis (T-RFLP) was used on a sulfate-reducing microbial consortium capable of benzene degradation to distinguish the microorganisms active during the processes of benzene degradation. The sulfidogenic consortium was originally established in 1993 from a deep-water hydrocarbon seep in Guaymas Basin, Gulf of California, Mexico (21). The original enrichment was found to completely mineralize benzene with sulfate as the terminal electron acceptor (21), and benzoate was determined to be a metabolic intermediate of degradation (24). Prior molecular characterization of this original enrichment suggested that the enrichment was made of diverse phylotypes in the domain Bacteria. 16S rRNA gene sequence analysis showed that the clones were distributed among γ , δ , and ϵ Proteobacteria, Cytophagales, low G+C Gram-positives, and a deeply rooted clone was also found. Amongst the 12 bacterial phylotypes identified then, four were sulfate reducers (22). Even though the diversity and phylogentic relationship of the microorganisms in the enrichment was understood in the earlier study, the specific microorganism(s), crucial for benzene degradation remained unidentified. Additionally, all attempts to obtain a pure culture of a sulfate-reducing benzene degrader have been unsuccessful. For this SIP experiment, the hypothesis was that the microorganism critical for benzene degradation processes would also be the one to derive cellular carbon from the mono-aromatic hydrocarbon for DNA replication and growth. As first reported (18), our results indicate that a bacterium previously characterized by 16S rRNA gene sequencing, SB-21, (AF029045 (22)) was the first to

derive the bulk of the ^{13}C carbon from benzene for DNA synthesis. This microorganism is related to the family *Desulfobacteriaceae* (3). This identification of the microorganism playing a key role in anaerobic benzene degradation is an important step towards advancing our understanding of *in situ* bioremediation of mono-aromatics in contaminated environments.

2.2 MATERIALS AND METHODS

Experimental setup

To perform the SIP experiment, all cultures were grown in a modified Widdel and Pfennig marine media (37) at 30 °C, with 4.0 g/L of Na_2SO_4 . A master culture was grown on $[\text{}^{12}\text{C}_6]$ benzene as the sole carbon source and then it was starved for 21 days. Undiluted 24 mL culture was amended with $[\text{}^{12}\text{C}_6]$ benzene (Chromasolv HPLC 99% \geq , Sigma, St. Louis, MO), or $[\text{}^{13}\text{C}_6]$ benzene (99 atom % ^{13}C , Isotec, Maimisburg, OH), to a concentration of 134 μM . Five replicates with $[\text{}^{12}\text{C}_6]$ benzene, and 10 replicates with $[\text{}^{13}\text{C}_6]$ benzene were prepared. Samples were taken on day 0, 4, 8, 11 and 15 to determine benzene concentrations by GC-FID analysis and the rest of the biomass was used for DNA extraction.

GC-FID analysis

Samples were extracted in 0.5 mL of pentane with 0.1 mM fluorobenzene as internal standard. The extracts were analyzed by GC-FID (Hewlett Packard Series II, 5890) with a DB-5 column (30 m with 0.25 mm internal diameter (J&W Scientific, Folsom, CA) using the following program: injection temperature was 250°C, detector temperature was 300°C, initial temperature was held at 35°C for 5.00 min (7).

DNA extraction and CsCl density gradients

DNA was extracted using a modified phenol chloroform procedure (13, 27). These DNA extracts were analyzed on 1% agarose gels and were quantified by comparing the ethidium bromide fluorescence with that of a λ *Hind* III ladder. Sample DNA (150 ng) was combined with 10-20 μ g of ethidium bromide, ^{12}C - and ^{13}C -archaeal carrier DNA (60 ng and 300 ng, respectively, of *Halobacterium salinarum* DNA (11)), and 30 ng *Escherichia.coli* DNA as internal indicator (29). DNA was separated by CsCl density gradient centrifugation as described by Gallagher et al. (11), and Tierney (31). The separated [^{12}C]- and [^{13}C]DNA bands were dialyzed (11) and equal volumes of dialyzed samples were used as template for PCR analysis.

16S rRNA gene PCR and T-RFLP analysis

16S rRNA gene PCR and T-RFLP analysis using bacteria-specific primers 27F (AGAGTTTGATCMTGGCTCAG) with 6-carboxyfluorescein label at the 5' end, and 1100R (GGGTTGCGCTCGTTG) was performed on all SIP samples. T-RFLP analysis of SIP samples was performed by digesting equal volumes of PCR product with *Hae* III restriction enzyme as described by McGuinness et al. (16). T-RFLP fingerprinting was carried out on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA) using Genescan software. Terminal restriction fragments (TRFs) between 50-500 bp with ≥ 50 unit area count were used for analysis. 16S rRNA gene PCR of SIP samples was also performed using *E.coli* species-specific primers ECA75F and ECR619R (26, 29), to test for cross-contamination of separated DNA bands.

Characterization of the Master Culture

The 16S rRNA genes from the master culture were amplified using 27F and 1525R (AAGGAGGTGWTCCARCC) and cloned into pCR4-TOPO (Invitrogen,

Carlsbad, CA). Sequence-ready plasmid DNA was purified using the Flexi Prep Kit (Amersham Biosciences, Piscataway, NJ). TRFs of individual insert were verified by T-RFLP analysis and 16S rRNA genes were sequenced on an ABI 3100 Genetic Analyzer (Foster, CA). Unambiguously assembled 500 bp sequences with unique TRFs were aligned with those from the SILVA 95 database (<http://www.arb-silva.de>) and a phylogenetic tree was constructed by using ARB tools (15). A 16S rRNA gene community fingerprint (T-RFLP) was also prepared with the genomic DNA from the Master culture.

2.3 RESULTS

2.3.1 Loss of benzene from the cultures

GC- FID analysis of the SIP samples showed that nearly half, 47 and 57 % of [$^{12}\text{C}_6$]- and [$^{13}\text{C}_6$]benzene, respectively, was utilized by day 4 and almost all substrate (87 and 95 % of [$^{12}\text{C}_6$]- and [$^{13}\text{C}_6$]benzene, respectively) was utilized within 8 days (Table 2.1). These data confirm that benzene was degraded by the cultures in the time frame chosen for the SIP experiment.

2.3.2 DNA extraction and CsCl density gradients

Analyses of the genomic DNA extracts by agarose gel electrophoresis indicated that all samples contained sufficient mass of DNA to be used in CsCl density gradient centrifugation. ^{12}C - and ^{13}C -labeled DNA in the SIP samples was separated using CsCl density gradient centrifugation. After centrifugation for 20-24 hours, two distinct DNA bands were observed in each CsCl gradient under UV trans-illumination. Figure 2.1 shows separated DNA bands in the gradients. The ^{12}C -archaeal carrier DNA, the *E.coli* [^{12}C]DNA, and the bacterial [^{12}C]DNA formed a separate [^{12}C]DNA band, while the

[^{13}C]DNA band contains the ^{13}C -archaeal carrier DNA and any ^{13}C -labeled bacterial DNA from the consortium.

2.3.3 *E.coli* specific 16S rRNA gene PCR

To test for contamination of the [^{13}C]DNA band with any [^{12}C]DNA, *E. coli* species-specific 16S rRNA gene PCR was performed on equal volumes of the separated DNA bands after dialysis. Figure 2.2 (A) shows the results obtained for *E.coli* specific 16S rRNA gene PCR with day 4 samples. In this gel, lanes 1 and 3 demonstrate PCR products obtained from [^{12}C]DNA bands with [$^{12}\text{C}_6$]- and [$^{13}\text{C}_6$]benzene amendments, respectively. No amplicons were detected in lane 2 and 4, from PCR amplification of the [^{13}C]DNA bands when cultures were amended with [$^{12}\text{C}_6$]benzene or [$^{13}\text{C}_6$]benzene, respectively. Thus, *E. coli* PCR product was only observed in the [^{12}C]DNA bands of the gradients. This is an important control, demonstrating that [^{13}C]DNA bands were satisfactorily separated in the CsCl gradients, with ^{12}C cross-contamination below the PCR detection limit (11), and that differences seen in the T-RFLP profiles of separated DNA bands are a consequence of the anaerobic degradation of benzene.

2.3.4 Bacteria specific 16rRNA gene PCR

Bacterial 16S rRNA gene PCR was performed on all DNA bands separated in the CsCl gradients during the course of the incubation, using equal volumes of dialyzed bands. This was done to identify the [^{13}C]DNA bands that contained any bacterial DNA, and secondly to ensure that the [^{13}C]DNA band from [$^{12}\text{C}_6$]benzene amended cultures were not contaminated with [^{12}C]DNA. The latter was an additional quality control, in addition to the *E.coli* specific PCR performed earlier. As expected, PCR product was

always obtained with all the [^{12}C]DNA bands. In the [^{13}C]DNA bands, detectable PCR product was obtained only with day 8, 11 and 15 day samples with [$^{13}\text{C}_6$]benzene amendment. Figure 2.2 (B) shows the results obtained for 16S rRNA gene PCR on day 11 with bacteria-specific primers. In this gel, lanes 1 and 3 demonstrate PCR products obtained from [^{12}C]DNA bands with [$^{12}\text{C}_6$]- and [$^{13}\text{C}_6$]benzene amendments, respectively. No amplicons were detected in lane 2, from PCR amplification of the [^{13}C]DNA band when cultures were amended with [$^{12}\text{C}_6$]benzene. This control indicates that any cross-contamination was below the detection limit. Lane 4 demonstrates that PCR product from [^{13}C]DNA band is only obtained when cultures are amended with [$^{13}\text{C}_6$]benzene, indicating that newly synthesized ^{13}C [DNA] is detected.

2.3.5 16S rRNA gene T-RFLP analysis

After amplification, all 16S rRNA gene PCR amplicons were subjected to T-RFLP analysis as shown in Figure 2.3. The panel labeled “Master culture” shows all of the members of the consortium. ^{12}C -control is the T-RFLP of [^{13}C]DNA band with [$^{12}\text{C}_6$]benzene amendment. It has no detectable TRFs and acts as an important control in this analysis as described earlier. Panels ^{13}C Day 4-15 show T-RFLP of [^{13}C]DNA bands from cultures with [$^{13}\text{C}_6$]benzene amendment and represent the changes in the active members of this consortium. No TRFs were obtained from the day 4 sample, which indicates that the level of ^{13}C -labeled DNA in the sample is below our PCR detection limits. On days 8, 11 and 15, eight, ten and seven different TRFs were detected in the samples, respectively. TRFs detected in more than one sample include: TRF 108, 110, 131, 237, 270, 272 and 421 bp. Of these, the 270 bp TRF dominates the T-RFLP profile at all time points. It accounts for 59, 54, and 46 % of the total area of the T-RFLP profile

on day 8, 11 and 15 respectively, and its appearance and decline is coincident with the decrease in the benzene concentrations in the samples taken during the course of the experiment (Table 2.1). Our SIP experiment indicates that this bacterium was the first to derive the bulk of ^{13}C -carbon from benzene for DNA synthesis and *is likely* playing a critical role in anaerobic benzene degradation.

2.3.6 16S rRNA gene phylogenetic analysis

16S rRNA gene T-RFLP analysis of the community genomic DNA (Figure 2.3 Master culture) and that of the 16S rRNA gene clones along with DNA sequence analysis was used to establish the phylogenetic identity of SSU genes identified in this study (Figure 2.4).

Analysis of the 500 bp DNA sequences of unique 16S rRNA gene clones showed that TRF 270, identified as the crucial member of the benzene-degrading consortium was 99% similar to previously reported phylotype SB-21 (AF029045), and it is 89% similar to a *Desulfobacterium* sp. (AJ237601). SB-21 is also 90% similar to phylotype BznS295, dominant in a sulfate-reducing benzene degrading enrichment culture (17). Similarly, TRF 272 and TRF 205 were 99% identical to SB-9 and SB-29 DNA sequences that were identified in the original consortium in 1998 (22). Phylotype TRF 272 is 95% and 96% identical to *Desulfotignum* sp. 7120 (EF442994) and BznS334 (17), respectively. Phylotype TRF 205 is 95% similar to a deltaproteobacterial clone VHS-B3-49 from polluted river sediment and 91% identical to *Desulfosarcinia variabilis* (M34407). The 16S rRNA gene sequence represented by TRF 421, was not identified in this consortium's earlier characterization (22) and is 99% similar to *Desulfotignum balticum*

DSM 7044, BznS333 and other 3 clones from the same sulfidogenic benzene degrading enrichment as described earlier (17).

2.4 DISCUSSION

The consortium used in this study has continually demonstrated the ability to use benzene as the sole carbon source with sulfate as the terminal electron acceptor for more than a decade. Although this consortium had been characterized by using molecular tools, and the diversity and phylogenetic relationship of its members was already established, the functional role played by the individual members in benzene degradation has been difficult to discern to date. Similar difficulties in understanding the function of organisms involved in anaerobic benzene degradation (especially under iron-reducing, sulfate-reducing, and methanogenic conditions) have been experienced by other researchers who relied primarily on molecular characterization to elucidate the important microbes. For example, Rooney-Varga et al. (25) investigated the microbial community composition associated with benzene degradation under Fe(III) reducing conditions in a petroleum-contaminated aquifer. Using DGGE analysis of 16S rRNA gene sequences with Bacterial and *Geobacteraceae* specific primers, a significant enrichment of a *Geobacter* cluster was seen in benzene degrading sediments. Although these results were also consistent with the MPN-PCR and phospholipids fatty acid analysis, no definitive linkage to benzene degradation could be established. Likewise, Ulrich and Edwards (36) identified five Bacterial and four Archeal 16S rRNA gene sequences in a methanogenic benzene-degrading enrichment, that was previously enriched under sulfate-reducing conditions, and was derived from an oil refinery in Oklahoma. Notably, one of their 16S rRNA gene clones (OR-M2) was related to our benzene mineralizing consortium clone

SB-21 (Figure 2.4) ((22), and this study). In another study (4) using 16S rRNA gene sequence analysis and restriction fragment length polymorphism analysis, it was found that in a benzene degrading methanogenic consortium the microbial community was affiliated with phyla as diverse as *Bacteroidetes*, *Euryarchaeota*, *Firmicutes*, and *Thermotogae*. However, which microorganism was responsible for anaerobic benzene degradation remained elusive.

In contrast, SIP is now enabling researchers to elucidate the role played by various organisms in benzene degrading environments (12, 14). Our data from DNA-SIP experiments indicates that phylotype SB-21 (TRF 270) affiliated with the family *Desulfobacteriaceae* plays a key role in metabolism of benzene as a sole carbon source in this consortium. This is supported by the following observations. First, SB-21 has been maintained in this benzene-degrading consortium over more than 10 years of subculturing ((22) and this study). Secondly, increases in the relative peak intensity of TRF 270 bp corresponded with almost complete loss of benzene from the cultures (Table 2.1). Finally, TRF 270 bp is the most prominent peak in the [^{13}C]DNA T-RFLP profiles (Figure 2.3) indicating that it has incorporated bulk of the ^{13}C carbon from [$^{13}\text{C}_6$]benzene into the DNA.

As such, all microorganisms present in this benzene degrading enrichment could be classified using the following conceptual models: i) strictly dependent, i.e., syntrophy (1, 10, 14, 38), sequential degradation of metabolites (mutualism) (28), or fastidious organisms inter-dependent for growth factors or nutrients. ii) no strict dependence, but co-existence: (synergy) (9, 30), possibly feeding off extracellular metabolites of degradation (20) and iii) all microorganisms degrade benzene, but with different

efficiencies. Considering that not all the TRFs identified in the master culture were identified in the [^{13}C]DNA (Figure 2.3, Day 8-15), model iii (all organisms are benzene degraders) can be eliminated. Although we could eliminate some of the unlikely functional models described above, a more definitive identification of the relationship between different players in this consortium needs more extensive investigation. However, in separate experiments we have also tested benzoate, phenol or toluene (proposed metabolites of benzene degradation (2, 24, 35)), as sole carbon sources for degradation in this consortium (19) (please refer section 3.2 and 3.3). Although benzoate and phenol could be degraded, the rate of degradation was considerably lower and there was a lag in the onset of degradation, as compared to benzene-amended cultures. Toluene was not utilized. These results suggest that the labeling of DNA in this SIP experiment, within an 8-day period, is not due to feeding off of these metabolites during benzene degradation (mutualism). Even if model i) or ii) is applied to this consortium, it can be definitively concluded that phylotype represented by TRF 270 (SB-21) is crucial to the process of benzene degradation in this consortium, since it has incorporated the bulk of the carbon from labeled benzene into its DNA, and the change in its relative peak intensity corresponds with loss of benzene from the cultures.

Our findings that a member of family *Desulfobacteriaceae* plays a key role in benzene degradation is also supported by a recent study (17) in which a dominant phylotype (clone BznS295), in a benzene degrading marine sulfate-reducing enrichment culture was related to SB-21 and SB-30 (Figure 2.4). Similarly, research in a column bioaugmented with a methanogenic enrichment (8), showed a correlation between benzene degradation activity and a *Desulfobacterium* like clone (OR-M2) (Figure 2.4).

In conclusion, these collective results are evidence that SB-21 (TRF 270 bp) like organisms are actively involved in benzene degradation in diverse sulfate-reducing and possibly methanogenic environments. Bacteria similar to SB-21 have been identified as one of the dominant microbes in benzene degrading enrichments established from widely dispersed environments such as a Mediterranean lagoon, France (17), and an oil refinery, OK, USA (36). Furthermore, the abundance of these 16S rRNA gene sequences could also be linked to benzene degradation (8, 17). Thus, SB-21 potentially could serve as a biomarker for *in-situ* biodegradation of benzene in environments under sulfidogenic and methanogenic conditions.

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Table 2.1. Utilization of benzene over the course of SIP incubations^a

Day	Mean conc (μM) of [$^{12}\text{C}_6$]benzene	SD	Amt of day 0 benzene degraded (%)	Samples used for analysis	Mean conc (μM) of [$^{13}\text{C}_6$]benzene	SD	Amt of day 0 benzene degraded (%)	Samples used for analysis
0	134.8	21.2		5	121.5	16.1		10
4	71.7	11.0	47	4	52.2	20.8	57	8
8	16.9	2.6	87	3	6.2	13.8	95	6
11	ND	0	100	2	15.7	—	87	1
15	—	—		—	ND	—	100	1

^a Means and standard deviations (SD) of benzene concentrations shown in samples taken over a 15-day period from [$^{12}\text{C}_6$]benzene and [$^{13}\text{C}_6$]benzene cultures. No samples were taken for analysis after the benzene concentrations in the previous samples were below the detection limit. ND: not detected, —: not determined.

Figure 2.1. CsCl density gradients of SIP DNA extracts. Two distinct DNA bands were seen in the CsCl density gradients after 20 to 24 hours of centrifugation. Picture of gradients containing DNA from [$^{13}\text{C}_6$]benzene fed cultures: 1: Day four, 2: Day eight, 3: Day Eleven, 4: Day Fifteen after centrifugation. Each gradient contains approximately 150 ng of DNA sample, 30 ng of *E.coli*, 60 ng of ^{12}C -*H. salinarum* DNA and 300 ng of ^{13}C -*H. salinarum* DNA, 500 μl CsCl (1g mL^{-1}) and 10 μg of ethidium bromide.

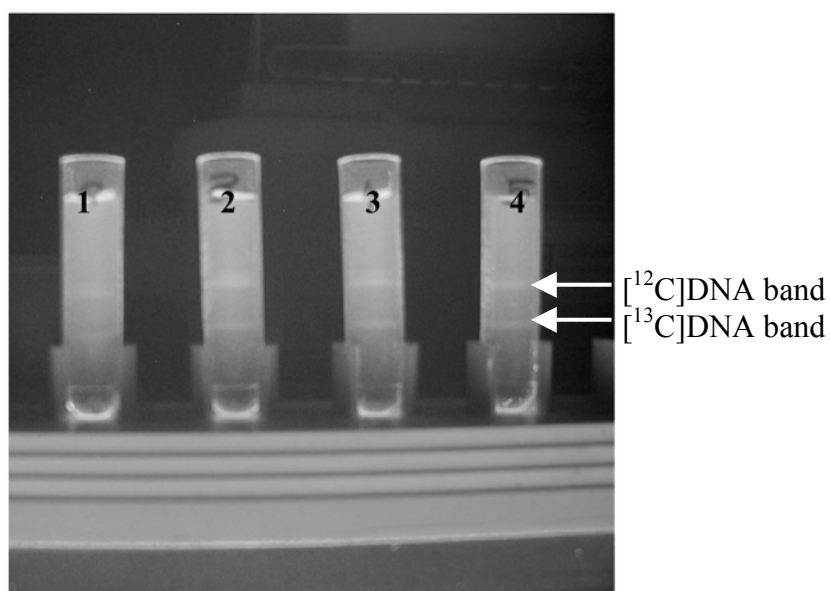
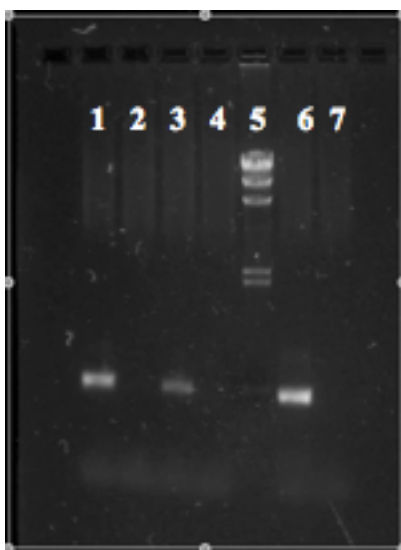


Figure 2.2. 16S rRNA gene PCR analysis with CsCl-separated DNA samples. Lanes represent different benzene amendments, as follows: 1: [^{12}C]DNA band, sample amended with [$^{12}\text{C}_6$]benzene; 2: [^{13}C]DNA band, samples amended with [$^{12}\text{C}_6$]benzene; 3: [^{12}C]DNA band, sample amended with [$^{13}\text{C}_6$]benzene; 4: [^{13}C]DNA band, sample amended with [$^{13}\text{C}_6$]benzene; 5: *Hind*III ladder; 6: positive PCR control; 7: negative PCR control.

