BIODEGRADATION OF AROMATIC HYDROCARBONS: MICROBIAL AND ISOTOPIC STUDIES

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Biodegradation of Aromatic Hydrocarbons: Microbial and Isotopic Studies

by

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Abstract

Aromatic hydrocarbon contamination of soil and groundwater is a widespread environmental problem. Among the compounds of interest is a range of low molecular weight aromatic hydrocarbons that includes the so-called BTEX compounds (benzene, toluene, ethylbenzene, xylenes). Aerobic biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated in the environment. Several indicators have been utilized to evaluate this process but their measurement (e.g., of hydrocarbon concentration, bacterial count, metabolites) maybe affected by other chemical and physical processes. Stable carbon isotope analysis is one technique that has been previously used in environmental studies particularly in tracing sources of organic pollutants. Compounds have characteristic carbon isotopic compositions that can be used to pinpoint their origins. Any process in which the compounds are involved may likewise impart significant isotopic fractionation. It is shown that abiotic processes affect the $^{12}C/^{13}C$ ratio but biological transformation is known to produce the largest fractionation.

The purpose of this study is to determine the magnitude and direction of transformation of stable carbon isotopes ($^{12}C, ^{13}C$) during microbial degradation of selected low molecular weight hydrocarbon compounds such as toulene, ethylbenzene, naphthalene, methanol and hexadecane. Coupled with this objective is the identification of the various species that make up the consortium used in the study and the metabolic pathways by which these

organisms degrade the compound. The overarching goal is to examine if the isotopic fractionation associated with such pathways can be employed for monitoring *in situ* bioremediation.

Replicate microbial biodegradation experiments modified from an earlier protocol were done using microbial cultures grown aerobically at room temperature. Optical density measurements during the course of the experiments were undertaken to establish microbial growth. In addition, hydrocarbon isotope analysis was conducted by periodically removing a specific headspace concentration from the culture flask and analyzing it by gas chromatography continuous flow isotope ratio mass spectrometry (GC-IRMS).

Laboratory biodegradation studies on toluene showed increase in microbial growth from increases in optical density measurements with corresponding decreases in hydrocarbon concentrations and no significant changes in the $\delta^{13}C$ values. Similar observations were obtained using a higher substrate concentration (10 μl of toluene) except for differences in incubation periods. Experiments conducted on ethylbenzene as the substrate likewise demonstrated the same effects on microbial biomass as well as in concentrations of the residual hydrocarbon. Carbon isotopic compositions also remained relatively constant during microbial growth.

Taxonomic identification of the microcosm resolved several strains that composed the different hydrocarbon-specific cultures. These bacterial strains consisted of Gram negative

rods as well as Gram positive cocci. Gram negatives included strains from the genera of *Pseudomonas*, *Stenotrophomonas*, *Oligella* and *Acidovorax* while Gram positives belonged to *Micrococcus*, *Staphylococcus*, *Dermacoccus* and *Kokuria* (or *Erythromyxa*).

Results of the present study were compared with other published works. Similarities and differences in the outcomes of the respective experiments indicate that the occurrence of isotopic fractionation depends on the degradative pathways utilized by the respective microbial consortia. In particular, the nature of the initial metabolic step (e.g., attack on methyl group versus scission of aromatic ring) could control the extent of carbon isotope fractionation.

Based on the results of the present study, application of stable carbon isotope analysis in aerobic degradation of aromatic hydrocarbons, particularly the BTEX compounds, does not appear promising for assessment of natural or engineered *in situ* bioremediation. Future studies should look more closely into the different degradative pathways and enzyme systems used by individual microorganisms as well as mixed populations and their effects on the magnitude of isotopic fractionation. Site-specific studies are also necessary to determine the inherent presence of (these) microbial consortia and quantify the associated biological isotope fractionation.