A) *E.coli* specific PCR



B) *Bacteria* specific PCR

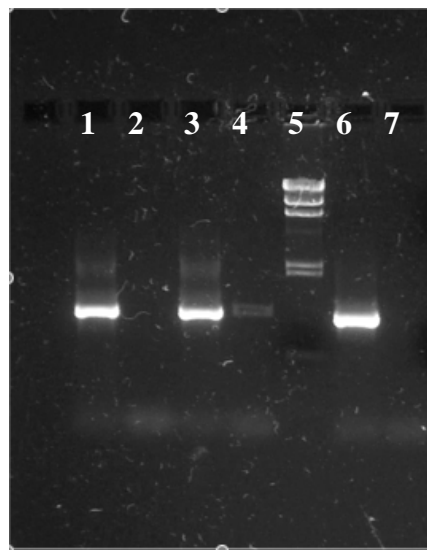


Figure 2.3. 16S rRNA gene T-RFLP analysis with HaeIII restriction digests. Master culture: T-RFLP profile of the culture used for setting up the experiment; ^{12}C control: T-RFLP profile of ^{13}C DNA with $^{12}\text{C}_6$ benzene amendment; ^{13}C day 4 to ^{13}C day 15: T-RFLP profile of ^{13}C DNA with $^{13}\text{C}_6$ benzene amendment. Axis x represents TRF size (values are bp), and y axis is relative peak intensity. Dominant TRFs have been identified with their sizes (bp).

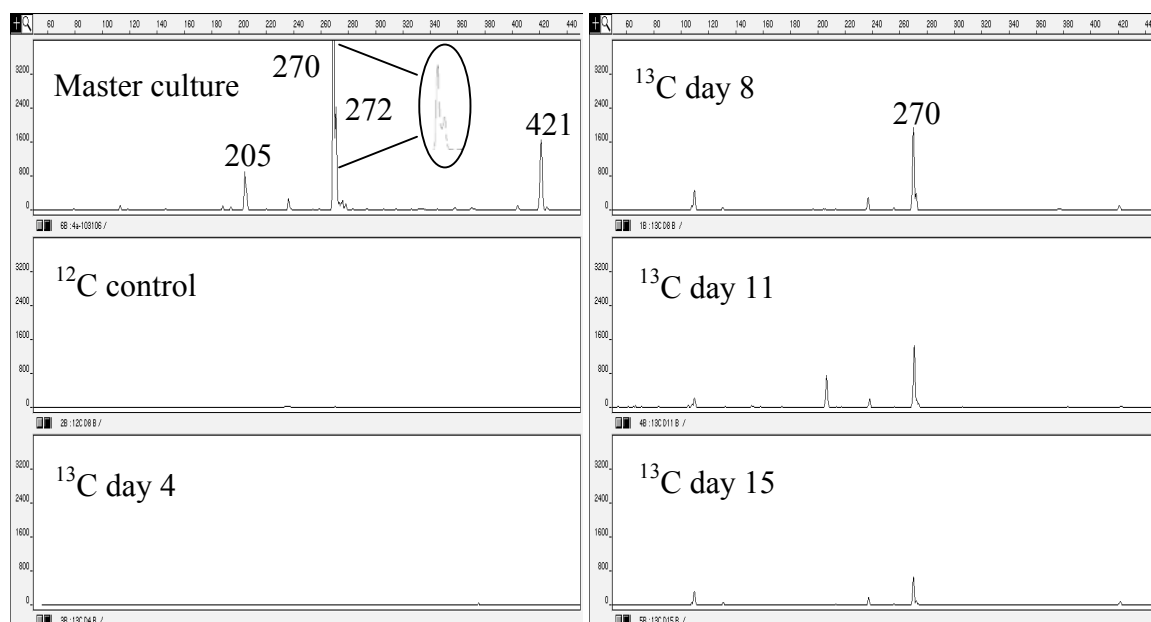
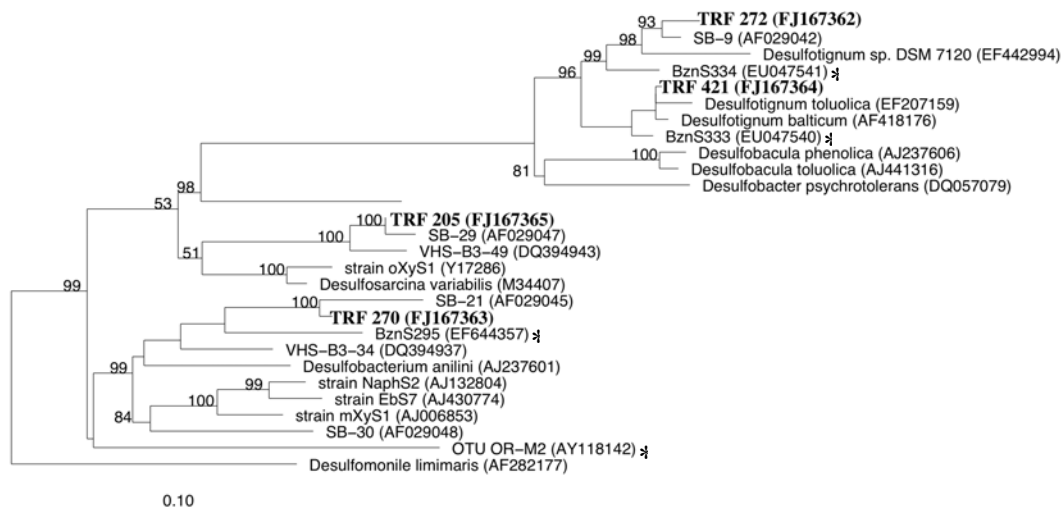


Figure 2.4. 16S rRNA gene based phylogenetic tree. Phylogenetic relationship between the clones identified in this study from the benzene degrading sulfate-reducing enrichment (boldface) and the members of Deltaproteobacteria is shown. The 16S rRNA gene tree was constructed using maximum likelihood analysis and 1,000 bootstrap iterations. Numbers indicate percentages of bootstrap values. Sequences represented by SB-9, SB-21, and SB-29 and SB-30 were identified in this culture's earlier characterization (22). Sequences from the study of other anaerobic benzene-degrading enrichments have been identified by an asterisk (*). GenBank accession numbers are shown in parentheses.



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CHAPTER 3

Anaerobic Benzene Degradation Under Sulfate-Reducing Conditions

ABSTRACT

The pathway of anaerobic benzene degradation has been proposed to include initial reactions such as hydroxylation (phenol pathway), methylation (toluene pathway), and carboxylation. A stable, benzene degrading sulfate-reducing consortium was examined in order to elucidate the metabolic pathways of benzene degradation. First, the proposed metabolites of anaerobic benzene degradation (phenol, benzoate, 4-hydroxybenzoate and toluene) were tested for their effect on benzene degradation, and also as sole carbon sources for degradation. Toluene was neither degraded nor did it inhibit benzene degradation suggesting that benzene degradation *via* toluene is unlikely in this consortium. Benzoate, phenol, and 4-hydroxybenzoate were all degraded, but only benzoate inhibited benzene degradation, indicating that benzoate is likely an intermediate of benzene degradation. Second, stable isotope labeling studies were used to identify downstream metabolites of degradation of benzene and phenol. Cultures and controls grown with [$^{13}\text{C}_6$]benzene or [$^{13}\text{C}_6$]phenol were extracted with solvents, derivatized and analyzed by GC-MS. Ten fold higher concentrations of universally labeled ([^{13}C -UL]) benzoate than ring labeled ([^{13}C -Ring]) benzoate were detected in the cultures amended with [$^{13}\text{C}_6$]benzene, while only trace quantities of [^{13}C -Ring]benzoate were detected in [$^{13}\text{C}_6$]phenol amended cultures. These data indicate that (i) Pathways for degradation of benzene and phenol exist in this consortium and both include a benzoate formation step (ii) The labeling pattern of benzoate derived from [$^{13}\text{C}_6$]benzene and [$^{13}\text{C}_6$]phenol amended cultures suggests that, in this consortium there are two different pathways of

benzoate formation. One pathway leads to the formation of [^{13}C -UL]benzoate, while the other pathway leads to formation of [^{13}C -Ring]benzoate. (iii) The pathway that leads to the formation of [^{13}C -UL]benzoate is dominant during benzene degradation. In this pathway one benzene ring is carboxylated by a carbon derived from another benzene ring. This pathway is different from the proposed phenol pathway of benzene degradation, as the labeling pattern of ^{13}C -labeled benzoate formed from [$^{13}\text{C}_6$]benzene or [$^{13}\text{C}_6$]phenol is not identical. In conclusion, anaerobic benzene degradation in this consortium likely proceeds via a novel pathway that activates one benzene ring through its reaction with the metabolic products of another benzene ring.

3.1 INTRODUCTION

Benzene is an important groundwater contaminant and is ranked 6th on the CERCLA priority list of hazardous substances (1) due to its carcinogenic properties. Remediation of impacted groundwater aquifers is necessary, as groundwater is an important source of drinking water in the U.S. An understanding of anaerobic degradation pathways of benzene can provide insight in evaluating different remediation strategies for contaminated sites, as these sites can rapidly turn anoxic after spills or leaks. Although it has been established that benzene can be anaerobically degraded under nitrate-reducing (5, 8, 15), sulfate-reducing (10, 16, 21, 24), methanogenic (14, 16) and Fe(III)-reducing conditions (16, 27), the understanding of the pathways of benzene degradation is very limited. This stems from the fact that only a few isolates degrading benzene anaerobically are available for study (8, 15). The dearth of pure cultures degrading benzene anaerobically, as compared to availability of pure cultures degrading other monoaromatics under anaerobic conditions, is partly due the stable nature of the benzene ring, and is partly due to the difficulties faced while growing anaerobes. The benzene ring lacks any functional groups, which can destabilize the resonating ring structure and facilitate activation during bacterial metabolism. Although nitrate-reducing benzene degrading pure cultures have been reported, the possibility that anaerobic benzene degradation may require a mixed culture under other electron accepting conditions cannot be ruled out. A possible syntrophic mechanism of benzene degradation in an iron-reducing culture has been postulated based on 16S rRNA gene based community characterization, though evidence to describe the mechanism of such a processes is lacking (18).

Studies using ^{13}C -, ^{14}C -, or ^2H -labeled benzene or ^{18}O -labeled water have been used to elucidate the pathways of anaerobic benzene degradation, and to our knowledge, to date such studies have been based only on enrichments or microcosms. Based on the identification of ^{13}C -, ^{14}C -, ^2H -, or ^{18}O -labeled metabolites in mixed cultures, three pathways of anaerobic benzene degradation have been proposed; A) hydroxylation to phenol (6, 14, 33, 35) (phenol pathway), B) methylation to toluene (33) (toluene pathway), or C) carboxylation to benzoate (which may be more than one step) (6, 17, 26, 33) (Figure 3.1), and as yet no consensus has been reached on a universal mechanism for benzene activation. Indeed there may not be a single universal mechanism, but several different pathways of anaerobic metabolism of benzene.

Although ^{13}C - or ^{14}C -labeled phenol has been detected in several studies (6, 14, 33-35), the mechanism of hydroxylation of benzene is not clearly understood. Free hydroxyl radical (7) or water is considered to be the potential hydroxyl group donor (34). Based on sequential formation of phenol and benzoate in cultures of nitrate-reducing *Dechloromonas* strain RCB amended with unlabeled benzene, Chakraborty and Coates (7) proposed hydroxylation as an initial step in benzene degradation. In this study 4-hydroxybenzoate was not detected in the cultures. In the same study, free hydroxyl radical was postulated as the hydroxyl group donor for conversion of the aromatic ring to phenol, as only 0 % to 30 % of benzene was degraded in the presence of free radical scavengers, as compared to 55 % degradation in the controls. An alternative mechanism of phenol hydroxylation has also been proposed earlier. Vogel and Grbic-Galic (34) used 9% ^{18}O -labeled water in a methanogenic, benzene degrading enrichment, and observed

that 8% of phenol had ^{18}O -label, suggesting that water was the source of oxygen for hydroxylation of benzene (34).

Methylation of benzene to toluene has been observed in only one study using nitrate-reducing and methanogenic enrichments (33), and the methyl group donor has also not yet been identified. Coates et al. (9) proposed methylation of benzene to toluene as an activation mechanism, since addition of toluene inhibited benzene degradation by *Dechloromonas* strain RCB, while addition of vitamin B₁₂ (a cobalt containing corrinoid generally involved in biomethylation) stimulated it, although no direct evidence was presented. Methylation of benzene to toluene has been observed in human bone marrow (9). S-adenosyl-methionine or methyl-tetrahydrofolate are considered to be the methyl group donors during methylation of benzene ring, since such a reaction is energetically favorable (9).

Benzene hydroxylation to phenol is proposed to proceed by ring reduction to cyclohexanone (14) (Figure 3.1), as observed in a methanogenic enrichment culture. Although benzene used in this study was ^{14}C -labeled, cyclohexanone detected in the cultures was unlabeled. No other studies based on benzene degrading cultures have noted production of labeled or unlabeled cyclohexanone. Lack of detection of labeled cyclohexanone in studies using labeled benzene, and moreover detection of unlabeled cyclohexanone negates the proposed pathway of benzene degradation by ring reduction to cyclohexanone (14). Phenol or toluene formed as a result of hydroxylation or methylation of benzene, respectively, can be metabolized via benzoyl CoA (4, 30) (Figure 3.1), a common intermediate of anaerobic degradation of monoaromatic hydrocarbons. Moreover, anaerobic metabolism of phenol in benzene degrading cultures

is considered to proceed via 4-hydroxybenzoate, to benzoate as has been observed in phenol degrading pure cultures (19, 20, 29).

The culture used for experiments in this study is a highly enriched, mixed culture that degrades benzene under sulfate-reducing conditions, and has been maintained on benzene as the sole carbon source, in our laboratory for 15 years. The sulfidogenic enrichment was originally established in 1993 using sediment obtained from a deep-water hydrocarbon seep from the Guaymas Basin, Gulf of California, Mexico (24). The enrichment was found to completely mineralize benzene with sulfate as the terminal electron acceptor (24). Prior molecular characterization of this original enrichment described it as having 12 dominant phylotypes in the domain Bacteria (25). Using stable isotope probing on this culture, we recently identified a member of this culture that is critical to anaerobic benzene degradation (23). Repeated attempts to isolate a pure culture from this enrichment have not been successful. In 2001, Phelps et al. (26) identified deuterated benzoate as an intermediate of deuterated benzene degradation in this enrichment culture. Phelps et al. (26) detected benzoate as the only metabolite of benzene. No other labeled or unlabeled metabolites were detected.

This enrichment is suitable for study of anaerobic metabolic pathways as it is a stable culture, free of sediment, it is well characterized in terms of its bacterial community, and it is dependent on sulfate-reduction for benzene mineralization. Therefore, this culture allows an interpretation of results that is not affected by the presence of extraneous carbon sources, electron donors, predatory microorganisms, or sporadic nature of benzene degradation as is observed in sediment cultures. In the present study, several lines of evidence were pursued to develop a more detailed

understanding of benzene degradation in this culture. These included i) use of proposed metabolites of benzene degradation as sole carbon sources, ii) use of proposed metabolites of benzene as feedback inhibitors of benzene degradation, and iii) comparison of metabolites of [$^{13}\text{C}_6$]benzene or [$^{13}\text{C}_6$]phenol, when used as sole carbon sources. We hypothesized that if benzene degradation in this mixed culture proceeds via the proposed phenol pathway (6, 14, 33), then the downstream metabolites produced as a result of [$^{13}\text{C}_6$]benzene or [$^{13}\text{C}_6$]phenol degradation would be identical.

Such a study, using different experimental methods to understand the same process, viz. benzene degradation, can provide more reliable data, as the results of one experiment can be validated by the outcome of another.

3.2 METHODS AND MATERIALS

Culture and culturing

Sediment free subcultures of a robust and stable benzene-degrading sulfate-reducing consortium established in 1993 from the Guaymas Basin (Gulf of California, Mexico) (24-26) were used as 20-25% inocula in all the experiments described here. Cultures were grown in a sulfate-reducing medium with vitamins and trace metal supplements, along with resazurin as a redox indicator. Please see section 2.2 for details. All sterile controls (killed cells) were prepared by autoclaving cultures on three consecutive days. All abiotic controls (media only) were prepared with sterile media. All cultures and samples were stored in serum bottles or vials with Teflon coated stoppers or septa respectively.

Analytical and extraction methods

HPLC analysis

For HPLC analysis 1mL of sample was filtered with syringe driven PVDF filters 0.45 μm (Millex-HV Japan) or Spin-X HPLC nylon filters, 0.2 μm , (Costar, Corning, NY). Samples were analyzed on HPLC (Beckman, Fullerton, CA) with 4.6 mm ID x 250 mm reversed phase Ultrapshere C-18 column (Beckman) and a UV detector (Beckman) at 270 nm. The mobile phase was 60:30:2 methanol: water: acetic acid, with 1mL/min flow rate, and the injection volume was 20 μL .

GC-FID analysis

Samples were extracted and analyzed as described earlier in section 2.2.

Solvent extraction and GC-MS analysis

For ethyl acetate extractions, 25-30 mL samples were alkalized to \sim pH 12 with 1N NaOH (2 mL) and incubated at room temperature for 30 min. Samples were then acidified to \sim pH 2 using 6N HCl (1mL) and incubated for another 30 min. Acidified samples were extracted with 10-15 mL of ethyl acetate by vigorous shaking for 90 sec. After the organic and aqueous phases had separated, the aqueous phase was carefully removed and the extraction was repeated. Traces of water in the ethyl acetate extracts were removed by running the extract through a column of anhydrous Na_2SO_4 . These samples were filtered using ashless Whatman filter paper # 41 (UK), and dried by rotary evaporation, under vacuum, by heating in a water bath at 50°C. Dried samples were resuspended in 4 mL ethyl acetate, by rinsing the round bottom flask with 2 mL ethyl acetate, two times, and the rinsate was dried under N_2 . The dried samples were derivatized using 0.1 mL of N,O-bis[trimethylsilyl] trifluoroacetamide (BSTFA) (Sigma, Saint Louis, MO), and heated in a water bath at 65°C for 10 min (7). Each sample was then analyzed by GC-MS.

For diethyl ether extractions, fifteen mL of samples were shaken with 5 mL of diethyl ether for 90 sec. The separated ether layer was collected and excess water was removed by running the samples through an anhydrous Na_2SO_4 column. Each sample was then analyzed by GC-MS (7).

GC-MS analysis

All samples were separated using an Agilent 6890N gas chromatograph with an HP-5MS column (30 m x 0.025 mm, I.D. 0.5 μm , Palo Alto, CA), fragmented using electron impaction, and the mass spectra were obtained by an Agilent 5973 mass selective detector. The injection volume was 3 μl unless otherwise mentioned. Samples were analyzed with a splitless mode and data from mass units 50-500 were scanned.

Diethyl ether extracts were analyzed using the following program. Initial temp: 30°C, for 5 min, ramp rate 4°C min⁻¹, oven temperature 80°C for 10 min, solvent delay 3.0 min. Ethyl acetate extracts were analyzed using the following program. Initial temp: 80°C for 5 min, ramp rate 4°C min⁻¹, oven temperature 230°C, solvent delay 10.0 min (7).

Experimental details

Degradation tests and rates of degradation of proposed metabolites

Three active cultures and 2 sterile controls, 36 mL each, were amended with 79 μM of toluene, 100 μM phenol, 100 μM of 4-hydroxybenzoate or 500 μM benzoate as the sole carbon source. Samples were taken over time for HPLC or GC-FID analysis of residual carbon source. Once the substrates that could be degraded were identified, another set of cultures and controls were set up to compare the rates of degradation of

different proposed metabolites, when equal concentrations of each substrate (50 μM) were used. Samples were taken for HPLC analysis of residual carbon source.

Inhibition of benzene degradation by its proposed metabolites

Three active cultures and 2 sterile controls, 36 mL each, were each amended with 52 μM toluene, 50 μM phenol, 50 μM 4-hydroxybenzoate or 50 μM benzoate along with 93 μM benzene. Benzene-only bottles were used as active controls. On day 0, 3, 7 and 10, one mL samples were taken from each replicate for GC-FID analysis of benzene.

Metabolites of [$^{13}\text{C}_6$]benzene

Replicate active cultures, sterile and abiotic controls, 100 mL each, were amended with 1 μL of [$^{13}\text{C}_6$]benzene (Sigma, Saint Louis, MO). Benzene concentration was monitored over time with GC-FID analysis, and all cultures as well as controls were re-amended with [$^{13}\text{C}_6$]benzene, when benzene concentration in active cultures was <20 μM . Samples were taken intermittently for solvent extraction and GC-MS analysis of the labeled metabolites.

Metabolites of [$^{13}\text{C}_6$]phenol

Replicate active cultures and sterile controls, 100 mL each, were amended with [$^{13}\text{C}_6$]phenol (Cambridge Isotope Laboratories Inc., Andover, MA), to a final concentration of ~ 11 μM . Phenol concentration was monitored by HPLC analysis. Active cultures and sterile controls were re-amended with [$^{13}\text{C}_6$]phenol when the residual concentration of phenol in active cultures was < 2 μM . Subsequently, these cultures and controls were split into smaller volumes (25 mL of each was mixed with 10 mL media) and were used for a time course experiment. Each 35 mL of culture and control was amended with 42 μM [$^{13}\text{C}_6$]phenol. On days 2, 5, 8, 10, and 14, one active and one

sterile control were sacrificed for solvent extraction. Three μl and 6 μl of concentrated samples were used for GC-MS analysis of labeled metabolites.

3.3 RESULTS

3.3.1 Degradation of proposed metabolites

Proposed metabolites of benzene degradation, benzoate, 4-hydroxybenzoate, phenol, and toluene, were tested as sole carbon sources for degradation. Benzoate, 4-hydroxybenzoate, and phenol, but not toluene could be degraded by the culture. Figure 3.2 shows change in concentration of the proposed metabolites with time, when equal concentrations of each metabolite were added separately to cultures and sterile controls (data for toluene is not included in this Figure). Although benzoate, 4-hydroxybenzoate and phenol, all could be degraded by the culture, the rates of degradation of these metabolites showed an apparent lag period of 10-20 days and the rates of degradation were always less than the rate of benzene degradation (compare Figure 3.2 with 3.3A). The relative rates of degradation were in the order; benzene > benzoate > 4-hydroxybenzoate > phenol. In cultures amended with 4-hydroxybenzoate, loss of the substrate was followed by formation of phenol (Figure 3.2), and subsequently degradation of phenol was observed (data not shown). The degradation rates of all substrates increased slightly when cultures were repeatedly re-amended (data not shown), and the lag, that was evident initially decreased on subsequent additions. These data suggest that the mixed culture was not initially well adapted for the degradation of any of the proposed metabolites of benzene degradation.

3.3.2 Inhibition experiment

Proposed metabolites of anaerobic benzene degradation (benzoate, 4-hydroxybenzoate, phenol and toluene) were also tested for their effect on benzene degradation. Each of these proposed metabolites were added separately to cultures and sterile controls along with benzene. Figure 3.3 shows loss of benzene in cultures and controls over time in the presence of the proposed metabolites. Loss of benzene in cultures when no additional substrate was added (Figure 3.3A) was used as an active control for the inhibition experiment. Figure 3.3(B) shows loss of benzene in 4-hydroxybenzoate amended cultures and controls. Although an equal concentrations of benzene were added to all active cultures, day zero concentration of benzene in these active cultures was about half that of the sterile controls. Loss of benzene continued without any lag, indicating that 4-hydroxybenzoate did not inhibit benzene degradation. When phenol or toluene was added to cultures and controls along with benzene (Figure 3.3C, D), benzene degradation continued without any lag. There was minimal loss of benzene in cultures relative to the sterile controls, when benzene and benzoate were added together (Figure 3.3E). Thus, inhibition of benzene degradation was only observed when benzoate was added to cultures along with benzene. This inhibition is evident when the benzene only active control (Figure 3.3A) is compared with benzoate amended cultures (Figure 3.3E). These data indicate that presence of benzoate affects the metabolism of benzene, which leads to almost complete lack of conversion of benzene to its downstream metabolites. Since benzoate can itself be degraded at higher concentration (0.5 mM) than that used in this test (data not shown), the observed effect of benzoate on benzene degradation is most likely due to feedback inhibition of substrate degradation. Presence of excess downstream metabolites can inhibit the enzymes that

catalyze the activation of the benzene ring, which leads to high residual substrate concentrations. Thus, inhibition of benzene degradation by benzoate indicates that benzoate is a downstream metabolite of benzene. This data supports the previously reported observation by Phelps et al. (26) based on a stable isotope labeling study that benzoate is a metabolite of benzene degradation.

Although the culture is capable of degrading phenol and 4-hydroxybenzoate (Figure 3.2), these proposed metabolites did not inhibit benzene degradation (Figure 3.3B and 3.3C). This suggests that they may not be metabolic intermediates of anaerobic benzene degradation. Toluene was not degraded by this culture (in multiple tests, data not shown), nor did it inhibit benzene degradation (3.3D), which suggests that toluene is an *unlikely* metabolite of benzene degradation. Although it seems that toluene has enhanced the rate of benzene degradation in the treatment (3.3D), its comparison with the benzene only control (3.3A) (high std. dev. on day 7) limits the conclusions that can be based on this data.

3.3.3 Metabolites of [$^{13}\text{C}_6$]benzene

Benzoate as a metabolite of benzene

Between 0 and 6 days after [$^{13}\text{C}_6$]benzene was added, samples of cultures and controls were taken for solvent extraction and GC-MS analysis of labeled metabolites. Figure 3.4(A) shows the mass spectra of [$^{12}\text{C}_6$]benzoate standard derivatized with BSTFA (Retention time (RT) = 13.24 mins), along with the structures of its aromatic fragments m/z 194, 179, 135, 105 and 77. Figure 3.4(B) is the mass spectra of a metabolite identified in [$^{13}\text{C}_6$]benzene amended cultures at the same RT. This mass spectra shows the presence of fragments m/z 201, 186, 141, 112, and 83, along with m/z 200, 185, and

111. Mass fragments m/z 201, 186 and 112 are 7 mass units heavier than fragments m/z 194, 179, 105 detected in the [$^{12}\text{C}_6$]benzoate standard, while mass fragment m/z 200, 185 and 111 are only 6 mass units heavier than the fragments in the [$^{12}\text{C}_6$]benzoate standard. This data indicates that the metabolites detected in cultures amended with [$^{13}\text{C}_6$]benzene are [^{13}C -UL]benzoate (fragments 7 mass units heavier) and [^{13}C -Ring]benzoate (fragments 6 mass units heavier).

The concentrations of [^{13}C -UL]benzoate detected in 4 samples of [$^{13}\text{C}_6$]benzene amended active cultures are given in Table 3.1(A). Concentrations of [^{13}C -Ring]benzoate identified in 3 samples of active cultures is also given in the same table. Comparison of the concentrations of two types of benzoate detected in [$^{13}\text{C}_6$]benzene amended cultures indicates that the concentrations of [^{13}C -UL]benzoate were always more than an order of magnitude higher than those of [^{13}C -Ring]benzoate. The maximum concentration of [^{13}C -UL]benzoate detected in the cultures is 0.3 % of the maximum substrate concentration in the cultures. These concentrations are consistent with the previous studies in which ^{13}C - or ^2H -labeled benzoate was detected as a metabolite of benzene (6, 26, 33) in mixed culture studies. No labeled benzoate was detected in the sterile controls in our study. A minute quantity of [^{13}C -UL]benzoate was detected in the abiotic controls. Since the low concentration of [^{13}C -UL]benzoate detected in the controls cannot account for the high concentration of [^{13}C -UL]benzoate detected in the active cultures, it can be concluded that both [^{13}C -UL]benzoate and [^{13}C -Ring]benzoate are metabolites of [$^{13}\text{C}_6$]benzene biodegradation.

Phenol and toluene as artifacts of extraction and analysis

[$^{13}\text{C}_6$]phenol was detected not only in the ethyl acetate extracts of the active cultures, but also in the sterile controls amended with [$^{13}\text{C}_6$]benzene (data not shown). These results were verified by repeating the solvent extraction of cultures and controls. The solvent extraction protocol was also modified (by eliminating the steps of pH changes), but phenol could still be detected in all the samples as described above. Potential contamination of the [$^{13}\text{C}_6$]benzene stock with [$^{13}\text{C}_6$]phenol was ruled out. From these results it can be concluded that phenol was abiotically formed during the incubation and/or analytical preparation of samples. The relative abundance of [$^{13}\text{C}_6$]phenol detected in [$^{13}\text{C}_6$]benzene amended sterile or abiotic controls (with $\sim 400\ \mu\text{M}$ of [$^{13}\text{C}_6$]benzene) was almost always higher (2-60 times) than that detected in [$^{13}\text{C}_6$]benzene grown cultures (data not shown). This was likely due to the fact that [$^{13}\text{C}_6$]benzene amended to the cultures was degraded and was low in concentration ($< 50\ \mu\text{M}$) before any abiotic transformation of benzene could occur.

^{13}C -labeled toluene was not detected in active cultures grown with [$^{13}\text{C}_6$]benzene, but it was detected in abiotic and sterile controls (data not shown). It was determined that the stock of [$^{13}\text{C}_6$]benzene was not contaminated with ^{13}C -labeled toluene. These data indicate that like phenol, toluene was also formed in the absence of live cells and thus was an artifact of the experimental processes. [$^{13}\text{C}_6$]benzene in active cultures was degraded ($< 50\ \mu\text{M}$) by the time samples were taken for diethyl ether extraction of toluene, while concentrations in sterile and abiotic controls did not change substantially ($\sim 400\ \mu\text{M}$, due to repeated addition). Detection of ^{13}C -labeled toluene only in controls and not in cultures indicates that detectable abiotic transformation of benzene to toluene occurred only at high concentration of substrate, as those found in sterile and abiotic

controls. It is unlikely that toluene was formed in cultures, but rather degraded during incubation, since previous tests with unlabeled toluene showed that toluene cannot be degraded by this culture (data not shown).

Abiotic formation of labeled phenol and toluene in our samples limits our efforts to use this data for identification of downstream metabolites of benzene degradation. This data also brings into question results from other studies that used similar analytical procedures (6, 7, 33).

Other proposed metabolites of benzene degradation

Neither cyclohexanone nor 4-hydroxybenzoate, were detected in any cultures or controls amended with [$^{13}\text{C}_6$]benzene. Cyclohexanone is a proposed downstream metabolite of benzene degradation via phenol (Figure 3.1) (14, 34).

Four-hydroxybenzoate is considered to be a transient intermediate of phenol degradation to benzoate (7) and has been observed in phenol degrading pure cultures (4). Lack of its detection in [$^{13}\text{C}_6$]benzene amended cultures is either due to its transient nature, or due to lack of its production during benzene degradation in this mixed culture.

3.3.4 Metabolites of [$^{13}\text{C}_6$]phenol

Phenol biodegradation was observed in cultures amended with [$^{13}\text{C}_6$]phenol (refer A3 in the appendix). Solvent extracts of [$^{13}\text{C}_6$]phenol amended cultures and controls were analyzed by GC-MS. A metabolite was detected in [$^{13}\text{C}_6$]phenol amended cultures at the RT same as that of the benzoate standard. Figure 3.4(C) is the mass spectra of BSTFA derivatized [$^{13}\text{C}_6$]phenol amended culture, and Figure 3.4(A) shows a mass spectra of a BSTFA derivatized [$^{12}\text{C}_6$]benzoate standard for comparison, along with the structures of its aromatic fragments. Mass fragment m/z 83, 111, 141, 185 and 200

detected in the cultures amended with [$^{13}\text{C}_6$]phenol, are 6 mass units heavier than mass fragments m/z 77, 105, 135, 179 and 194 detected in [$^{12}\text{C}_6$]benzoate standard. These data indicate that the metabolite detected in [$^{13}\text{C}_6$]phenol amended cultures was [^{13}C -Ring]benzoate.

No ^{13}C -labeled benzoate was detected in sterile controls (data not shown), indicating that [^{13}C -Ring]benzoate is a metabolite of [$^{13}\text{C}_6$]phenol biodegradation in this consortium. No [^{13}C -UL]benzoate was identified in any of the [$^{13}\text{C}_6$]phenol amended cultures or controls. This suggests that the dominant pathway of phenol degradation in this consortium leads to [^{13}C -Ring]benzoate, and that the carboxyl carbon for phenol carboxylation is not derived from the aromatic ring. These results are consistent with previous studies in which bicarbonate (carbon dioxide) has been identified as a carboxyl group donor for phenol metabolism (19, 29).

The concentration of [^{13}C -Ring]benzoate in the two samples is given in Table 3.1 (B). Since degradation of ^{13}C -, ^{14}C - or ^2H -labeled phenol has not been studied in any of the other benzene degrading cultures, no reference values are available for comparison of benzoate concentrations. Chakraborty et al. (7) reported that when phenol (45 μM) was added to cultures of *Dechloromonas* strain RCB, benzoate (>30 μM) was detected in the cultures, but these studies were carried out with unlabeled phenol.

Neither cyclohexanone nor 4-hydroxybenzoate were detected in any cultures or controls amended with [$^{13}\text{C}_6$]phenol. All mass spectra were also scanned to detect any 4-hydroxybenzoate, which is a reported downstream metabolite of phenol degradation before its conversion to benzoate (4, 12, 13, 31, 32). ^{13}C -labeled or unlabeled 4-hydroxybenzoate was not detected in cultures or controls amended with [$^{13}\text{C}_6$]phenol,

which may be due to the transient nature of this metabolite—lack of detection of these metabolites does not necessarily imply lack of their formation in the cultures.

3.4 DISCUSSION

In this study a stable, sediment free benzene degrading, sulfate-reducing enrichment was used to investigate the pathway of anaerobic benzene degradation. At present, anaerobic benzene degradation has been proposed to occur through methylation to toluene, hydroxylation to phenol, or carboxylation to benzoate (Figure 3.1) (6, 14, 33).

To elucidate the pathway of benzene degradation under sulfidogenic conditions in this consortium, 4 different lines of experimentation were carried out. Use of multiple lines of evidence allowed the comparison and validation of our conclusions. Use of a stable culture for these studies was crucial, as it facilitated visualization of a simple metabolic network that was useful for interpretation of data, otherwise not possible in sediments or natural systems.

Benzene degradation via phenol

A phenol pathway of benzene degradation has been proposed as ^{13}C - or ^{14}C -labeled phenol was detected in mixed cultures amended with ^{13}C - or ^{14}C -labeled benzene respectively, in various studies (6, 14, 33, 35). Since phenol is proposed as an intermediate of benzene degradation, it is important to understand the fate of phenol in our benzene culture. To our knowledge, degradation of labeled phenol has not been studied in any benzene degrading culture to date, and thus the downstream pathway of phenol in a benzene degrading culture is not clearly understood. Chakraborty et al. (7) documented that when phenol was added as a sole carbon source, it was degraded to benzoate in a denitrifying pure culture also capable of benzene degradation, but the

substrates used in that study were not labeled. Based on studies in phenol degrading cultures, it is understood that phenol degradation can proceed either via ring reduction to cyclohexanone (2, 3) (mixed cultures) or by carboxylation to benzoate (pure cultures) (4). In addition, 4-hydroxybenzoate has been identified as an intermediate of phenol degradation during its conversion to benzoate (4, 12, 13, 31, 32), and carbon dioxide/bicarbonate is the source of the carboxyl group (28, 29) in pure cultures. Thus, detection of cyclohexanone and phenol in a benzene amended culture is indicative of a ring reduction pathway of phenol, while detection of 4-hydroxybenzoate and benzoate is indicative of carboxylation of phenol. It should be noted, however, that benzoate could be formed by direct carboxylation of benzene, similar to naphthalene and phenanthrene (37). Therefore, detection of benzoate along with phenol does not necessarily indicate benzene degradation via phenol carboxylation.

To understand if benzene degradation in this enrichment culture proceeds through phenol, the following steps were taken: (i) phenol was tested as a sole carbon source for degradation. (ii) the effect of phenol on benzene degradation was evaluated, and (iii) a comparison of metabolites identified in [$^{13}\text{C}_6$]benzene and [$^{13}\text{C}_6$]phenol amended cultures was done. A difference in the labeling pattern of the metabolites of [$^{13}\text{C}_6$]benzene and [$^{13}\text{C}_6$]phenol in this enrichment would indicate that benzene and phenol do not share a common metabolic pathway in this consortium.

We observed that phenol could be degraded by the culture (Figure 3.2), which indicates that a phenol degradation pathway exists in this consortium. Phenol did not inhibit benzene degradation (Figure 3.3C), thus it does not exert a feedback inhibition and suggests that it is not a metabolite of benzene. In the [$^{13}\text{C}_6$]phenol amended cultures

$[^{13}\text{C}_6]$ benzoate was detected as the only intermediate (Figure 3.4C). Neither cyclohexanone nor 4-hydroxybenzoate was detected in this study. These data suggest that phenol degradation in this consortium proceeds via benzoate. In $[^{13}\text{C}_6]$ benzene amended cultures both $[^{13}\text{C}\text{-Ring}]$ benzoate and $[^{13}\text{C}\text{-UL}]$ benzoate were detected in the cultures and $[^{13}\text{C}\text{-UL}]$ benzoate was the dominant form of benzoate detected. On closer examination of the metabolites identified in $[^{13}\text{C}_6]$ benzene or $[^{13}\text{C}_6]$ phenol amended cultures, it was observed that the labeling pattern of the dominant form of benzoate detected in these cultures was different. These data suggests that although benzene and phenol degradation proceed via a common intermediate (i.e. benzoate), the earlier steps in the pathways that form benzoate during degradation of these different substrates are different. If we assume that phenol - whether amended externally or formed as an intermediate of benzene degradation – is expected to be degraded by the same degradation pathways, then identically labeled benzoate would be produced in each case. Given that differently labeled metabolites are produced if benzene or phenol is the substrate, the results strongly suggest that the dominant pathway of benzene degradation in this culture does not proceed via phenol.

Low concentrations of $[^{13}\text{C}\text{-Ring}]$ benzoate were detected in cultures amended with $[^{13}\text{C}_6]$ phenol (0.28-0.47 nM) and $[^{13}\text{C}_6]$ benzene (up to 1nM). This suggests that the pathway leading to $[^{13}\text{C}\text{-Ring}]$ benzoate formation also exists in this consortium, and was working during degradation of phenol as well as during degradation of benzene. Considering that phenol is a proposed metabolite of benzene degradation the observation that $[^{13}\text{C}\text{-Ring}]$ benzoate is formed in cultures amended with $[^{13}\text{C}_6]$ phenol or

[$^{13}\text{C}_6$]benzene suggests that the phenol pathway of benzene degradation also takes place in this consortium, although only as a minor component of the processes.

Abiotic formation of phenol was noted in our studies, when [$^{13}\text{C}_6$]benzene was added as a substrate to cultures and controls. Therefore, phenol could not be directly verified or eliminated as a metabolite of benzene degradation in our study. Similar observations on abiotic phenol formation were made by Kunapuli et al. (17). In that study it was suggested that phenol was formed due to autooxidation of the benzene ring by free hydroxyl radicals that are produced after reduced media components are exposed to air. ^{13}C -labeled phenol and ^{13}C -labeled benzoate have been reported in cultures amended with [$^{13}\text{C}_6$]benzene under sulfate reducing and methanogenic conditions in mixed cultures (6, 33). None of these studies noted detection of cyclohexanone, and concluded that benzene degradation in cultures proceeds through phenol and benzoate. As noted in our study, and also described by Kunapuli et al. (17), phenol was formed abiotically likely during sampling and sample extraction in anaerobic media amended with benzene. Thus, detection of phenol in the previous studies as an intermediate of anaerobic benzene degradation needs to be revisited and verified. Chakraborty and Coates (7) reported that phenol was formed in cultures of *Dechloromonas* RCB, amended with unlabeled benzene with nitrate as an electron acceptor. This data was compared with cultures without electron acceptor, as a control. Evidence of biotic phenol formation in these cultures needs to be substantiated by demonstrating lack of phenol formation in sterile and abiotic controls amended with benzene. Absence of such data puts the conclusions made Chakraborty and Coates into question.

We noted that phenol was degraded by our consortium after a lag, at approximately 1/10 the rate of benzene degradation, when phenol was amended to the cultures grown only on benzene. On subsequent additions the rate of phenol degradation increased slightly. Musat and Widdel (22) also noted that a sulfate-reducing benzene degrading mixed culture degraded phenol after a lag. It was suggested that the lag indicates that the population of cells in the mixed culture was not previously enriched for phenol degradation. Our results are in agreement with the results obtained by Musat and Widdel (22). In addition to the similarity in these observations (made in this study and that by Musat and Widdel (22)), it should be noted that the sulfate-reducing enrichments used in these 2 studies have some similarity in the bacterial community composition. The dominant SB-21 phylotype in this consortium (23) is phylogenetically related (90-99% similar) to the members of the sulfate-reducing enrichment studied by Musat et al. (22). This similarity in the members of the 2 enrichments may be responsible for similar metabolism.

Pathway via toluene

Only one study so far has documented conversion of ^{13}C -labeled benzene to ^{13}C -labeled toluene. Ulrich et al. (33) first provided evidence for methylation of benzene, as ring [$^{13}\text{C}_6$]toluene (ring labeled) was detected in nitrate-reducing and methanogenic enrichment cultures amended with [$^{13}\text{C}_6$]benzene. Musat and Widdel (22) noted that toluene could not be degraded by their sulfate-reducing benzene degrading enrichment, while Kunapuli et al. (17) observed that their iron-reducing benzene degrading enrichment lost the capability to degrade toluene after subsequent transfers.

To understand if benzene degradation in this enrichment culture proceeds through toluene, the following steps were taken: (i) toluene was tested as a sole carbon source for degradation, (ii) the effect of toluene on benzene degradation was evaluated; and (iii) metabolites of [$^{13}\text{C}_6$]benzene in cultures and controls were analyzed.

We observed in this study that toluene was not degraded as a sole carbon source (data not shown), although in our earlier study (23) one of the clones in this benzene degrading sulfate-reducing consortium was noted to be 99% similar to *Desulfobacterium toluolica* (EF207159), which is a sulfate-reducing toluene degrading pure culture. In addition, toluene did not inhibit benzene degradation (Figure 3.3D), and neither ^{13}C -labeled nor unlabeled toluene was detected in [$^{13}\text{C}_6$]benzene amended active cultures. It cannot be conclusively stated whether toluene enhanced the rate of benzene degradation (Figure 3.3D). Based on the fact that toluene was not degraded by this enrichment, and that [^{13}C]labeled toluene was not detected in active cultures, along with the lack of an inhibitory effect of toluene on benzene degradation, we conclude that it is unlikely that the toluene pathway of benzene degradation is functional in this consortium.

In our experiments we observed that ^{13}C -labeled toluene was formed abiotically in sterile and abiotic controls amended with [$^{13}\text{C}_6$]benzene. ^{13}C -labeled toluene was not detected in active cultures. The concentration of [$^{13}\text{C}_6$]benzene in active cultures was low (<50 μM) as compared to controls (sterile and abiotic) (~ 400 μM) when samples were withdrawn for diethylether extractions of toluene. This data suggests that abiotic transformation of benzene to toluene occurred only when a high concentration of (~ 400 μM) substrate was present. It is unlikely that toluene was actually formed during incubation in culture, but was degraded before we took samples for GC-MS analysis,

since our previous analysis shows that toluene cannot be degraded by this culture (data not shown).

Our data shows that detection of labeled toluene in anaerobic cultures amended with labeled benzene does not necessarily imply that benzene was transformed to toluene. Thus, detection of toluene as a metabolite of benzene degradation in previous studies (33) needs to be revisited and verified.