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List of Abbreviations and Symbols

acidity	pH
benzene, toluene, ethylbenzene, xylene	BTEX
Canadian Centre for Occupational Health Service	CCOHS
carbon dioxide	CO_2
carbon 13	^{13}C
carbon 12	^{12}C
coenzyme A	CoA
compound specific isotope analysis	CSIA
degree Celsius	°C
degree Celsius per minute	°C/min
delta carbon 13	$\delta^{13}C$
dissolved inorganic carbon	DIC
flavin adenine dinucleotide	FAD
gas chromatography-isotope ratio mass spectrometry	GC-IRMS
Gram negative non-enteric	GN-ENT
Gram negative/Gram positive inoculating fluid	GN/GP-IF
Gram negative/Gram positive inoculating fluid with thioglycollate	GN/GP-IF + T
gram per kilogram	g/kg
hydrocarbon degrader medium	HDM
hydrogen sulfide	H_2S
lethal concentration to kill 50% of the test population	LC_{50}
lethal dose to kill 50% of the test population	LD_{50}
methane	CH_4
microgram per liter	µg/l
microliter	µl
micrometer	µm
milligram per kilogram	mg/kg
milligram per liter	mg/l
milligram per cubic meter	mg/m^3
milliter per kilogram	mL/kg
millimeter	mm
National Research Council	NRC
nicotinamide adenine dinucleotide	NAD^+
nicotinamide adenine dinucleotide, reduced form	NADH
nitrogen	N_2
optical density	OD
oxidation-fermentation test	OF test
parts per million	ppm
Peedee Belemnite	PDB

per cent	%
personal computer	PC
per mil (parts per thousand)	‰
polycyclic aromatic compound(s)	PAH(s)
pounds per square inch	psi
rounds per minute	rpm
sodium chloride	NaCl
standard deviation (sigma)	σ
Thermodynamic Research Center	TRC
2,3,5-triphenyltetrazolium chloride	TTC
trypticase soy agar	TSA
United States Department of Health and Human Services	USDHHS
United States Public Health Service	USPHS
World Health Organization	WHO

Chapter 1

Introduction

The widespread dispersal of hazardous and toxic organic compounds in virtually all areas of the environment resulting from increased industrial activities of humans has become one of the major problems facing the world today. Among the compounds of interest are low molecular weight hydrocarbons used primarily as solvents and fuels. Their occurrence in the surface and groundwater environments is due to accidental spills and leakage of underground storage tanks, or through inadvertent releases during use, transport or disposal. Because of the great concern for their relatively high pollution potential and high toxicity (Gibson and Subramanian, 1984; Barker, *et al.*, 1987; Alexander, 1994), the need for immediate remediation becomes apparent.

Aerobic biodegradation, catalyzed by natural populations of microorganisms, represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants are eliminated in the environment. Indicators that have been used to evaluate and monitor this process involve the measurement of changes over time in the concentration of hydrocarbon, number of bacteria, rate of bacterial activity, adaptation, metabolic by-products, intermediary metabolites, growth-stimulating materials and ratio of nondegradable to degradable compounds (NRC, 1993; Aggarwal and Hinchee, 1991; Madsen, 1991). However, such measurements may be affected not only by biodegradation

but by other processes such as volatilization, dissolution, dilution, migration off the site, sorption to the soil or transformation via abiotic chemical reactions (Riser-Roberts, 1992).

Previous works demonstrated the application of stable carbon isotope analysis to environmental studies, particularly in establishing sources of organic pollutants (O'Malley, 1994; Hunt, 1996; Santiago, 1997). These studies showed that compounds have characteristic stable carbon isotope compositions that can be used to establish their origins. In the same manner, any process in which a compound is involved will impart a significant isotopic fingerprint (Abrajano and Sherwood Lollar, 1999). Abiotic processes (volatilization, reductive halogenation) have been shown to affect the $^{13}C/^{12}C$ ratio but fractionation associated with biological transformation is known to produce the largest fractionation in natural systems (Stahl, 1980; Galimov, 1985; Abrajano and Sherwood Lollar, 1999). Therefore, stable carbon isotope analysis may be used to monitor biodegradation of organic compounds.

1.1. Purpose of the Study

The present paper primarily focuses on the applicability of stable isotope analysis to determine the magnitude and direction of fractionation of carbon isotopes (^{12}C, ^{13}C) during microbial degradation of selected hydrocarbon compounds such as toluene, ethylbenzene, naphthalene, methanol and hexadecane. It also attempts to look at various factors that brought about such fractionation and that play a major role in the

transformation of these compounds into less harmful forms. Coupled with this objective is the characterization of the various microorganisms that make up the consortium used in the study and this in turn will give us insight into the different metabolic pathways by which these organisms degrade the compounds. The overarching goal is to determine if the isotopic fractionation associated with such pathways can be used for monitoring *in situ* bioremediation.