Source of carboxyl group in [^{13}C -UL]benzoate

The source of the carboxyl group of benzoate formed from benzene is important as it can give some indication as to which activation mechanism is used for benzene degradation. Kunapuli et al. (17) observed that the carboxyl group of benzoate was labeled when unlabeled benzene was added to iron-reducing mixed cultures along with ^{13}C -labeled bicarbonate. In our study at least 10 fold higher concentrations of [^{13}C -UL]benzoate were detected in [$^{13}\text{C}_6$]benzene amended cultures, as compared to [^{13}C -Ring]benzoate, indicating that the carboxyl carbon of benzoate is actually derived from the benzene ring itself. The possibility that ^{13}C carbon was used for carboxylation of benzene instead of ^{12}C carbon as a random event can be ruled out as the cumulative total concentration of [^{13}C]CO₂ (calculated) in [$^{13}\text{C}_6$]benzene amended cultures was 3.15 mM as compared to 30 mM of [^{12}C]CO₂ (calculated as released from bicarbonate buffer). Thus, the bicarbonate in the system is not likely to be responsible for carboxylation of the benzene ring, but rather the carboxyl carbon of the benzoate ring must originate from benzene itself. In earlier work by Phelps et al. (26), ^{13}C -labeled bicarbonate and ^{13}C -acetate were both tested as sources of carboxyl carbon with deuterated benzene, and

yielded negative results. This further supports our conclusions that the carboxyl carbon of benzoate is derived from the benzene ring and not from bicarbonate in the system.

Thus, we propose that two pathways of benzene degradation are at work in this consortium (Figure 3.5). One pathway leads to the formation of [^{13}C -Ring]benzoate, while the second pathway leads to formation of [^{13}C -UL]benzoate. These two pathways differ in the carbon source used for the carboxyl carbon of benzoate formed as a result of benzene degradation. Our data clearly indicates that the carboxyl carbon of [^{13}C -UL]benzoate originates from [$^{13}\text{C}_6$]benzene, and [^{13}C -UL]benzoate is the dominant form of labeled benzoate formed during benzene degradation.

The dominant pathway of benzene degradation in this consortium does not appear to follow either of the previously proposed mechanisms including methylation to toluene (6, 17, 26, 33), or hydroxylation to phenol (6, 14, 33, 35). Carboxylation has been proposed earlier, and is considered to occur subsequent to benzene activation through either methylation (33) or hydroxylation (6, 7, 33). Direct carboxylation of benzene to benzoate is also possible (17), similar to what has been observed during naphthalene and phenanthrene degradation in sulfate-reducing enrichment cultures (37). Bicarbonate has been shown to be the source for benzene carboxylation (17) in an iron-reducing enrichment culture, but it is not the carboxyl group donor in the consortium used for our study, as reported earlier by Phelps et al. (26).

Data from our study shows that none of the proposed pathways (Figure 3.1) are functioning in this consortium. Instead, as illustrated in figure 3.5, a novel pathway leading to carboxylation of benzene ring by the products of another benzene ring is proposed in this consortium. As shown in the figure, the proposed pathways of benzene

degradation in this consortium involve a dominant pathway (bold arrow) in which benzene reacts with an organic compound derived from another benzene ring to form benzoate. The carboxyl carbon of this benzoate is thus derived from products of another benzene ring. A minor pathway also likely exists in this consortium and it involves hydroxylation of benzene to phenol, with its subsequent conversion to benzoate. The carboxyl carbon of this benzoate is likely derived the cellular pool of carboxyl group donors, including bicarbonate. This activation mechanism can be compared to a fumarate addition reaction observed during anaerobic toluene degradation. Although such a reaction has been considered to be unlikely to activate benzene to phenyl radical due to the high activation energy needed for fumarate addition (36), it should be tested as a carboxyl group donor. In addition, a similar reaction with other organic compounds derived from the benzene ring may be occurring. Acetate and bicarbonate have already been tested as potential carboxyl group donors for benzene carboxylation in this consortium (26), and yielded negative results. Other metabolic intermediates such as fumarate and products of benzoate degradation can be tested as likely donors of carboxyl group to further investigate the mechanism of benzene activation in this consortium. Stable isotope labeling studies using a combination of deuterated or unlabeled benzene and ^{13}C -labeled intermediates of benzoate degradation can be used to specifically identify the carboxyl group donor and likely mechanism of benzene activation in this culture.

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Table 3.1. Concentrations of ^{13}C -labeled benzoate detected in samples. The concentration of benzoate was calculated by using the area of the base peak m/z 185 and 186 for $[^{13}\text{C}\text{-Ring}]$ - and $[^{13}\text{C}\text{-UL}]$ benzoate, respectively. All samples were derivatized with BSTFA before GC-MS analysis. A: active culture, S: sterile control and AB: abiotic control, ND: not detected, QL: quantification limit. The numbers represent samples taken subsequently on different days.

A) ^{13}C -labeled benzoate in $[^{13}\text{C}_6]$ benzene amended cultures and controls.

Active	$[^{13}\text{C}\text{-Ring}]$ (nM)	$[^{13}\text{C}\text{-UL}]$ (nM)	Control	$[^{13}\text{C}\text{-Ring}]$ (nM)	$[^{13}\text{C}\text{-UL}]$ (nM)
1A	ND	2	1S	ND	ND
3A	9	140	3AB	ND	ND
4A	14	235	4AB	<QL	1
5A	18	337	5AB	<QL	1

B) ^{13}C -labeled benzoate in $[^{13}\text{C}_6]$ phenol amended cultures and controls.

Active	$[^{13}\text{C}\text{-Ring}]$ benzoate (nM)	Control	$[^{13}\text{C}\text{-Ring}]$ benzoate (nM)
2A	0.5	2S	ND
3A	0.3	3S	ND

Figure 3.1. Proposed pathways of anaerobic benzene degradation (Adapted from (11)).

A) hydroxylation to phenol (6, 14, 33, 35), B) methylation to toluene (6, 17, 26, 33), and C) carboxylation to benzoate (6, 17, 26, 33). Square brackets indicate presence of transient intermediates.

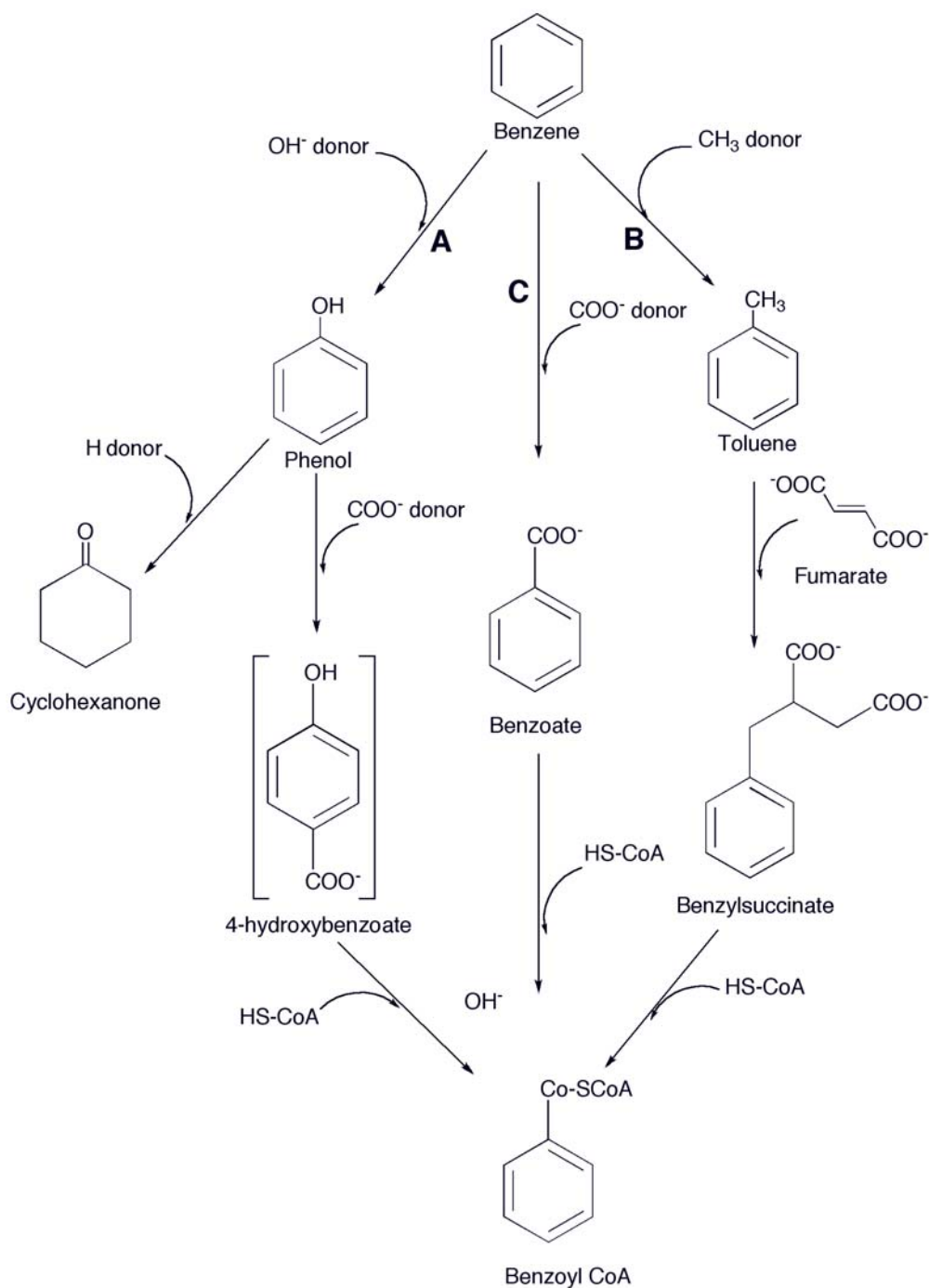


Figure 3.2. Rates of degradation of proposed metabolites of benzene. The graph shows the concentrations of benzoate (\blacktriangle), 4-hydroxybenzoate (\blacksquare) or phenol (\bullet) when added as sole carbon sources to active cultures (—) and sterile controls (---). Concentrations of phenol detected in 4-hydroxybenzoate amended cultures (\blacklozenge) are also shown. Values are average of duplicate for active cultures and sterile controls. X-axis; sampling day, Y-axis; concentration of carbon sources (μM).

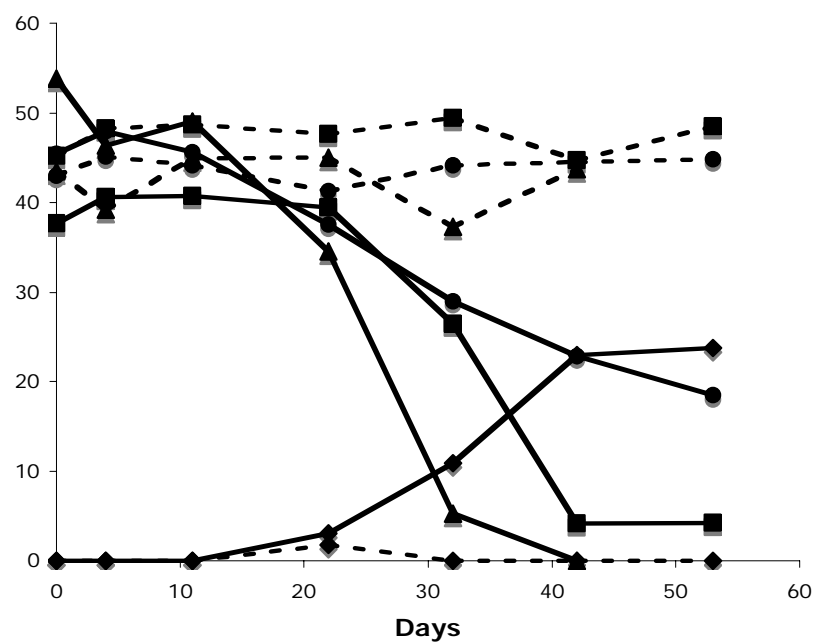


Figure 3.3. Effect of the proposed metabolites on benzene degradation. The graphs show concentration of benzene in active cultures (—) and in sterile controls (---). Benzene only amendment was used as a control (A). Tests included addition of 4-hydroxybenzoate (B), phenol (C), toluene (D), and benzoate (E) to cultures and controls along with benzene. Values are averages of 3 active cultures or 2 sterile controls. X-axis: sampling day; Y-axis: benzene concentration (μM). Error bars show (± 1) standard deviation.

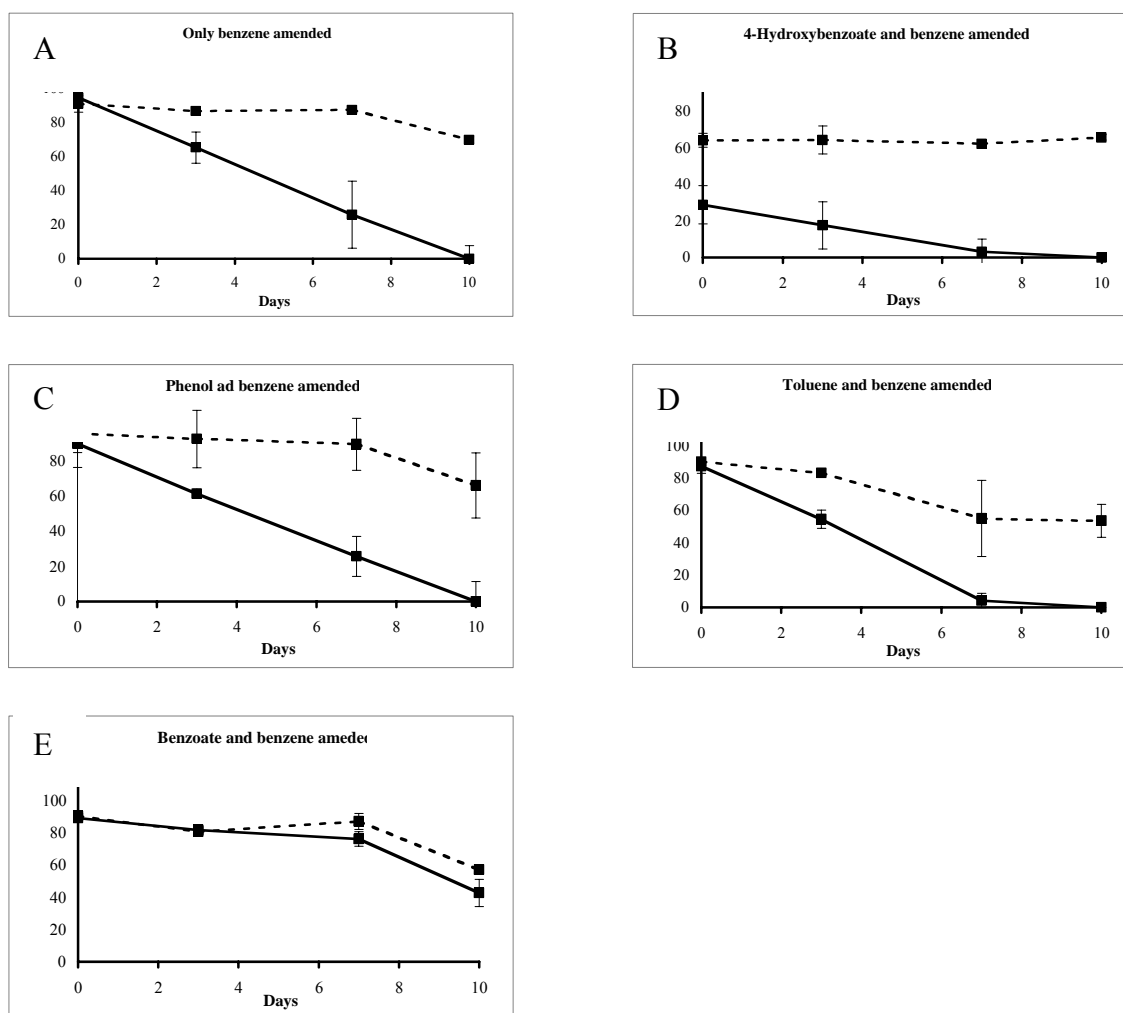


Figure 3.4. GC-MS mass spectra A) [$^{12}\text{C}_6$]benzoate standard, B) [^{13}C -UL]benzoate detected in [$^{13}\text{C}_6$]benzene amended cultures and, C) [^{13}C -Ring]benzoate detected in [$^{13}\text{C}_6$]phenol amended cultures. All samples were derivatized with BSTFA before GC-MS analysis.

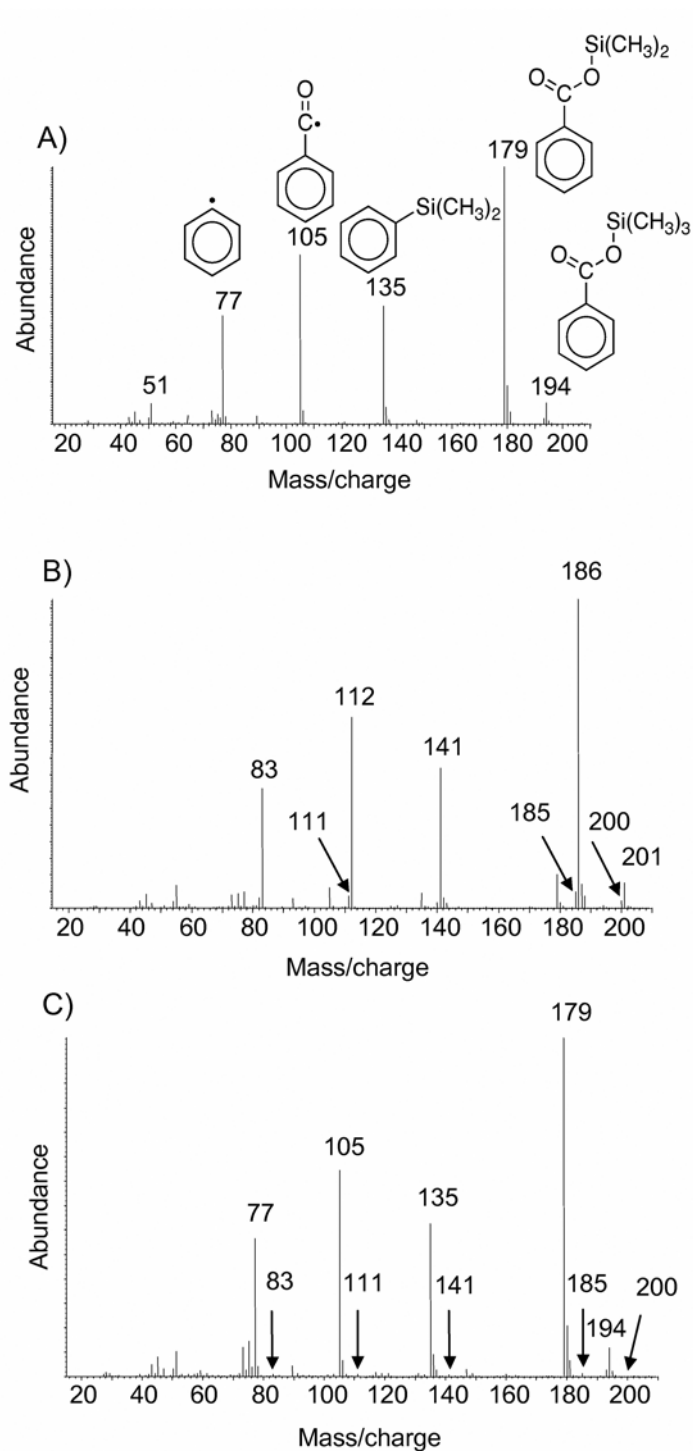
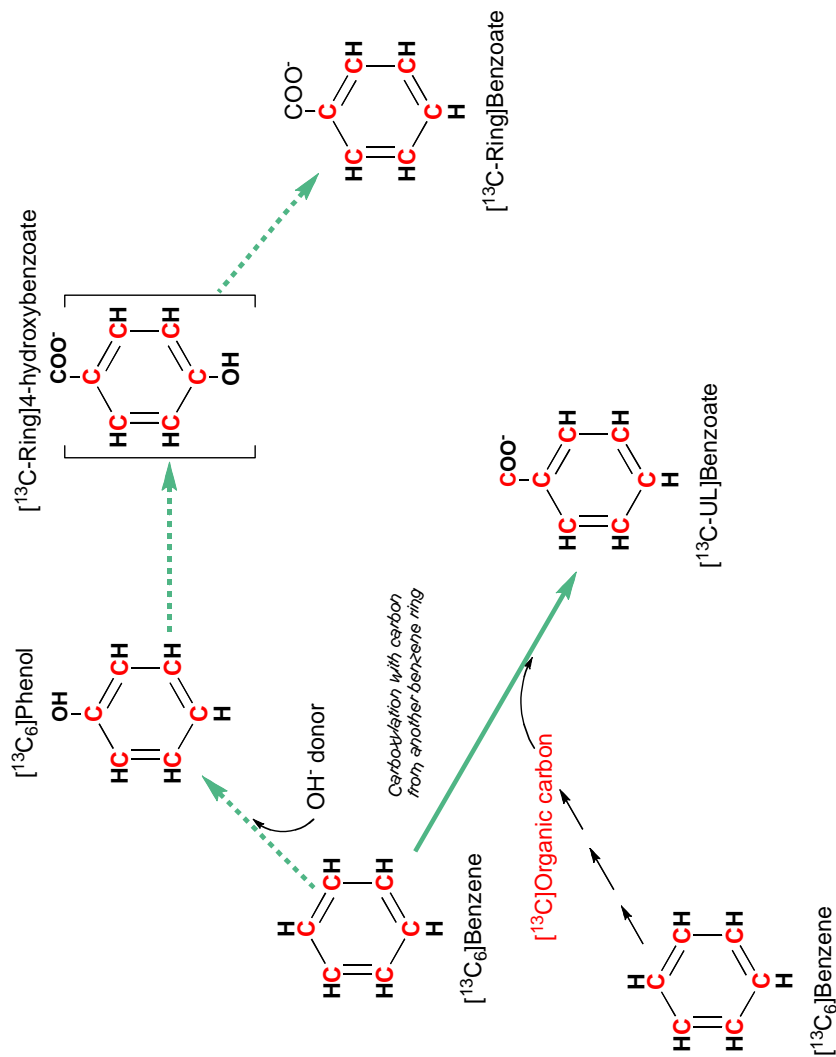


Figure 3.5. Proposed pathway of benzene metabolism and carboxylation with carbon from another benzene ring. Bold arrow indicates dominant pathway. Dashed arrows indicate minor pathway in benzene degradation. Note that carboxylation of benzene to benzoate in the dominant pathway may occur in multiple steps. Red color of carbon atom represents ^{13}C label. Square brackets indicate presence of transient intermediates.



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CHAPTER 4

Evidence of Anaerobic Biodegradation of Hydrocarbons in the Subsurface

Environment

ABSTRACT

Contamination of subsurface sites due to hydrocarbons can impact groundwater resources. Anaerobic conditions can develop and persist in the groundwater at contaminated sites due to growth of aerobic bacteria. Thus, anaerobic biodegradation in the subsurface may be crucial for remediation of the impacted subsurface. In this study, groundwater samples from a manufactured gas plant impacted site were used for qualitative and quantitative analysis of metabolic intermediates of polycyclic aromatic hydrocarbons (PAHs) and gene encoding analogues of the alpha subunit of benzylsuccinate synthase (*bssA*). Metabolic intermediates of naphthalene and 2-methylnaphthalene degradation including, 2-naphthoic acid (NA), tetrahydro-2-NA, and hexahydro-2-NA, were detected in the groundwater of monitoring wells within the plume. *BssA* gene analogues were also detected specifically in the impacted wells. Quantitative analysis of metabolic intermediates and the *bssA* genes showed that the monitoring wells within the plume were enriched for anaerobic hydrocarbon degradation. MW-24, within the plume, had the highest concentration of metabolic intermediates and *bssA* gene copies, as compared to all other wells in this study. Copies of *bssA* genes in sulfate-reducing or denitrifying bacteria were not above our detection limit in the non-impacted wells. Thus, 2 different biomarkers of anaerobic hydrocarbon degradation were simultaneously detected at the impacted site, and with quantitative analysis of these biomarkers, it was possible to identify an area within this site that was enriched for

hydrocarbon degradation. Detection of not just one but two different indicators specific to the presence and activity of anaerobic microorganisms provides strong evidence of anaerobic microbial processes at the site that are responsible for natural attenuation of the contaminants.

4.1 INTRODUCTION

Subsurface environments can be contaminated by petroleum hydrocarbons originating from modern day activities related to petroleum refining and use, along with residues of historic contamination originating from coal-refining processes. Remediation of subsurface environments can be challenging due to their structural heterogeneity. *In situ* biodegradation of hydrocarbons by microorganisms naturally present at the site is an important component of site remediation through processes of natural attenuation. Since subsurface environments can rapidly turn anoxic, anaerobic degradation processes can play an important role in site cleanup.

To establish *in situ* remediation at impacted sites, three steps are suggested by the U.S. Environmental Protection Agency (48): (i) groundwater chemistry data that demonstrate a clear and meaningful trend of decrease of contaminant mass and/or concentration over time, (ii) hydrogeologic and geochemical data that demonstrate indirectly the types of natural attenuation processes active at the site, and (iii) data that provide direct evidence for degradation taking place in actually contaminated site media (microcosms) or *in situ* degradation (aquifer). Assessment of *in situ* anaerobic biodegradation of hydrocarbons at impacted sites can be achieved by (a) study of signature metabolites or metabolic intermediates of hydrocarbons in the field samples (referred to here as metabolic biomarkers) (53), (b) compound specific isotope analysis (CSIA) of the target compounds (31), and (c) study of functional genes involved in anaerobic biodegradation of target compounds (referred to here as genetic biomarkers) (5, 6). Metabolic intermediates of anaerobic degradation of target compounds can serve as indicators of *in situ* biodegradation if these fulfill a set of prerequisites (3): (1)

unequivocal and unique biochemical relationship to the parent hydrocarbon, (2) release from the microbial cells to the extra cellular medium, (3) no commercial or industrial production or uses, and (4) generation as an intermediates of mineralization rather than a product of co-metabolism. Young and Phelps (53) suggested that a metabolic intermediate could be used as a biomarker if it is biodegradable rather than stable, as this ensures that the intermediate is formed during active biodegradation of the target compound, and is not a result of processes in the past. In addition it was also suggested that the metabolic biomarker should be normally absent in unimpacted environments, and should be water soluble for ease of sampling.

The Young lab has pioneered in the study of anaerobic biodegradation of aromatic hydrocarbons such as benzene (27, 34-37), toluene (11-13, 17-20, 44), ethylbenzene (34, 36), xylenes (17, 20, 34, 36), naphthalene (54, 55), 2-methylnaphthalene (46), and phenanthrene (55), along with alkanes (8-10, 40-43) under different electron accepting conditions.

Metabolic intermediates such as benzylsuccinate, and 2-, 3- or, 4- methyl benzylsuccinate are products of anaerobic degradation of toluene, *o*-, *m*-, and *p*-xylene, respectively, and are specific to these compounds (please see review (45) for details). Beller et al. (4) first demonstrated the use of benzylsuccinates as indicators of *in situ* biodegradation in field tests in which BTEX contaminants were added to unimpacted groundwater and samples were withdrawn periodically from the subsurface for detection of metabolic intermediates. Young and Phelps (53) demonstrated that benzylsuccinates can be used as indicators of anaerobic alkylbenzene degradation at sites that were historically contaminated with mixed waste. They detected 2-methylbenzylsuccinate

(degradation product of *o*-xylene) in the groundwater samples from a manufactured gas plant impacted site. The abundance of these metabolites also corresponded with the concentration of aromatic hydrocarbons present at the site. Since their first detection in the environmental samples, metabolic intermediates of anaerobic degradation of alkylbenzenes have been detected in several contaminated subsurface environments (6, 16, 22-24, 30, 53).

Anaerobic degradation of polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, 2-methylnaphthalene, and phenanthrene has also been investigated in the laboratory studies. Zhang and Young (55) demonstrated that the initial reaction in anaerobic degradation of naphthalene and phenanthrene degradation was carboxylation, leading to formation of 2-naphthoic acid (2-NA) and phenanthrene carboxylic acid (PHE-CA), respectively, and that the carboxyl carbon for this reaction is derived from carbon dioxide/bicarbonate. These studies were based on sulfate-reducing enrichments. Further investigations by Zhang et al. (54) on these enrichments established that degradation of naphthalene after initial carboxylation proceeds through ring reduction steps. Metabolic intermediates such as 2-naphthoic acid (2-NA), dihydro-2-NA (DH-2-NA), tetrahydro-2-NA (TH-2-NA), hexahydro-2-NA (HH-2-NA), and decalin-2-carboxylate were identified in sulfate-reducing enrichment cultures. The degradation of 2-methylnaphthalene can proceed through two different pathways. Annwelier et al. (1, 2) identified that one pathway involves oxidation of the methyl group of 2-methylnaphthalene to a carboxyl group through a fumarate addition reaction to form 2-naphthoic acid. Activation of 2-methylnaphthalene through fumarate addition was demonstrated by detection of succinyl adducts naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid in a

sulfate-reducing enrichment culture growth with 2-methylnaphthalene (1). Sullivan et al. (46) identified that anaerobic degradation of 2-methylnaphthalene can proceed via a alternative pathway which involves carboxylation of the unmethylated ring of 2-methylnaphthalene (46) to form carboxylated 2-methylnaphthalene (methylnaphthoic acid (MNA)). Both these pathways further involve sequential ring reduction steps like those observed during naphthalene degradation (54, 55). Metabolic intermediates of naphthalene and 2-methylnaphthalene, including TH-2-NA, HH-2-NA, MNA, naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid, satisfy the above mentioned prerequisites for indicators of *in situ* degradation (unique to anaerobic biodegradation, released outside cells, no industrial use, and products of degradation, not cometabolism). In addition, these metabolic intermediates are not produced by background natural processes. These properties make them excellent candidates to be used as biomarkers of *in situ* anaerobic degradation of PAHs.

Phelps et al. (33) first demonstrated the application of metabolic intermediates of anaerobic PAH degradation as biomarkers of *in situ* biodegradation. Phelps et al. analyzed groundwater samples from maintenance yard impacted site in NJ. Metabolites including 2-NA, TH-2-NA, and MNA were detected in ground water samples, and the concentration of 2-NA could also be related to the zones of contamination. Phelps et al. also analyzed two additional sites (one impacted by a manufactured gas plant (MGP), and the other impacted by creosote) for metabolic intermediates of PAHs. 2-NA was detected at both sites, while TH-2-NA, HH-2-NA and MNA were detected at the creosote-impacted site. Young and Phelps (53) investigated the same MGP site in NJ for the presence of metabolic intermediates of both PAHs and alkylbenzenes. Both 2-NA and 2-

methylbenzylsuccinic acid were detected at the site with high abundance in the wells near and downgradient of the source. These studies demonstrated that metabolic intermediates of PAHs can be detected at the field sites and their presence can be related to the parent compounds. Thus, metabolic intermediates can be reliably used as biomarkers for detection of *in situ* biodegradation. Subsequent to the initial findings by Phelps et al. (33) metabolic intermediates of PAHs have been detected in other contaminated sites (23, 24, 32, 33, 53).

Investigations into the metabolic pathways have also lead to the identification of enzymes involved in metabolism of substrates. Benzylsuccinate synthase (Bss) (29) and analogous enzymes (10) are the only known enzymes that are involved in anaerobic activation of hydrocarbons. Bss is a member of the glycyl radical family of enzymes (39). In anaerobic toluene degradation Bss catalyzes the activation of toluene by a fumarate addition reaction to the methyl group generating benzylsuccinate (26). Bss catalyzes toluene degradation in all known isolates (26, 50). Bss like enzymes also catalyze the fumarate addition reaction to several hydrocarbon substrates including *o*-, *m*- and *p*-xylenes, *o*-, *m*-, and *p*-cresols, ethylbenzene, 2-, 3- and 4-fluorotoluenes, 1-methyl-1cyclohexane, *n*-hexane and hexadecane (7, 10, 25, 28, 49). Bss like enzymes are considered to be active also in 2-methylnaphtahlene (1, 2) and naphthalene degradation (38). Since Bss-like enzymes catalyze hydrocarbon degradation in phylogenetically diverse microorganisms only under anaerobic conditions, the presence of *bssA* gene (encoding the alpha subunit of Bss) in anoxic environments is a strong and specific indicator for the presence of microorganism that have the potential to degrade hydrocarbons under anaerobic conditions. A 16S rRNA gene based assay, as a measure

of bacteria capable of hydrocarbon degradation, is not feasible as the diversity of microorganisms associated with anaerobic degradation of hydrocarbons is vast (50).

Identification of genes encoding Bss like enzymes in the environment as an indicator of anaerobic degradation of hydrocarbons recently has been applied in a handful of studies (6, 51, 52). Quantification of the *bssA* gene has been coupled with analysis of metabolic intermediates of alkylbenzenes (6). CSIA together with analysis of the *bssA* genes and analysis of metabolic intermediates of alkylbenzenes has been demonstrated only once (6). To our knowledge, to date, the analysis of the *bssA* gene in the environment has never been demonstrated along with analysis of metabolic intermediates of PAHs.

In this study we collected groundwater samples from a MGP impacted site in NJ, analyzed them for the presence of metabolic intermediates of PAH degradation, and analyzed all samples quantitatively for *bssA* gene analogues. The MGP site is located in Glassboro, NJ. The contamination present at this site was the result of waste disposal at the now abandoned MGP that was operated for over 40 years prior to 1951. In the 1980's all surface and subsurface structures were removed during remediation of the unsaturated soil. The contaminant plume originating from the MGP extends northward, through a residential area (Figure 4.1). Since the subsurface structure is very heterogeneous, the flow of water is not expected to be uniform (53). This site was investigated by and Young and Phelps (53). At that time 2-NA was detected in several groundwater samples, but it was not related to any genetic biomarker at that time.

In the present work the aim was to study the distribution of metabolic intermediates as well as genetic biomarkers of anaerobic hydrocarbon degradation when

contaminants are present as mixed waste. This also was an opportunity to reexamine the site after 9 years, since our laboratory first studied it. This site was suitable for our purpose as it was exposed to a mixture of contaminants from the MGP site for decades, likely affecting the distribution and activity of microorganisms in the subsurface. Also, earlier study at this site by Young and Phelps (53) had identified metabolic intermediates of BTEX and PAHs in the subsurface. In the current study we hypothesized that metabolic as well as genetic biomarkers of anaerobic hydrocarbon degradation would be found at this site in areas that were still exposed to hydrocarbons. Secondly, we expect that this continual exposure of the site to hydrocarbons would have lead to enrichment of microorganisms specifically in the areas where the hydrocarbons were present, and this would be evident by the differences in concentration of *bssA* gene copies present at the site, as well as concentrations of metabolic intermediates detected. Detection and enrichment of two different biomarkers at the site would provide compelling evidence of *in situ* biodegradation of hydrocarbons. To our knowledge, to date our study is the first one to document enrichment of PAH degradation *in situ* by detection of metabolic intermediates of PAHs and genetic biomarkers for bacteria involved in hydrocarbon degradation.

4.2 MATERIALS AND METHODS

Groundwater Sampling

Groundwater samples from three pairs of monitoring wells were collected for this study (Figure 4.1). Each pair consisted of one well within the contaminant plume and one outside it. Monitoring wells (MW) 24, 29 and 40 are within the plume, while MW-15, 25 and 30 are outside the plume. MW-40 is located within the source area, while

MW-15 is close by. The second pair (MW-24 and MW-25) is further down gradient from the source and the last pair (MW-29 and MW-30) is near the down-gradient edge of the contaminant plume. Samples were collected from each of these wells in July 2008.

Groundwater sampling was conducted with the assistance of personnel from TestAmerica (Edison, NJ) and GZA (Boston, MA). Prior to collecting any samples, wells were purged at a low flow rate, until the parameters measured (pH, temperature, dissolved oxygen (D.O.), turbidity, oxidation reduction potential (ORP) and conductivity) had stabilized. The last set of readings from each well was recorded (Table 4.1), and then the contracted testing laboratory, Test America, collected samples for analysis of organics. The concentrations of select organics is provided in Table 4.2. The pump flow rate was later increased to collect samples for analysis of biomarkers. From each well two types of samples were collected, samples for analysis of genetic biomarkers and samples for analysis of metabolic intermediates.

Sample collection for analysis of genetic biomarkers

Two to four Liters of groundwater samples were filtered under vacuum through 0.22 μ M sterile filters using a sampling manifold. Only 1 L of water was filtered through each filter disk. Filters were placed in sterile 50 mL falcon tubes and were frozen immediately on dry ice. For each well at least 2 L of water was filtered. If the water sample had low turbidity (likely indicating low biomass), 4 L was filtered to collect enough biomass for DNA extraction. Between sampling different wells, the filtration assembly was rinsed with 1 L clean water. This was achieved by drawing clean water under vacuum through the filtration assembly, without the filter disk.

Sample collection for analysis of metabolic intermediates

From each well 4 L of water sample was directly collected into acid cleaned 4 L amber colored glass bottles. Samples were acidified immediately with 6N HCl to pH 2, and stored in a cooler packed with ice for transportation back to the laboratory.

Upon arrival in the laboratory, the water samples were stored at 4 °C, while the filters were frozen at -20°C until further processing.

Extraction and analysis of metabolic intermediates

One L of acidified water sample was transferred to a 2 L separatory funnel. The sample was extracted with methylene chloride two times. First, 60 mL of methylene chloride was added to the water sample. The sample was shaken vigorously for 1 min and then was allowed to separate for 10 min. The organic phase was transferred to a 500 mL round bottom flask and dried under vacuum using a rotary evaporator and a warm water bath. This was the first extraction of the sample. For the second extraction, the protocol was repeated with another 60 mL of methylene chloride with the 1 L water sample remaining in the separatory funnel. The second solvent extract was transferred to the round bottom flask with the first dry extract, and the drying procedure was repeated as above. To the dried sample 10 mL of methylene chloride was added and the round bottom flask was rinsed thoroughly. This concentrated extract was passed through a column of anhydrous sodium sulfate to remove any residual water, and was then dried under argon. The dry extract was dissolved in 3-4 mL of methylene chloride and transferred to a 7 mL HPLC vial. The sample was again dried under argon and resuspended in 100 µL of methylene chloride and vortexed. The sample was derivatized by adding 100 µL of bis(trimethyl-silyl)trifluoro-acetamide (BSTFA) and incubated at 60 degrees for 10 min. The extracted samples were stored at -20°C (33).

Extracted samples were analyzed as described by Phelps et al. (33). The samples were compared against standards of 2-NA, TH-2-NA (synthesized according to protocol in (54)), decalin-2-carboxylate and PHE-CA. Standards for HH-2-NA, and MNA were not available, and the identification of these metabolites was done by comparison with published mass spectra (46, 54).

DNA extraction and PCR analysis

DNA was extracted from filters by using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Extract from each filter disk was eluted using eight elution columns and all extracts were pooled together. The DNA concentrations in the extract were measured by U.V. spectrophotometer measuring the absorption at 260 nm. The DNA extracts were stored at -20°C until further use. Although use of internal standard is preferred for extraction of DNA from environmental samples to quantify the extraction efficiency, it was not used in our study. This is because we observed that the *bssA* gene PCR, not the 16S rRNA gene PCR, was inhibited when *Halobacterium* DNA was added to the filters as an internal standard before extractions (data not shown).

End-point PCR analysis of the 16S rRNA gene and *bssA* (52) gene was performed on each DNA extract. *Bacteria* specific 16S rRNA gene primers were used for this analysis to ensure the presence of eubacteria in the water samples. Sequences of primers used for end-point PCR analysis is given in Table 4.3(A). The PCR program for 16S rRNA genes included denaturation at 95°C for 5 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min 30 s, followed by 10 min extension at 72°C. The *bssA* gene PCR protocol and program was as described by Winderl et al. (52). It included a denaturation at 94 ° for 5 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and

72°C for 1 min, followed by extension at 72 °C for 5 min. The 16S rRNA gene PCR included 1X PCR buffer, with 0.2 mM of dNTPs, 0.2 µM of each primer and 1.5 units of Red Taq in a 50 µL reaction. The *bssA* gene PCR included 1X PCR buffer, with 0.1 mM of dNTPs, 0.5 µM of each primer, 10 µg of BSA and 1.5 units of Red Taq in a 50 µL reaction (52). All reagents were from Sigma (St. Louis, MO) unless specified otherwise. All PCRs were performed with equal volume and equal mass of DNA to account for any potential inhibitors in the DNA extract and the mass of DNA template that may affect the PCR results.

The qPCR analysis of 16S rRNA gene and *bssA* gene was performed on all DNA extracts for quantitative analysis of molecular biomarkers using SYBR green assay. For 16S rRNA gene analysis, primers by Suzuki et al. (47) were used. For *bssA* gene analysis primers developed by Beller et al. (5, 6) were used. This included two sets of primers, one for toluene-degrading denitrifying bacteria (5), and the other for sulfate-reducing /methanogenic toluene-degrading bacteria (6).

A modification of the qPCR protocol by Beller et al. (5, 6) was used for 16S rRNA gene and *bssA* gene analysis. For qPCR analysis 2X PCR buffer, 0.5 mM dNTPs, 3 mM MgCl₂, 1.25 units of Taq, 0.5 units of Amperase UNG, and 5 µL of diluted or undiluted DNA extract was used. In addition, 300 nM of primers (Table 4.3B) for *bssA* gene PCR in denitrifying toluene-degrading bacteria and 16S rRNA gene PCR analysis was used, while 400 nM of primers for *bssA* gene PCR in sulfate-reducing/methanogenic toluene-degrading bacteria was used. For each *bssA* gene qPCR 10 µg of BSA was also used. Primers were from Sigma (St. Louis, MO), BSA was from New England Biolabs (Ipswich, MA), and all other reagents were Applied Biosystems (Foster City, CA). The

program for qPCR analysis included incubation for 2 min at 50°C, followed by denaturation at 94°C for 10 min followed by 45 cycles of 94°C for 15 s and 58°C for 1 min. This was followed by a dissociation stage, 30 s at 94 °C, 1 min at 58°C and 30 s at 94°C. For 16S rRNA gene analysis only 35 cycles were used. All samples and standards were analyzed in triplicates.

Calibration curves for quantification of *bssA* gene in toluene-degrading bacteria was prepared by using dilution of *Thauera aromatica* T1 genomic DNA, while the calibration curve for *bssA* gene in sulfate-reducing/methanogenic bacteria was made by using dilutions of *Desulfobacterium cetonicum* DSM7627 (*Desulfosarcina cetonica* DSM7267) genomic DNA. The concentrations of genomic DNA extracts were measured by U.V. spectrophotometer measuring the absorption at 260 nm. The number of gene copies in the genomic DNA extracts was calculated by using the equation,

$$\text{Gene copies } \mu\text{L}^{-1} = (\mu\text{g DNA } \mu\text{L}^{-1} / \text{bp genome}^{-1}) (\text{bp } \mu\text{g}^{-1} \text{ DNA}) (\text{genes genome}^{-1})$$

For qPCR analysis we assumed that (i) the size of the genomic DNA of each *Thauera aromatica* T1, and *Desulfobacterium cetonicum* DSM7627 was 4.6 Mbp. (ii) the genomic DNA of *Thauera aromatica* T1 had only one 16S rRNA gene and 1 *bssA* gene, and genomic DNA of *Desulfobacterium cetonicum* DSM7627 has only 1 *bssA* gene. (iii) the 16S rRNA gene calibration curves prepared with genomic DNA of *Thauera aromatica* T1 and the *bssA* calibration curve prepared with genomic DNA of *Desulfobacterium cetonicum* DSM7627 were representative of the other bacteria in the environment.

4.3 RESULTS

4.3.1 Characteristics of the water samples

Before collecting samples for analysis of organic contaminants and biomarkers of anaerobic hydrocarbon degradation, each monitoring well was purged until parameters such as pH, temperature, dissolved oxygen, turbidity, ORP, and conductivity had stabilized. The last set of stable readings prior to sampling is summarized in Table 4.1.