1.2. Environmental Chemistry

The low molecular weight hydrocarbons selected for the study with their corresponding chemical and physical characteristics are listed in Table1.1 and their chemical structures shown in Fig. 1.1. The following section briefly describes their occurrence in the atmosphere, terrestrial and aquatic environments, and their impact on ecosystems and humans.

1.2.1. Toluene

Toluene (C_7H_8) is one of the monocyclic petroleum hydrocarbons known as the BTEX (benzene, toluene, ethylbenzene and xylene) compounds. This organic compound is a clear, colourless liquid with a sweet smell at room temperature (Environment Canada, 1984b). It is commonly found as solvents in many industrial products such as cleaners, inks, paints, lacquer, resins and adhesives as well as in pharmaceutical products (CCOHS,

Table 1.1. Chemical and physical properties of the selected hydrocarbons.

Compound	Chemical Composition	Molecular Weight (amu)	Melting Point (°C)	Boiling Point (°C)	Solubility in Water (mg/L)	Density (g/ml at 25°C)	Henry's Constant (atm-m^3/mole)
Toluene	C_7H_8	92.13	-95	110.6	534.8	.862	5.74×10^{-3}
Ethylbenzene	C_8H_{10}	106.16	-94.97	136.2	161	.863	8.44×10^{-3}
Naphthalene	$C_{10}H_8$	128.16	80.2	217.9	31.7	——	4.83×10^{-4} 5.83×10^{-4}
Methanol	CH_4O	32.04	-97.8	64.7	miscible	.786	1.35×10^{-4}
Hexadecane	$C_{16}H_{34}$	226.45	18.34	187.0	.0009	.764	

Note: Hexadecane data taken from Gallant and Yaws (1993), other compounds from Howard (1990a and 1990b) except density of toluene and ethylbenzene from TRC (1990) and that of methanol from Yang (1994).