All wells within the plume area (MW-24, MW-29, and MW-40) were characterized with very low dissolved oxygen (0.3 to 0.4 mg/L) and negative ORP (-169.5 to -47.9 mV), while two of the unimpacted wells (MW-15 and MW-25) had positive ORP (46.0 to 76.9 mV) and relatively higher dissolved oxygen concentration (2.5 to 4.4 mg/L). These results indicate that the wells within the plume area had depleted levels of dissolved oxygen and had developed highly reducing conditions. Thus, microbial activity in this area is expected to be occurring mainly under anaerobic conditions. MW-30, which is outside the plume area, had high positive ORP, but low dissolved oxygen concentration suggesting the presence of microbial activity or redox reactions in this area. The differences in turbidity and conductivity of the water samples reflect the heterogeneous nature of the subsurface.

4.3.2 Contaminants present in the water samples

The concentrations of volatile organic compounds and semi-volatile organic compounds were reported by GZA, Boston. Concentrations of select organic contaminants are listed in Table 4.2.

MW-40, which is located within the source area, had a high concentration of BTEX and naphthalene (1724 µg/L and 19.0 µg/L respectively). The concentration of phenanthrene in this well was very low (0.047 µg/L). MW-24 is downstream of the source and had no detectable BTEX, while a low concentration of naphthalene was

present (0.5 µg/L). MW-29 is also within the impacted area and had no detectable naphthalene and phenanthrene, while BTEX was detectable at this well (121.9 µg/L). Since, MW-29 is down gradient of MW-24, and the source (near MW-40), loss of BTEX due to physical processes such as dilution and dispersion are expected to decrease the concentration of BTEX from the source (MW-40). Thus, BTEX from MW-40 does not seem to account for contaminants at MW-29, and therefore BTEX at MW-29 may represent contamination from a separate source. Alternatively, the groundwater flow may not be directly from the area of MW-40 to MW-24 to MW-29.

MW-15, MW-25 and MW-30 are outside the plume area, and BTEX was not detected in these wells during the July 2008 sampling event (or in 2007, data provided by GZA). Phenanthrene was detected in MW-30 in July 2008, and comparable concentrations were also detected in MW-15 and MW-25 at least once in the last 9 years. Since BTEX, naphthalene and 2-methylnaphthalene were not detected in monitoring wells MW-15 and MW-25, which are outside the plume, these wells can be used as control wells in our study.

4.3.3 End-point PCR analysis

16S rRNA gene PCR

DNA extracts from all monitoring wells were amplified using a primer pair (Table 4.3A) specific for *Bacteria* and were analyzed using agarose gel electrophoresis (Figure 4.2A). 16S rRNA gene PCR products of expected size (~1100 bp) were obtained from all samples. These results indicate that all the samples contained eubacterial DNA, and that the effect of PCR inhibitors in the DNA extract, if any, was not evident.

***BssA* gene PCR**

DNA extracts from all monitoring wells were amplified using *bssA* gene primers (Table 4.3A). PCR product of the expected size (~794bp) was obtained from all impacted wells (MW-24, MW-29 and MW-40), while no detectable PCR products were obtained from non-impacted wells (MW-25 and MW-30) (Figure 4.2B). Detection of the *bssA* genes specifically in the impacted wells clearly indicates that bacteria capable of anaerobic hydrocarbon degradation are present in the groundwater at these wells. A weak amplification of *bssA* gene was observed with DNA extract of groundwater from MW-15 (is not readily visible in the gel, Figure 4.2B). This signal of *bssA* gene in the non-impacted groundwater might be explained by cross contamination during processing of the samples. Another possibility is that groundwater at MW-15, which is somewhat upgradient and to the side of the source area (MW-40), has come in contact with groundwater from MW-40 due to localized flow patterns. This mixing may be transporting bacteria or hydrocarbon substrates to the area of MW-15.

Some amplicons of smaller size were also observed in PCR products when DNA extracts from MW-29 and MW-40 were amplified with degenerate *bssA* gene primers (Figure 4.2B). These results indicate non-specific amplification of genes in the environmental genomic DNA extracts. A summary of end-point PCR results is provided in Table 4.4 (A).

4.3.4 qPCR analysis

qPCR analysis was used to quantify the 16S rRNA gene and *bssA* gene copies in the groundwater samples. *Thauera aromatica* T1 genomic DNA was used to construct a calibration curve for quantification of 16S rRNA gene copies and *bssA* gene copies in toluene-degrading, denitrifying bacteria. *Desulfobacterium cetonicum* DSM7627

genomic DNA was used to construct a calibration curve for quantification of *bssA* genes with primers for toluene-degrading, sulfate-reducing/methanogenic bacteria. The calibration curve for *bssA* assay in denitrifying bacteria and sulfate-reducing/methanogenic bacteria had a linear range from 9.9 to 1.9×10^5 ($r^2 = 0.999$) gene copies and 1.98×10^2 to 1.98×10^5 gene copies ($r^2 = 0.998$), respectively. (A linear *bssA* calibration curve for sulfate-reducing/methanogenic bacteria ($r^2 = 0.995$) with a range of 1.98 to 1.98×10^5 could be obtained, but the lowest concentration standards had high standard deviations in the cycle threshold (Ct) values and melting temperature (T_m), and were therefore not used for analysis). The standard curve for 16S rRNA gene assay in eubacteria had a linear range from 1.98×10^2 to 1.9×10^5 gene copies ($r^2 = 0.998$). Fluorescence due to primer dimer formation was not evident in the qPCR analysis. The efficiencies of amplification of 16S rRNA gene in *T. aromatica* T1 genomic DNA, *bssA* gene in *T. aromatica* T1 and *bssA* gene in *D. cetonicum* DSM7627 were 78.8%, 81.1% and 79.3%, respectively. All these data indicate that qPCR analysis could be satisfactorily used for analysis of samples, and that primer dimer formation has minimal effect on the results of our analysis.

***bssA* gene analysis**

The qPCR assay for *bssA* genes in denitrifying toluene-degrading bacteria in the groundwater samples yielded positive results only when DNA extracts from MW-24 were used for analysis. The melt curve analysis showed that the fluorescence in the sample was due to the presence of only one major peak similar to that in the standards (T_m = 83.3°C), and not due to the presence of primer dimers. The negative control did not have any detectable PCR product. In MW-24, 2.55×10^3 copies/L of *bssA* gene were

detected (Table 4.4 B). These results indicate that toluene-degrading denitrifying bacteria were highly enriched in MW-24, which is downstream from the source (MW-40), while similar bacteria were not detectable at the other impacted or non-impacted wells.

The result of qPCR assay for *bssA* genes in sulfate-reducing/methanogenic, toluene-degrading bacteria in the groundwater samples were unlike those obtained by analysis of the *Desulfobacterium cetonicum* DSM7627 standards. Fluorescence above the background was obtained in all samples, but some samples did not amplify consistently. The melt curve analysis did not show presence of any primer dimer, but PCR products with $T_m \pm 1.9^\circ\text{C}$ (as compared with the standard) were obtained. These results indicate that PCR products similar to the standard, but likely differing in the length or G + C content were obtained from the environmental samples. Consistent T_m and C_t values were only obtained from samples of MW-24 (std. dev. 0.56 and 0.23 respectively). T_m values of samples from MW-29 were also consistent (std. dev. 0), but the C_t (std dev. 0.84) values were below our quantification limit (data not shown). The deviation in the C_t values of other samples increased with the increase in the C_t values, and this is consistent with results obtained with standards of low concentration (data not shown). C_t values obtained with samples from MW-24 had low std. dev. (± 0.23). We calculated that MW-24 contained 1.66×10^4 copies/L of *bssA* gene (Table 4.4 B). Our results indicate that MW-24 contains a relatively high abundance of the analogues of *bssA* genes in toluene-degrading sulfate-reducing/methanogenic bacteria. These results provide evidence that the microbial community in MW-24 is enriched for anaerobic hydrocarbon degradation under sulfate-reducing or methanogenic conditions. Low

concentrations of similar genes are likely present in MW-29, but are below the quantification limit of our assay.

16S rRNA gene analysis

qPCR analysis was used to quantify eubacterial 16S rRNA genes in the water samples. Fluorescence above the background was obtained in all the groundwater samples, and melt curve analysis did not show presence of a primer dimer. The abundance of 16S rRNA genes was quantified in all the samples (Table 4.4 B). The results confirm the findings of our endpoint 16S rRNA gene PCR analysis, that a bacterial population was detectable in all the groundwater samples. Comparison of gene copy numbers in various wells shows that the highest density of 16S rRNA genes was detectable in MW-29, which was an impacted well farthest from the source. The relative abundance of the 16S rRNA genes in the samples was in the order MW-29> MW-24> MW-40>MW-25> MW-15> MW-30. These results indicate that bacterial population density in all the impacted wells (MW-24, MW-29 and MW-40) was 1-2 orders of magnitude higher than in the non-impacted wells (MW-15, MW-25 and MW-30). These results are consistent with the site characteristics, which show continual exposure of the impacted wells to organic contaminants over the decades. Thus, the higher abundance of 16S rRNA genes in the impacted wells strongly indicates growth in bacterial density due to the effect of organic carbon from the contaminants.

4.3.5 Analysis of metabolic biomarkers

Solvent extracts of all water samples were derivatized with BSTFA before GC-MS analysis. Metabolic intermediates in the samples were compared with the retention time (RT) and mass spectra of standards of 2-NA, TH-2-NA, decalin-2-carboxylate and

PHE-CA, that were also analyzed similarly. Identification of HH-2-NA, and MNA was done by comparison with published mass spectra. Mass spectra of standards and metabolites detected in the samples are provided in Figures 4.3-4.6. In all of the samples a metabolite was detected at RT and mass spectra similar to 2-NA standard (Figure 4.3A). All samples showed the presence of the diagnostic fragments (m/z 115, 127, 155, 185, 229, 244) (Figure 4.3B-G) indicating that the metabolite detected in the samples was 2-NA. With a similar analysis TH-2-NA (m/z 159, 189, 233, 248) was identified in samples from MW-24 and MW-40 (Figure 4B, D). In samples from MW-15, MW-25 and MW-29, all of the diagnostic fragments of TH-2-NA could not be identified due to low abundance (Figure 4C, E and F). HH-2-NA (m/z 161, 191, 235, 250) was detected only in 3 water samples, MW-24, MW-40, and MW-30 (Figure 5A, B, C).

Detection of 2-NA, TH-2-NA, and HH-2-NA – metabolic intermediates of anaerobic naphthalene, and 2-methylnaphthalene degradation – at the impacted wells MW-24, and MW-40 indicates that degradation of naphthalene and 2-methylnaphthalene by anaerobic bacteria is taking place in the impacted subsurface. Although 2-NA can also be produced aerobically from 2-methylnaphthalene (15), its presence along with the detection of ring-reduction intermediates (TH-2-NA, and HH-2-NA), which are formed only under anaerobic conditions, indicates anaerobic microbial degradation of naphthalene and 2-methylnaphthalene is dominant in these wells. In MW-24, which is downgradient of the source area (MW-40), MNA (a metabolic intermediate of anaerobic 2-methylnaphthalene degradation) was also detected, but it was not detected in MW-40, in the source area. These results indicate that the microbial community in the area of

MW-24 is actively degrading naphthalene and 2-methylnaphthalene anaerobically. A summary of all the metabolites detected in the water samples is provided in Table 4.5.

It is not surprising that 2-NA and TH-2-NA were also detected in the wells outside the plume, as well as in wells downgradient of the source (within the plume area, but without detectable naphthalene or 2-methylnaphthalene). The products of metabolism of naphthalene and 2-methylnaphthalene, including ring reduction intermediates 2-NA, TH-2-NA, and HH-2-NA are more water soluble (e.g, water solubility of 2-NA is 47 mg/L at 25°C) than the parent compounds (e.g, water solubility of naphthalene is 31 mg/L at 25°C) and can explain their presence in wells without detectable substrates. The water soluble metabolites can migrate downgradient with the groundwater flow and also can be distributed through physical processes such diffusion and dispersion. Therefore, physical characteristics of the metabolic intermediates, and physical processes at the site are most likely responsible for detection of metabolic intermediates in wells without detectable PAHs.

Abundance of 2-NA and TH-2-NA was quantified by comparison with a calibration curve. Base peaks m/z 229 of 2-NA and m/z 233 of TH-2-NA were used for quantification of the metabolites. Table 4.6 gives concentrations of 2-NA and TH-2-NA detected in the samples. Although TH-2-NA was detected in all the samples, except MW-30, its abundance could not be quantified in all samples as the concentrations were below the quantification limit. A comparison of the concentrations in Table 4.6 shows that the highest concentrations of 2-NA and TH-2-NA were detected in MW-24. This indicates that MW-24, which is downgradient of the source area (MW-40), contains a microbial community highly enriched for anaerobic PAH degradation. This is also

underscored by the fact that MNA (a metabolic intermediate of anaerobic 2-methylnaphthalene degradation) was only detected in MW-24. Since HH-2-NA could not be quantified directly, we compared the abundance base peak m/z 235 in MW-24, MW-30 and MW-40. The relative abundance was in the order MW-24>MW-40>MW-30. These data further indicate that MW-24 has microbial populations actively degrading PAHs.

4.4 DISCUSSION

This study gave an unique opportunity to revisit the MGP impacted site at Glassboro, NJ, after about 8 years since we first evaluated the site for its potential to undergo natural attenuation. In the earlier study at this site, metabolic intermediates of BTEX and PAH degradation were used as biomarkers of *in situ* biodegradation (33, 53). In the present work we were able to apply qualitative and quantitative analysis of metabolic intermediates of hydrocarbon degradation, as well as of the catabolite gene *bssA*. We compared groundwater samples from within and outside the plume at the site. Quantitative analysis of metabolic intermediates of anaerobic PAH degradation was performed along with qualitative and quantitative analysis of the 16S rRNA gene specific for *Bacteria*, and *bssA* gene analogues.

Such an analysis allowed the examination of the site for any changes that might have occurred during the last 8 years, and most importantly we were able to co-relate the results obtained this time by the analysis of 2 different types of biomarkers amongst themselves, as well as with the earlier data. Metabolic intermediates are present in the subsurface as result of microbial activity, while specific genes are a more direct indication of the presence of bacteria with potential to degrade hydrocarbons

anaerobically. Detection of metabolic intermediates along with genes specific for bacteria involved in microbial hydrocarbon degradation provides strong evidence for the anaerobic biodegradation processes in the subsurface.

In our study we observed that the impacted wells (in the plume area) had detectable BTEX and/or naphthalene concentrations (Table 4.2). The impacted wells at the site had also developed highly reducing conditions, indicated by negative ORP and low dissolved oxygen concentrations (Table 4.1). Thus, most microbial activity at the active was expected to be anaerobic.

Metabolic intermediates

Several metabolic intermediate were detected in the impacted wells that are specific to anaerobic hydrocarbon degradation. These include 2-NA and TH-2-NA, in the groundwater samples of almost all of the wells that were sampled (except MW-30, see Table 4.5), while HH-2-NA was detected in 2 wells within the plume (MW-24, MW-40) and one well outside the plume (MW-30). 2-NA, TH-2-NA and HH-2-NA have been identified as metabolites of anaerobic naphthalene (54, 55) and 2-methylnaphthalene (1, 46) degradation in laboratory enrichments under sulfate-reducing conditions.

The ring reduction metabolites are unique to anaerobic degradation processes — these are not produced during aerobic degradation. They are not found in groundwater as a result of other background natural processes, and they also satisfy other prerequisites for metabolic indicators (uniquely related to parent compounds, released outside microbial cells, products of degradation, no commercial use, degradable, and water soluble, (3) (53)). Therefore, the presence of these intermediates can be used as unequivocal indicators of anaerobic PAH biodegradation.

Thus, the detection of metabolic intermediates (2-NA, TH-2-NA, and HH-2-NA) in the groundwater provides evidence for anaerobic degradation of naphthalene and 2-methylnaphthalene in the subsurface. Although 2-NA can be produced from 2-methylnaphthalene by aerobic degradation processes (15), detection of 2-NA along with the ring reduction intermediates (TH-2-NA, HH-2-NA) provides evidence that these intermediates were produced as a result of anaerobic not aerobic degradation processes.

Evidence specific for 2-methylnaphthalene degradation at the site was also obtained. In our analysis MNA was detected in MW-24. Sullivan et al. (46) identified MNA as a metabolite of anaerobic 2-methylnaphthalene degradation. Although 2-methylnaphthalene was not detected in the groundwater samples collected in July 2008, it was detected in MW-24 in Sept 2007 (4.2 $\mu\text{g/L}$). Comparison with earlier data (courtesy of GZA, Boston) shows decreasing 2-methylnaphthalene concentrations at MW-24. MNA is not considered a dead-end product of 2-methylnaphthalene, and thus, it is unlikely that MNA has accumulated in the groundwater as a result of degradation in the past. Therefore, we conclude that MNA was produced as a result of active degradation of 2-methylnaphthalene.

PHE-CA has been identified as a product of anaerobic phenanthrene degradation under sulfate-reducing conditions in enrichment cultures (14, 54). We did not detect PHE-CA in any of our samples. This can be explained by the very low concentrations of phenanthrene (0.026-0.072 $\mu\text{g/L}$) in the July 2008 samples that were used for analysis. Earlier data (provided by GZA) shows that in 1999, phenanthrene concentrations were in the range 0.9-1.4 $\mu\text{g/L}$, and in 2007 they were 0.048-0.5 $\mu\text{g/L}$. Thus, it appears that phenanthrene concentrations at this site have been decreasing, and this is likely

responsible for the lack of detectable PHE-CA, which is a product of initial carboxylation of phenanthrene, in the groundwater samples (55).

The parent compounds, naphthalene, 2-methylnaphthalene, and other PAHs have low water solubility (31 mg/L and 24 mg/L at 25°C respectively for naphthalene and 2-methylnaphthalene), compared to the metabolites (2-NA, 47 mg/L at 25 °C) that are produced by anaerobic degradation processes. This can be applied to interpret some of our observations. We detected 2-NA, TH-2-NA and HH-2-NA in MW-15, MW-25, MW-29, and MW-30 (Table 4.5) without detectable naphthalene (Table 4.2). The solubility of the metabolites, together with the location of the monitoring wells (Figure 4.1), and the direction of groundwater flow suggests that the detection of the metabolites in the wells without detectable naphthalene was likely due to the migration and distribution of soluble metabolites due to physical processes.

The abundance of 2-NA and TH-2-NA in the samples was quantified (Table 4.6) by using available standards, but the abundance of HH-2-NA could not be quantified due to lack of suitable standards. The highest concentrations of both 2-NA and TH-2-NA (6.66 µg/L and 2.56 µg/L respectively) were detected in MW-24, which was downgradient of the source. TH-2-NA could be quantified only in 2 wells (MW-24 and MW-40), while it was below the quantification limit in other wells. The relative abundance of HH-2-NA in the monitoring wells was in the order MW-24>MW-40>MW-30, while MNA was only detected in MW-24. Detection of not one, but four different metabolites in MW-24, at high relative abundance along with low concentration of naphthalenes (0.5 µg/L of naphthalene), provides strong evidence that MW-24, within the

plume, has been enriched for anaerobic biodegradation of PAHs as compared to other wells in this study.

Concentrations of metabolites detected at this site in July 2008 samples are consistent with the observations made by Phelps et al. and (33) and Young and Phelps (53). In our study it was observed that the highest concentrations 2-NA and TH-2-NA were 6.66 µg/L and 2.56 µg/L respectively. Phelps et al. observed that the highest concentration of 2-NA was 4 µg/L, while Young and Phelps noted that it was 5.1 µg/L. Other studies have noted higher concentrations of metabolic intermediates of naphthalenes. Griebler et al. (24) noted that 2-NA was detected all over a field site with concentrations ranging 0.2-45 µg/L, which was likely due to higher naphthalene concentrations in this area (14 µg/L -86 mg/L).

In our study the ratios of 2-NA/TH-2-NA in MW-40 and MW-15 were 4.2 and 2.6, respectively. This was in the range of ratios observed by Griebler et al. (24) (1.4 to 9, $n = 3$). These data indicate that the metabolite 2-NA was degraded to TH-2-NA, which was degraded further to down stream metabolic intermediates in the subsurface, at rates comparable to those observed at other sites.

Over all distribution of the metabolites observed by Young and Phelps (53) at the NJ site, and observations made in our study indicate that enrichment of microbes for PAH degradation at this site has taken place within 30-500 feet from the source area. In addition, our data shows that enrichment of PAH degradation was in the area downgradient of the source, and not at the source. This was likely due to the presence of easily metabolized hydrocarbons (BTEX) at the source well, which limited the degradation of PAHs.

Young and Phelps (53) also investigated the site for *in situ* degradation of alkylbenzenes. It was observed that concentrations of 2-methylbenzyl succinate (fumarate addition product of *o*-xylene) in the wells were related to the concentrations of toluene, and the highest concentration was found in MW-24 (<500 ft down gradient, 200 µg/L of toluene). Although we did not analyze samples for metabolic intermediates of alkylbenzenes, our study identified the highest concentrations of the PAH metabolites in MW-24, indicating high microbial activity in the same well as identified by Young and Phelps (53).

If a comparison of concentrations of parent hydrocarbons in MW-40, MW-15, MW-24, MW-29, MW-30 and MW-40, in 1999, 2007 and July 2008 is done (data provided by GZA), then a trend of decreasing concentrations can be observed. Decreases in the concentrations of the parent compounds can be due to physical processes, chemical transformations, and processes of biodegradation. Our data from analysis of July 2008 samples clearly shows enrichment of two different biomarkers specific to anaerobic hydrocarbon biodegradation in the impacted wells, as compared to the non-impacted wells. In addition Phelps and Young (53) also detected metabolic intermediates specific to anaerobic degradation of PAHs and BTEX at this site. These data from independent studies, clearly provides evidence that contaminant loss at the site over the years is also due to processes of biodegradation.