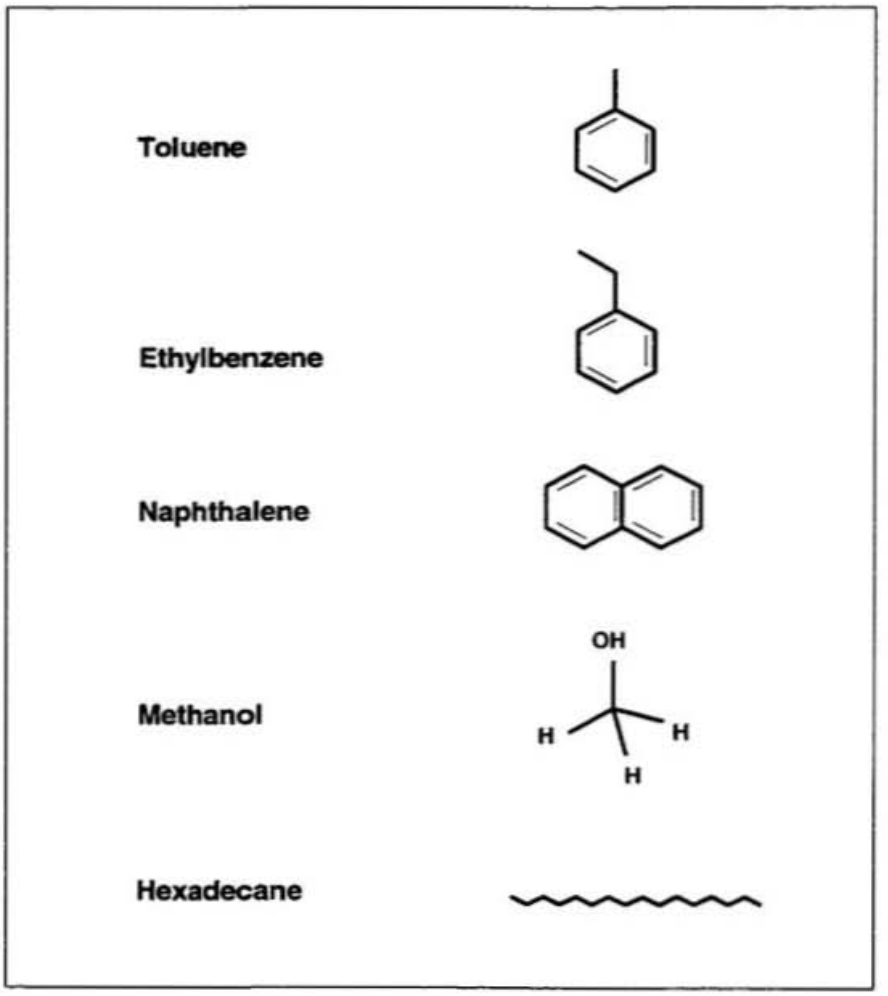

Fig. 1.1. Chemical structures of selected hydrocarbons.

1988; Environment Canada, 1984b; WHO, 1985). In Canada and the United States, its main use is in the production of benzene and other chemicals and in lesser amounts, as a major component of automobile gasoline and aviation fuels (Environment Canada, 1984b; Moore and Ramamoorthy, 1984). Toluene is also employed in the manufacture of explosives and dyes (CCOHS, 1988).

Toluene enters the environment chiefly during volatilization of petroleum fuels and toluene-based solvents and thinners and from motor vehicle exhaust. A substantial amount is discharged into waterways and on land during storage, transport, and disposal of fuels and oils (Howard, 1990b). Toluene released on soil may dissipate because of evaporation from near-surface soils, slow biodegradation, and leaching into groundwater. Releases in water will be diminished by evaporation (half-life of days to several weeks) or biodegradation which can take several weeks depending upon temperature, mixing conditions, and acclimatization of microorganisms. In addition, toluene will not adsorb onto sediment or bioconcentrate in aquatic organisms. In the atmosphere, this compound is removed by reaction with hydroxyl radicals giving it a half-life of three hours to slightly over a day. It may also be washed out by rain.

A considerable amount of information on the toxicological effects of toluene on test animals and humans are found in the literature (WHO, 1985; Environment Canada, 1984b; USPHS, 1989). Humans are primarily exposed to toluene by inhalation of contaminated air near congested traffic or gas stations, or in areas where toluene-based solvents are used

(Howard, 1990b). The most important hazard to humans upon acute inhalation exposure is its effect on central nervous system function (Environment Canada, 1984b; WHO, 1985). Toluene vapour can also cause mild irritation to the eyes, nose, throat and skin upon contact. On the other hand, swallowing of this compound can cause nausea, diarrhea and loss of consciousness (CCOHS, 1988).

Toluene can also affect aquatic and terrestrial life. It can be deleterious to marine animals through ingestion and contact at levels ranging from 3.7 to 1180 mg/l (WHO, 1985) but has no food chain concentration potential (Environment Canada, 1984b). Toxicological studies on test animals showed that LD_{50} (lethal dose to kill one-half of the population of these animals) varies from 5,000 to 7,500 mg/kg by ingestion (rats) and 1,100 to 8,700 mg/kg by weight by absorption through skin. The LC_{50} or the concentration in air which kills half of the organisms following an exposure after an indicated time period is placed between 26,000 to 72,000 mg/kg by weight by inhalation (CCOHS, 1988; Speijers, 1993). Toxic effects include tremors, elevated neurotransmitters and loss of coordination.

Various bacteria can degrade toluene (Atlas, 1978; Gibson, 1984). Increase in growth rate of some bacteria was observed with low level of toluene (20mg/l) but toxic effects occurred at higher concentrations (200 mg/l) (Environment Canada, 1984b). Degradation by *Pseudomonas fluorecens* was inhibited at about 30 mg/l whereas that by *Escheria coli* at 200 mg/l.

The concentrations of toluene in Canadian drinking water supplies averaged from 2.0 µg/L (Government of Canada, 1992). Groundwater near landfill sites in Ontario ranged from 0.2 µg/L to 730 µg/L. Concentrations in soils and sediments have not been identified but measurable concentrations in soil would be expected to occur in case of spills and around waste disposal sites.