Metabolic intermediates of PAHs have been detected at other sites impacted with hydrocarbons. Phelps et al. (33) studied two sites, one impacted by gasoline (maintenance yard at Galssboro, NJ) and other impacted by creosote from a wood treatment facility. 2-NA was found in groundwater at both sites, and TH-2-NA, HH-2-

NA and, and MNA were detected at the creosote site. Similarly Griebler et al. (24), Ohlenbusch et al. (32) and Gieg et al. (23) detected metabolic intermediates of naphthalene and 2-methylnaphthalene at different contaminated sites, 2-NA being the more common. A common finding in these studies is that metabolic intermediates of anaerobic naphthalene and 2-methylnaphthalene degradation (2-NA, TH-2-NA, HH-2NA, MNA) could be detected in the subsurface environments with substrate concentrations $< 1\mu\text{g/L}$. These metabolic intermediates could be detected even when PAHs were present in combination with BTEX contaminants, which are relatively easily metabolized. Our findings and these data indicate that metabolic intermediates of PAHs can be detected at contaminated sites, which are different in terms of their physical and chemical characteristics, structural features and groundwater flow patterns, but all of which have developed anaerobic conditions. This indicates that metabolic intermediates of PAHs can be reliably used as specific biomarkers of anaerobic hydrocarbon degradation, irrespective of the subsurface characteristics.

16S rRNA gene analysis and *bssA* gene analysis

End-point as well as qPCR analysis of the 16S rRNA gene and *bssA* gene analogues was performed on all samples. Although a bacterial community was detectable in all the samples (Figure 4.1A), *bssA* genes were detectable by end-point PCR analysis only in wells within the plume area. This indicates enrichment of hydrocarbon degrading bacteria particularly within the plume as an effect of the contaminants.

Eubacterial 16S rRNA gene and *bssA* gene copies were quantified in all of the wells. We observed that wells within the plume area (MW-24, MW-29, and MW-40) had 16S rRNA gene copies 1-2 orders of magnitude higher in concentration, as compared to

the wells outside the plume area (MW-15, MW-25, MW-30) (refer to Table 4.4 B), which indicates enrichment of *eubacterial* populations specifically in the contaminated wells due to nutrients from the plume. The highest concentration of 16S rRNA gene copies was detected in MW-29 (Table 4.4B), which is downgradient of the source area. MW-29 had high concentration of BTEX (121.9 µg/L) (Table 4.2), and earlier data (from 1999 and 2007 provided by GZA, not shown) show a decreasing trend in concentrations. PAHs were not detected at the site in 1999, 2007 or 2008. Thus, the high concentrations of microorganisms in this area are likely due to the presence of more easily metabolized BTEX compounds. However, the abundance of 16S rRNA gene copies in MW-29 is comparable to other wells (MW-40 and MW-24), which are within the plume. This data indicates that bacterial cell densities in the impacted wells were equally enhanced.

Quantitative PCR analysis of the *bssA* gene with primers for toluene-degrading, denitrifying, and sulfate-reducing/methanogenic bacteria was performed for all of the samples. The highest concentrations of *bssA* gene copies were detected in MW-24, with both primers for toluene-degrading, denitrifying or sulfate/reducing and methanogenic bacteria (refer Table 4.4 B). Since a signal was not detected when samples from other wells, within and outside the plume area, were analyzed with primers for toluene-degrading bacteria, it can be concluded that denitrifying hydrocarbon degrading bacteria at these wells were below our detection limit. Low concentrations of genes in sulfate/reducing and methanogenic bacteria were likely also present in MW-29 but could not be quantified by our assay. A comparison of 16S rRNA gene and *bssA* gene qPCR analysis shows that although bacterial cell density was enhanced at all monitoring wells, bacteria capable of anaerobic hydrocarbon degradation were specifically enriched at

MW-24 (Table 4.4 B). The enrichment of anaerobic hydrocarbon degradation at MW-24 was also underscored by detection of the highest concentrations of metabolic intermediates of naphthalene and 2-methylnaphthalene (Table 4.6) at this site. These data indicate that the microbial community at MW-24 was highly enriched for hydrocarbon degradation and was actively degrading PAHs.

Only a few studies have done quantitative analysis of *bssA* genes in environmental samples, and therefore limited data are available for comparison. We detected 2.55×10^3 copies/L and 1.66×10^4 copies/L of *bssA* genes in toluene-degrading denitrifying and sulfate-reducing/methanogenic bacteria respectively, in MW-24. Feris et al. (21) used *bssA* gene primers for toluene-degrading denitrifying bacteria to study the effect of ethanol on natural attenuation of monoaromatics in a sulfate-reducing aquifer, and did not detect any *bssA* genes.

Beller et al. (6) used quantitative *bssA* gene analysis on groundwater samples obtained in a field test. This study involved addition of BTEX with or without ethanol to the groundwater, and the changes in *bssA* gene copies were analyzed with respect to time. In comparison, the site that we studied in NJ has historic contamination from a MGP that operated for over 40 years. Therefore our study, and study performed earlier at this site (33, 53) are representative of *in situ* remediation processes at aged sites with ‘natural’ contamination events. A direct comparison of results in our study with that of Beller et al. is therefore not appropriate, but is presented here for academic purposes (6).

The abundance of *bssA* genes in our study (Table 4.3B) was comparable to the lower range detected by Beller et al. (6). It was reported that background concentration of *bssA* genes in toluene-degrading sulfate-reducing/methanogenic bacteria (detected

with the same primers as used in our study) in an aquifer under study was 2×10^4 gene copies/L ($n = 2$ out of 40, other samples exhibited PCR inhibition) (6). It was observed that after the release of BTEX in the subsurface, the *bssA* gene concentration ranged from 10^4 to $>10^6$ copies/L. In addition, the *bssA* gene copies in the BTEX with ethanol treatment were higher (3-63 fold) in 3 out of the 4 transects studied. In our study, we detected the highest abundance of *bssA* genes (2.55×10^3 and 1.66×10^4 gene copies/L in toluene-degrading denitrifying or sulfate-reducing/methanogenic bacteria, respectively) in a monitoring well with a low concentration of naphthalene ($0.5 \mu\text{g/L}$) and no detectable BTEX. In contrast, in the study by Beller et al., 1-3 mg/L of each monoaromatic hydrocarbon (BTEX) was used with or without 500 mg/L of ethanol. Thus, the low abundance of *bssA* gene copies detected in our study as compared to Beller et al. is likely a result of low substrate concentration. Although investigations by Beller et al. have demonstrated the application of the qPCR protocol to environmental samples, our study actually illustrates the application of this molecular tool for evaluation of an unmanipulated contaminated subsurface site and its changes over a 9 year period of time.

Even though the *bssA* genes were detected in all of the wells within the plume with end-point PCR analysis, we were unable to detect *bssA* gene copies from denitrifying and sulfate reducing/methanogenic bacteria with qPCR analysis in 2 of 3 wells within the plume. All of the wells within the plume had developed negative ORP indicating anaerobic conditions, therefore it is expected that wells with BTEX would contain detectable *bssA* genes from denitrifying or sulfate-reducing bacteria. The results of the *bssA* assay for denitrifying bacteria clearly indicated enrichment of denitrifying toluene-degrading bacteria in MW-24. This assay could detect 7.93×10^2 gene copies/L,

but did not yield detectable PCR product in any other well other than MW-24. This data indicates that it was lack of enrichment of denitrifying toluene-degrading bacteria at these wells that was likely responsible for absence of detectable fluorescence in the samples, and the detection limit of the qPCR assay (for *bssA* gene in denitrifying toluene-degrading bacteria) was not a limiting factor.

In our *bssA* gene assay for sulfate-reducing/methanogenic bacteria, we were able to detect *bssA* gene copies only in MW-24, while *bssA* the gene concentrations in other wells were below our detection limit. The detection limit of this assay was higher (1.59×10^4 copies/L), which was likely responsible for the lack of detectable *bssA* gene copies in samples from monitoring wells other than MW-24. Data from Beller et al. (6) supports this point, namely that the detection limit of the assay can be a problem.

Another possible reason for the apparent lack of detection of *bssA* gene copies in monitoring wells within the plume other than MW-24, is that the *bssA* gene diversity at the site was not captured by these two primer sets. These primers were designed (6) by using *bssA* gene sequence from only toluene-degrading denitrifying/sulfate-reducing bacteria, and thus may not be representative of all possible bacterial *bssA* gene populations. Limited coverage of *bssA* gene diversity at this site is likely affecting our results, since we were able to detect *bssA* genes in all of the impacted wells by end-point PCR analysis. We used degenerate primers developed by Winderl et al. (51), who noted that these primers could detect *bssA* genes in bacteria other than denitrifying bacteria, including sulfate reducers, although the primers were based on denitrifying bacteria. In spite of these limitations our results clearly show that toluene-degrading sulfate-

reducing/methanogenic bacteria were specifically enriched in MW-24 as compared to other wells.

In conclusion, our study provides conclusive evidence for *in situ* anaerobic hydrocarbon biodegradation at this MGP impacted site in NJ. The analysis provides strong evidence using not one but two different biomarkers of anaerobic hydrocarbon degradation. Several metabolic intermediates of anaerobic PAH degradation were detected at the site. In addition we also detected *bssA* gene analogues specifically within the impacted wells. High concentrations of metabolites as well as high concentrations of 16S rRNA gene copies and *bssA* gene copies were detected in MW-24, which is within the plume and downgradient of the source. Thus, abundance and distribution of genetic biomarkers is correlated with the abundance of the metabolic intermediates. Our data clearly demonstrates enrichment of *in situ* anaerobic hydrocarbon degradation by using dual biomarker analysis: metabolic intermediates of PAH degradation and *bssA* gene as a genetic biomarker.

Detection of *in situ* biodegradation of anaerobic hydrocarbons has progressed substantially because of the understanding of several anaerobic degradation processes that have been developed through laboratory studies. Isolation of pure cultures has played a crucial role in this development. Many more questions about anaerobic degradation of hydrocarbons still remain unanswered, and research in this area will greatly benefit from field studies, like this one. We have demonstrated that metabolic intermediates and genetic biomarkers can be detected at hydrocarbon impacted sites. It is important to understand how soon such biomarkers can be detected in the subsurface after a contamination event. Some studies with alkylbenzenes have already investigated

this question to some extent, but biomarkers of PAH degradation have not been studied as a function of time after a contamination event. Such studies are important and can potentially make valuable contributions to the field.

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Table 4.1. Characteristics of the groundwater samples

Well	MW-40	MW-15	MW-24	MW-25	MW-29	MW-30
pH	5.4	6.1	6.8	10.0	4.9	6.5
Temp (°C)	17	16.3	20.2	18.8	18.7	18
D.O. (mg/L)	0.4	2.5	0.4	4.4	0.3	0.4
ORP (mV)	-169.5	76.9	-9.3	46.0	-47.9	118.4
Turbidity (NTU)	2.1	2.7	33.5	2.3	6.5	21.1
Conductivity (µs/cm)	433	487	696	199	176	75
Depth to water (ft)	8.43	10.35	8.62	13.57	5.46	5.15
Pumping rate (mL/min)	200	180	160	100	120	220

D.O.: Dissolved oxygen, ORP: Oxidation-reduction potential

Table 4.2. Concentration (µg/L) of contaminants in the monitoring wells

	MW-40	MW-15	MW-24	MW-25	MW-29	MW-30
BTEX	1724	-	-	-	121.9	-
2-Methylnaphthalene	-	-	-	-	-	-
Naphthalene	19	-	0.5	-	-	-
Phenanthrene	0.047	-	0.049	-	-	0.024

BTEX: benzene, toluene, ethylbenzene and xylenes

Table 4.3. Primers used in this study

A) Primers used for end-point PCR analysis

Target	Primer	Sequence (5' to 3')
16S rRNA gene	27 F	AGA GTT TGA TCM TGG CTC AG
	1100 R	GGG TTG CGC TCG TTG
<i>bssA</i> gene ^a	7772 F	GAC ATG ACC GAC GCS ATY CT
	8546 R	TCG TCG TCR TTG CCC CAY TT

^a Primers by Winderl et al. were used (52)

B) Primers used for qPCR analysis

Target	Specificity	Primer	Sequence (5' to 3')
<i>bssA</i> gene ^a	Denitrifying bacteria	F	ACGACGGYGGCATTTCCTC
		R	GCATGATSGGYACCGACA
	SRBs	F	GTSCCATGATGCGCAGC
		R	CGACATTGAACTGCACGTGRTCG
16S rRNA gene	Bacteria	F	CGGTGAATACGTTTCYCGG
		R	GGWTACCTTGTTACGACTT

F: Forward primer, R: Reverse primer, SRB; Sulfate reducing bacteria

^a Primers by Beller et al. were used (5, 6)

Table 4.4. Results of PCR analysis

A) Detection of genes in the monitoring wells using end-point PCR analysis

	MW-40	MW-15	MW-24	MW-25	MW-29	MW-30
16S rRNA gene	+	+	+	+	+	+
<i>bssA</i>	+	+ ^a	+	+	+	-

A: a very weak *bssA* amplification product was obtained

B) Quantification of genes in the groundwater samples with qPCR analysis

	MW-40	MW-15	MW-24	MW-25	MW-29	MW-30
16S rRNA gene	3.50×10^7	3.44×10^5	1.83×10^7	5.84×10^5	3.91×10^7	8.99×10^4
<i>bssA</i> in Denitrifying bacteria	-	-	2.55×10^3	-	-	-
<i>bssA</i> in SRB/methanogenic bacteria	N/A	N/A	1.66×10^4	N/A	N/A	N/A

(-): No product detected, N/A: Fluorescence from a PCR product was detected, but could not be quantified. Ct values of these samples had high std.dev., and were out outside the calibration curve.

Table 4.5. Summary of metabolites detected in the monitoring wells

	MW-40	MW-15	MW-24	MW-25	MW-29	MW-30
2-NA	+	+	+	+	+	+
TH-2-NA	+	+	+	+	+	-
HH 2-NA	+	-	+	-	-	+
Deacilin-2-carboxylate	-	-	-	-	-	-
MNA	-	-	+	-	-	-
PHE-CA	-	-	-	-	-	-

Table 4.6. Concentration of 2-NA and TH-2NA in the groundwater samples (µg/L)

	MW-40	MW-15	MW-24	MW-25	MW-29	MW-30
2-NA	0.94	0.80	6.66	0.43	0.33	0.20
TH-2-NA	0.22	<QL	2.56	<QL	<QL	-

<QL: detected, but less than quantification limit; (-) not detected.

Figure 4.1. Location of the monitoring wells (MWs). MW-40, MW-24 and MW-29 are within the plume, while MW-15, MW-25 and MW-30 are outside the plume. Scale bar represents 250 ft.

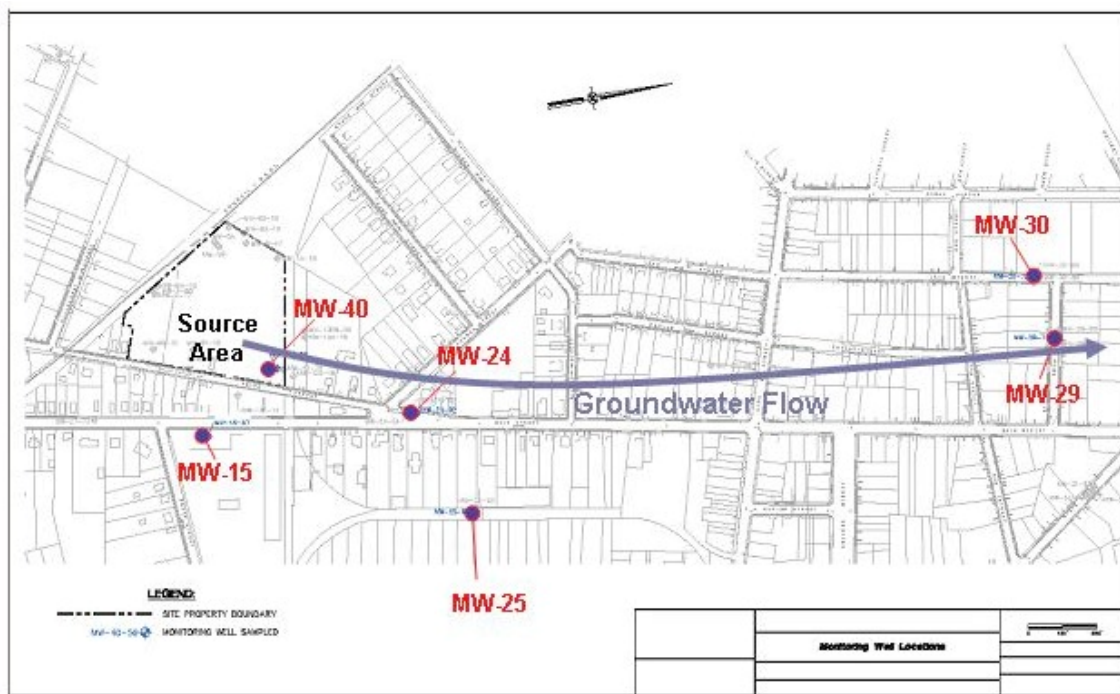


Figure 4.2. End-point PCR analysis. All DNA extracts obtained from groundwater samples were PCR amplified and analyzed on 1 % agarose gel. L1: Lamda Hind III ladder, L2: Kb + ladder. Arrows indicate the correct size of the PCR products. The numbers refer to the monitoring wells while (+) and (-) refer to the PCR controls.

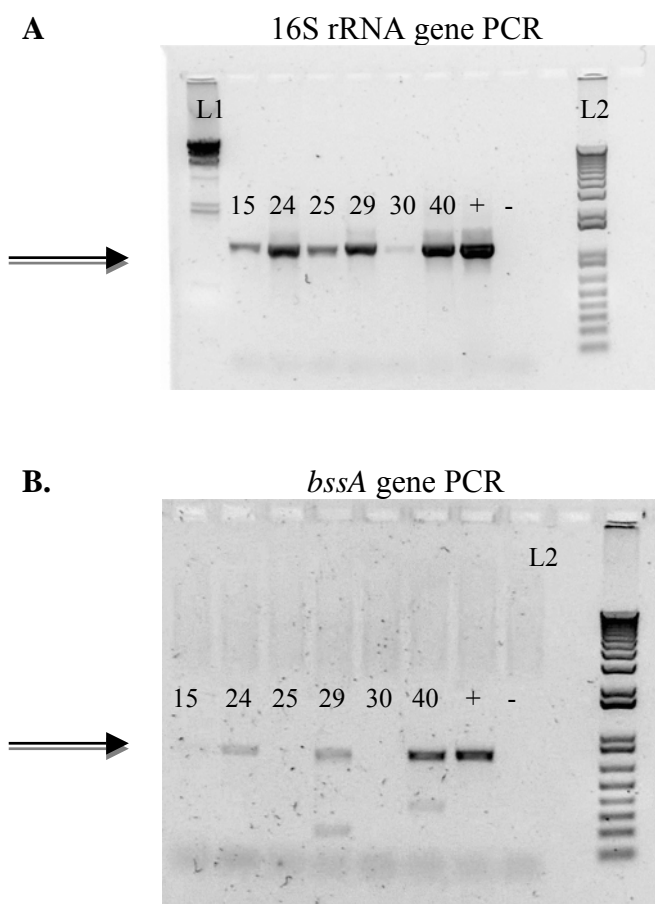


Figure 4.3. Mass spectra of 2-NA standard and metabolites detected in the groundwater samples. A) standard, B) MW-24, C) MW-15, D) MW-40, E) MW-25, F) MW-29, G) MW-30

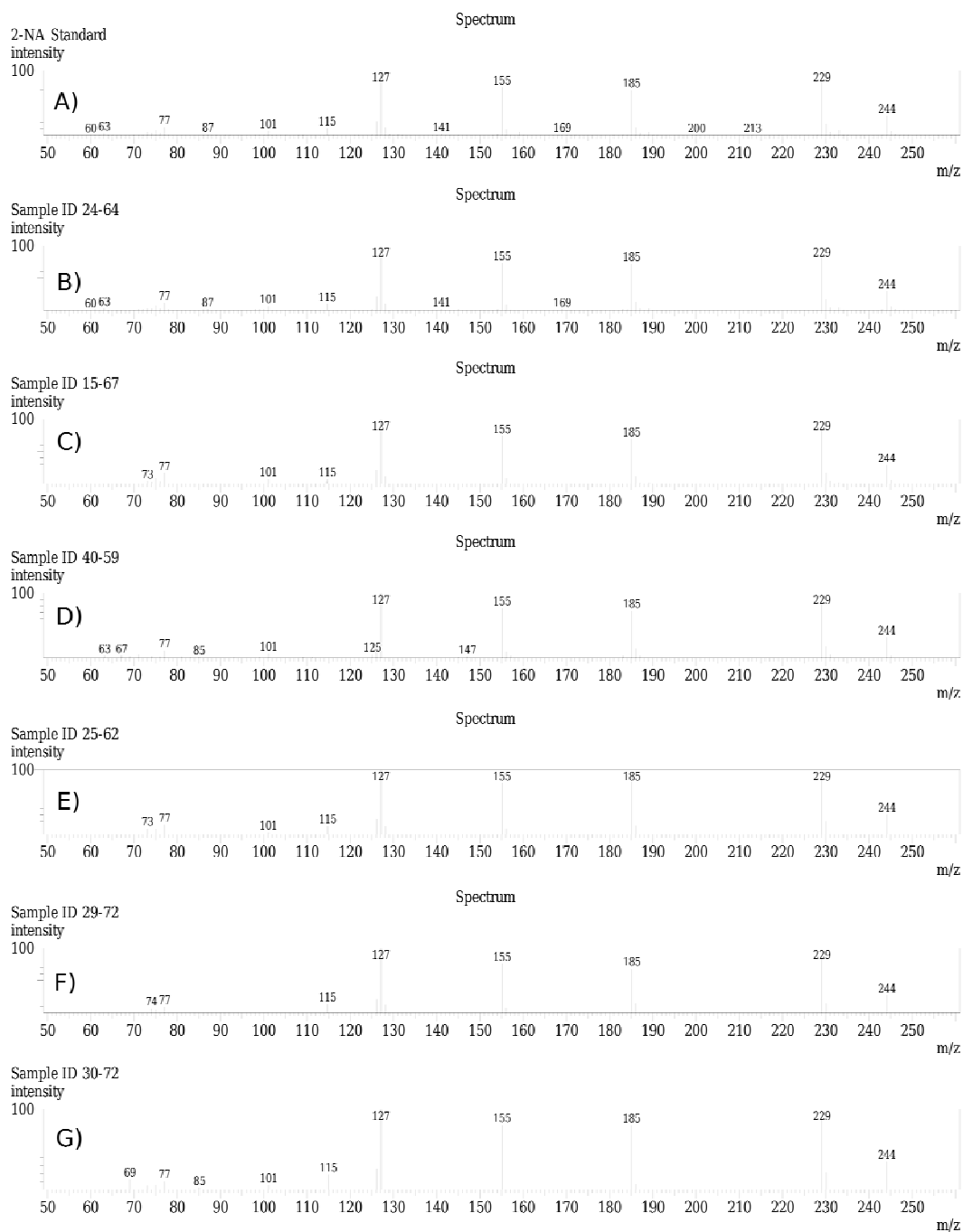


Figure 4.4. Mass spectra of TH-2-NA standard and metabolites detected in the groundwater samples. A) Standard, B) MW-24, C) MW-15, D) MW-40, E) MW-25, F) MW-29

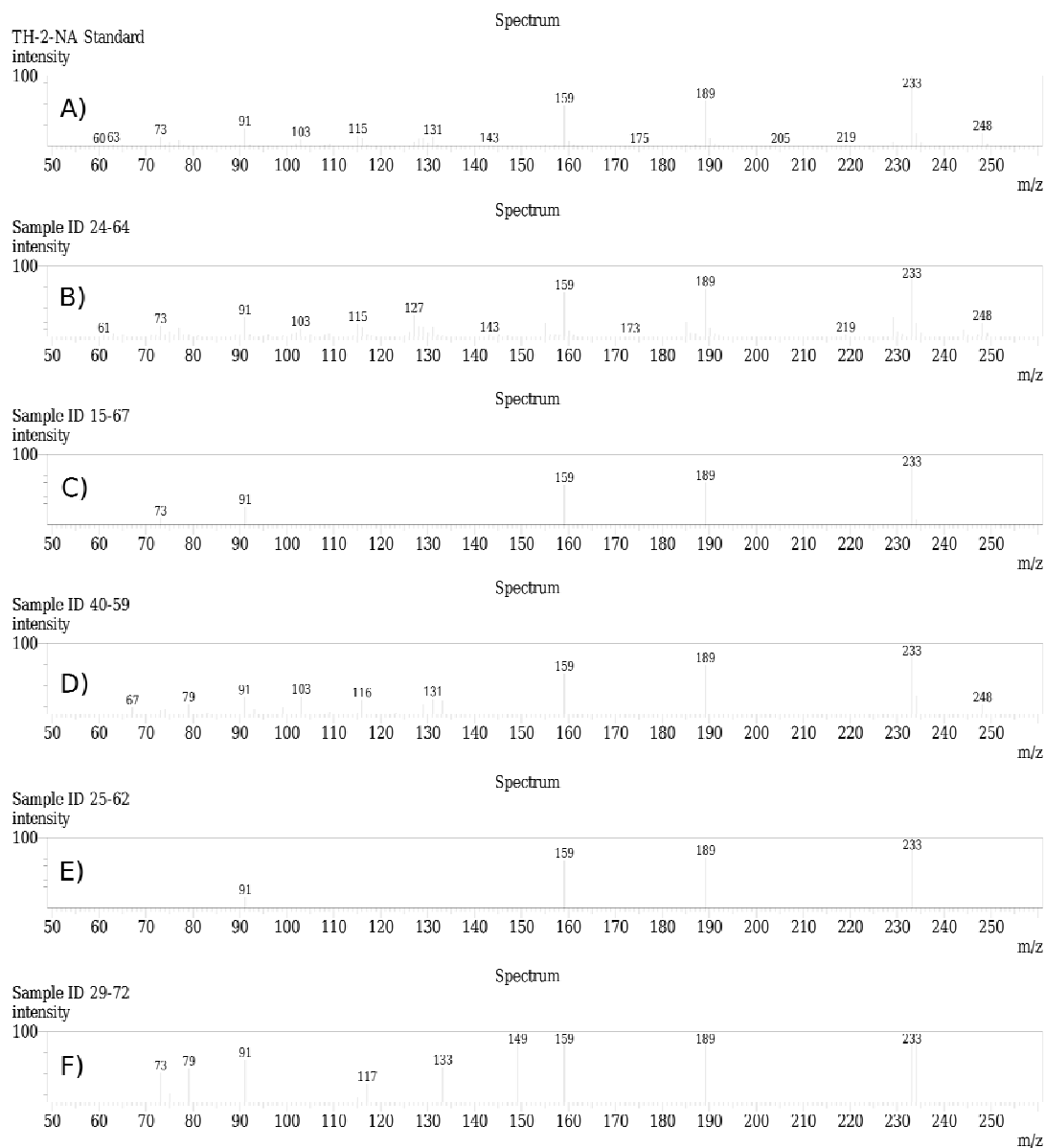


Figure 4.5. Mass spectra of metabolites identified as HH-2NA in groundwater samples.

A) MW-24, B) MW-40, C) MW-30

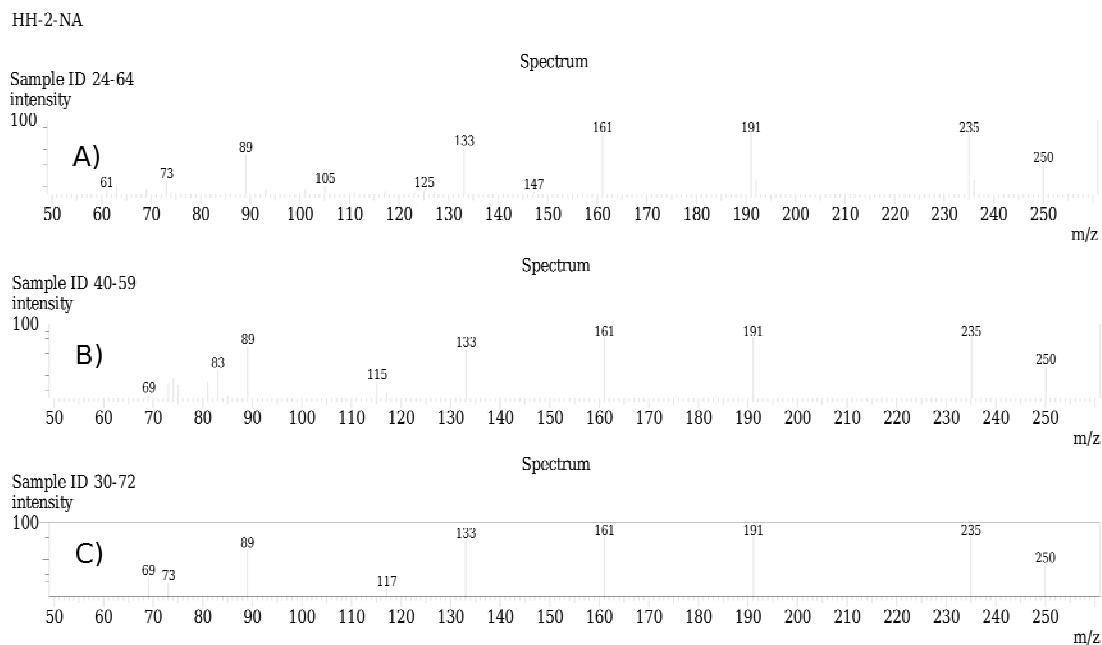
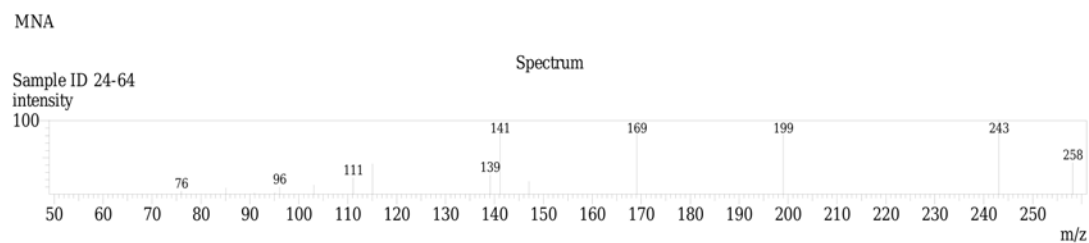


Figure 4.6. Mass spectra of metabolites identified as MNA in the groundwater sample of MW-24.



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CHAPTER 5

Conclusions and future research

Biodegradation plays an important role in the remediation of sites that have been contaminated by inputs of hydrocarbons. Several laboratory and field studies have shown that bacteria can transform and even mineralize hydrocarbons under different electron accepting conditions (5). The work presented in this thesis has added to our fundamental understanding of the processes of anaerobic benzene biodegradation. In addition, it has demonstrated that metabolic intermediates and genetic biomarkers of anaerobic hydrocarbon degradation that have been identified through laboratory studies, can be applied for evaluation of *in situ* anaerobic hydrocarbon degradation potential of impacted sites.

For studies of anaerobic benzene degradation we used a stable consortium that has been enriched in the laboratory for 15 years with benzene as a sole carbon source. Through previous studies it was understood that benzene mineralization in this consortium was sulfate dependent (10). The community present was characterized earlier, but the organism that catalyzes the initial attack on the benzene ring was not identified (11). We used DNA based stable isotope probing (DNA-SIP) studies to identify the bacteria in the sulfate-reducing benzene degrading consortium that play a crucial role in benzene degradation. By using terminal restriction fragment length polymorphism analysis, along with sequence analysis of 16S rRNA genes as molecular tools, we identified that a *Desulfobacterium*-like phylotype is most dominant in this consortium during the processes of anaerobic benzene degradation, and therefore likely initiates the process of benzene degradation (9). The presence of similar bacteria in other

mixed cultures from diverse environmental sources (sections 2.3 and 2.4) suggests that this phylotype is important for anaerobic benzene degradation not only in our consortium, but also in other mixed cultures and environments. This phylotype can therefore be developed into a genetic biomarker and used for evaluation of bioremediation potential of impacted environments. This would be a significant development, since as yet genetic biomarkers or metabolic intermediates unique to anaerobic benzene degradation have not been identified, and biomarkers of anaerobic degradation of other hydrocarbons are not applicable to evaluate the *in situ* anaerobic benzene degradation potential of a site.

One of the questions that has arisen through our DNA-SIP studies is: what is the possible role of other bacteria in the consortium in the processes of benzene degradation? This question is tied to the metabolism of benzene, including initial attack on the benzene ring and metabolism of intermediates. We have identified that at least four different 16S rRNA gene based phylotypes are present in this consortium (section 2.3). Full length 16S rRNA gene sequence analysis of these phylotypes along with the development of 16S rRNA gene specific primers can be used to elucidate the role of different organisms in benzene degradation. 16S rRNA gene expression can be analyzed as a function of time after addition of a pulse of benzene to a starved culture. The flow of carbon in the community can be followed by comparing the relative expression of different 16S rRNA genes with time. This study can be coupled with stable isotope labeling of intermediates of benzene. A parallel analysis of 16S rRNA gene expression and different ^{13}C -labeled metabolites formed during benzene degradation will not only elucidate the flow of carbon through the community, but also indicate the form of carbon that is being exchanged between different community members.

Although different pathways of anaerobic benzene degradation have been proposed, a consensus on a universal mechanism of benzene degradation has not been reached. Earlier studies on this consortium had identified benzoate as a metabolite of benzene (12). To develop a deeper understanding of the benzene degradation processes, we applied stable isotope labeling studies, in addition to feedback inhibition experiments and degradation tests to the sulfate-reducing benzene degrading consortium. Using ^{13}C label we were able to identify that the dominant pathway of benzene degradation in this consortium involves carboxylation of the benzene ring to benzoate by its reaction with an organic form of carbon derived from another benzene ring. These conclusions were supported by the results obtained from the degradation tests and inhibition experiments, which identified benzoate as a true metabolic intermediate of benzene degradation. Isolation of a pure culture that can degrade benzene anaerobically would help elucidate the genetic mechanisms and enzymes involved in activation of benzene, as well as to evaluate the downstream metabolism. Using this, mixed culture studies can be setup to test different organic substrates as potential carboxyl group donors for conversion of benzene to benzoate. Acetate and bicarbonate have been previously tested as carboxyl group donors and have yielded negative results (12). Fumarate and metabolic intermediates of benzoate degradation should be tested as carboxyl group donors. Although the fumarate addition reaction is considered unlikely in the case of benzene, the possibility of novel enzymes catalyzing such a processes cannot be eliminated (15).

The characterization of this culture has established that it can degrade benzene, phenol, 4-hydroxybenzoate and benzoate. It is not fully understood, though, what range of hydrocarbons can be degraded by this culture in addition to benzene? We have tested

toluene and naphthalene as potential substrates, and these tests yielded negative results. Interestingly, the 16S rRNA gene based characterization of this consortium (sections 2.3 and 2.4) indicates that the dominant *Desulfobacterium*-like phylotype (TRF 270) that is present in this consortium is related to *m*-xylene and naphthalene degrading sulfate-reducing strains mXyS1 and NaphS2, respectively. In addition, another 16S rRNA gene phylotype, TRF 205, in this consortium is related to *o*-xylene degrading sulfate-reducing strain oXyS1. These data indicate that microorganisms related to bacteria capable of degrading other hydrocarbons are present in this consortium. Therefore, other monoaromatic hydrocarbons (xylenes and ethylbenzene) should be tested as carbon sources along with polycyclic aromatic hydrocarbons such as naphthalene and 2-methylnaphthalene. The cometabolic transformation potential of this consortium should also be tested using mixed substrates. These studies would be interesting not only from an academic point of view, but will also elucidate the bioremediation potential of this mixed culture, since contaminants in the environment are more often present as mixtures.

Another approach to understand the metabolic capabilities of this consortium is to understand its genetic makeup that encodes different enzymes for anaerobic metabolism. The only enzyme that is known to be involved in anaerobic activation of hydrocarbons is benzylsuccinate synthase (Bss). *BssA* encodes the alpha subunit of the enzyme (8) that is involved in toluene activation. Bss like enzymes also catalyze the fumarate addition reaction to several hydrocarbon substrates including toluene, *o*-, *m*- and *p*-xylenes, *o*-, *m*-, and *p*-cresols, ethylbenzene, 2-, 3- and 4-fluorotoluenes, 1-methyl-1-cyclohexane, *n*-hexane and hexadecane (3, 4, 6, 7, 14). Bss like enzymes also are considered to be active in 2-methylnaphthalene (1, 2) and naphthalene degradation (13). We used degenerate

bssA gene primers (16) as probes for PCR amplification of the genomic DNA extracted from the consortium. A PCR product of expected size was obtained. It was cloned and sequenced, and the gene sequence obtained was used to compare against a protein database using blastx search of the GenBank database. The gene sequences were 75 to 80 % similar to *bssA* gene sequences of *Geobacter* sp. These results are intriguing, especially considering the fact that this consortium did not degrade toluene in repeated tests, and toluene was also not detected in [$^{13}\text{C}_6$]benzene amended cultures. Further investigations are needed to understand the role of the putative *bssA* gene in our consortium.

Studies presented in this thesis have established a strong foundation based on which many avenues of research can be perused to develop a deeper understanding of anaerobic benzene degradation as well as other metabolic capabilities of this consortium. In addition, the 16S rRNA gene identity of the dominant phylotype SB-21, in this mixed culture can be developed into a biomarker for evaluation of *in situ* anaerobic bioremediation potential of impacted sites.

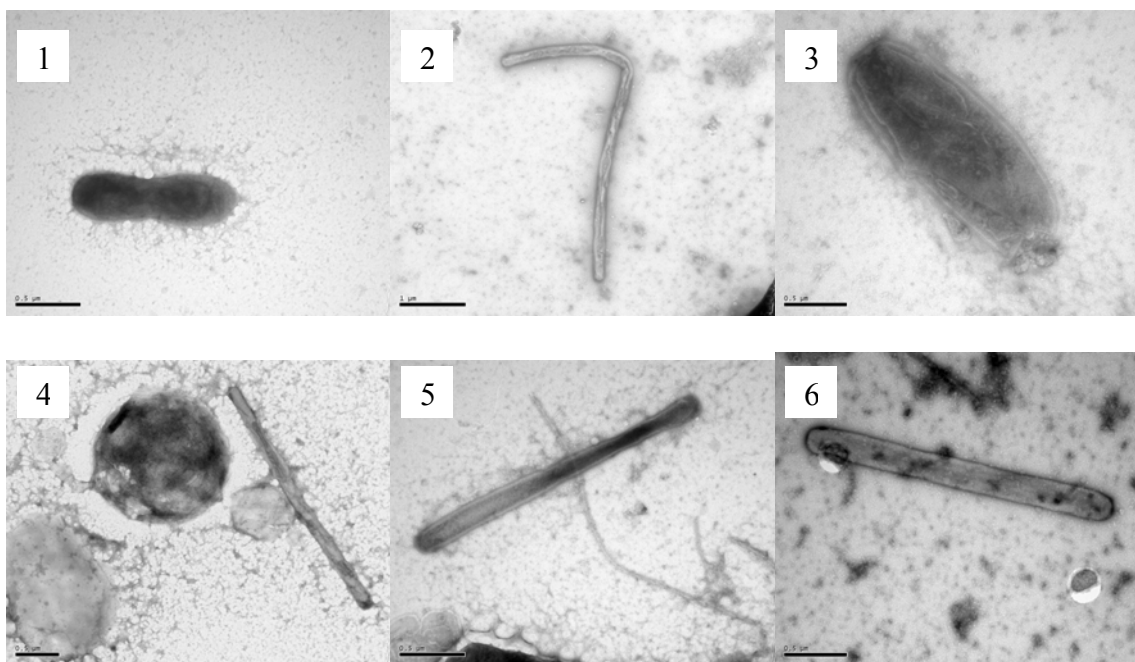
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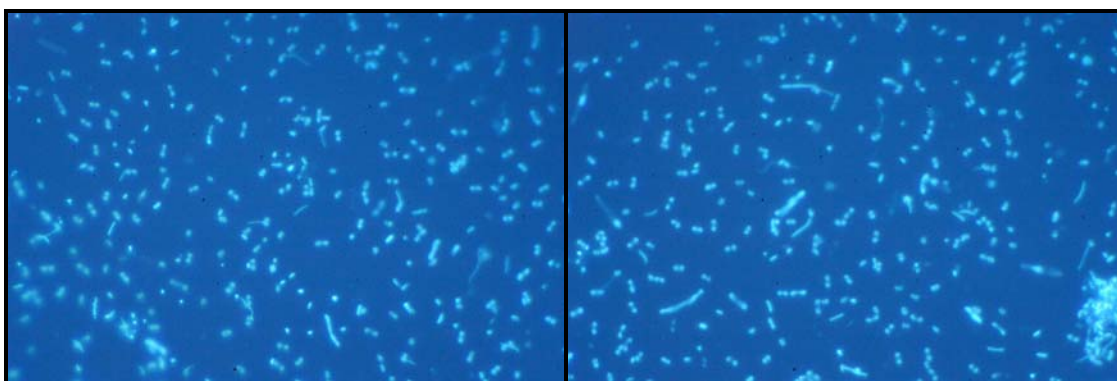
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APPENDIX

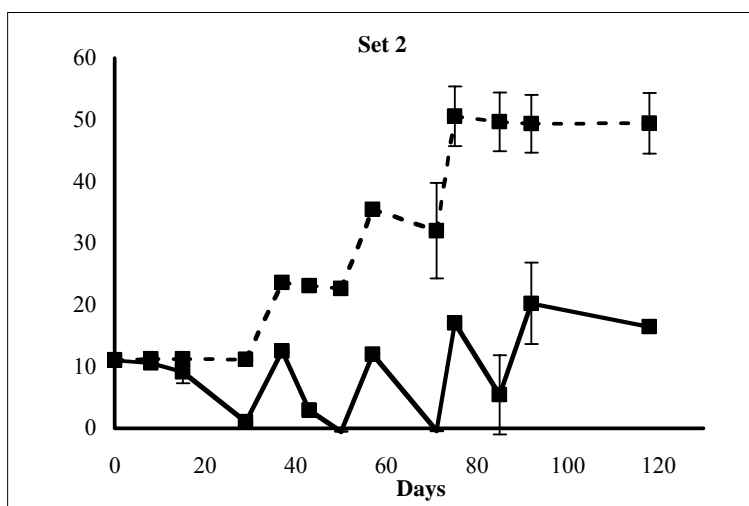
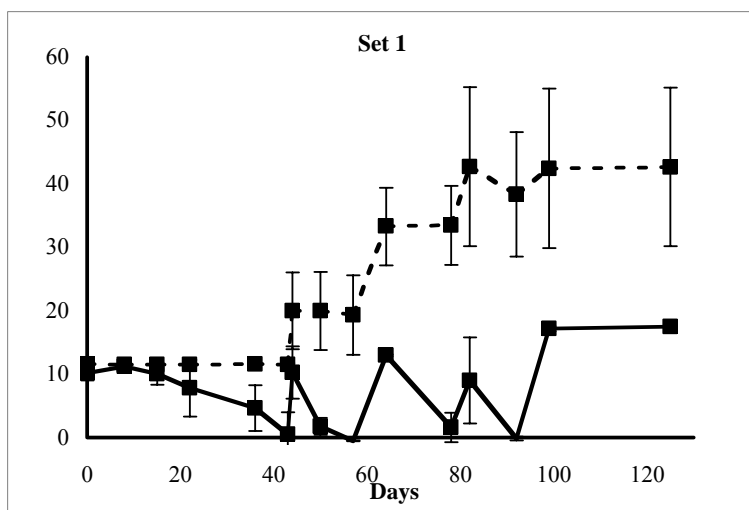
A1. Electron micrographs of different morphologies in the sulfate-reducing enrichment culture. Scale bar in 1 and 3-6 is 0.5 μm , and it is 1 μm in 2.



A2. Photomicrographs of cells in sulfate-reducing mixed culture stained with DAPI at 1000X magnification.



A3. Change in concentration of [$^{13}\text{C}_6$]phenol in sulfate-reducing enrichment cultures (—) and sterile controls (---). Data from 2 different sets are shown, each with at least 2 active and 2 sterile controls. Error bars are ± 1 standard deviation. Cultures and controls were repeatedly reamended with [$^{13}\text{C}_6$]phenol.



Curriculum Vita

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Publications:

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