1.2.2. Ethylbenzene

Ethylbenzene (C_8H_{10}) is also a monocyclic compound and a colourless liquid with a gasoline-like odour. It is used primarily for the production of styrene. It is an important solvent and chemical intermediate in the chemical, paint and rubber manufacturing industries. It is also an additive for motor fuel formulations (USDHHS, 1992a; WHO, 1996).

This hydrocarbon is released to the atmosphere mainly from fugitive emissions and exhausts, wastewater and spills, related to the use of gasoline and manufacture of styrene. It exists in the atmosphere mainly in gaseous phase due to its vapor pressure and degrades by reaction with hydroxyl radicals with a half-life of a few hours to two days (Howard, 1990a). Spills into water form slicks that dissolve and diminish by evaporation and degradation. Ethylbenzene may be adsorbed by sediment but is not usually bioaccumulated or bioconcentrated (Howard, 1990a).

Human exposure to ethylbenzene chiefly occurs by inhalation, particularly in areas of traffic. Inhalation of vapor causes irritation of mucous membranes, dizziness, headache, and depression of the central nervous system (Environment Canada, 1984a). Contact with the liquid irritates eyes and skin. After short single exposures, threshold level values that affect the human central nervous system and mucous membrane were calculated to be approximately 430-860 mg/m^3 (100-300 ppm) (WHO, 1996).

A 13-week inhalation toxicology study of pure ethylbenzene (99%), conducted on rats and mice, at varying concentrations from 0 to1000 ppm showed, except for increased weights of liver and kidney, no evidence of toxicity (USDHHS, 1992a).

The acute toxicity of ethylbenzene to some species of aquatic organisms is moderate with the lowest values at 4.6 mg/l, 1.8 mg/l and 4.2 mg/l for algae, invertebrates and fish, respectively (WHO, 1996).

1.2.3. Naphthalene

Naphthalene ($C_{10}H_8$) is a white, crystalline powder with a characteristic odor. Industrial applications include the manufacture of various organic acids such as phthalic and anthranilic acids and sulfonic acids (USDHHS, 1992b). Naphthalene is also used as an insecticide, antiseptic and vermicide. Commercial moth repellants and toilet bowl cleaners contain this compound as a major ingredient.

Release of naphthalene to the environment is from accidental emissions and exhausts related to production and use of gasoline and fuel oil as well as from spills on land and water during storage, transport and disposal of these materials. Rapid degradation, however, occurs immediately upon contact with hydroxyl radicals in the atmosphere. In water, naphthalene is lost by volatilization, photolysis, adsorption, and biodegradation (Howard, 1990a). When discharged on land, naphthalene concentration is reduced by moderate adsorption and biodegradation.

People are generally exposed to naphthalene by inhalation of ambient air near heavy traffic areas, gasoline stations and from tobacco smoke (Howard, 1990a). Spills on hand, moderate ingestion through drinking water supplies and consumption of contaminated food may also be some sources of exposure.

Inhalation toxicological effects of naphthalene to humans include headache, confusion, eye irritation, nausea, profuse perspiration with vomiting, optic neuritis, hematuria and edema. Ingestion of this substance gives rise to abdominal pain, nausea, vomiting, diarrhea, darkening of the urine, irritation of the bladder, jaundice, anemia and hyperthermia (USDHHS, 1992b). Possible evidence of its carcinogenic potential was observed in East Germany where four cases of laryngeal carcinoma, a case of gastric carcinoma, a case of colon carcinoma and a case of lupus erythematosus were found among 7 to 15 employees involved in naphthalene manufacture (USDHHS, 1992b).

For animal toxicity, the oral LD_{50} value is 490 mg/kg for rats while lethal dose values for mice are 533 mg/kg (oral), 969 mg/kg (subcutaneous), 100mg/kg (intravenous), and 100 mg/kg (inhalation) (USDHHS, 1992b).

1.2.4. Methanol

Methanol (CH_3OH) is described as a clear, colourless, volatile flammable liquid with a mild alcoholic odour when pure. It is a chief constituent of a large number of commercially available solvents and consumer goods. It is also utilized as a chemical intermediate for production of formaldehyde and other important industrial organic chemicals (Environment Canada, 1985; CCOHS, 1986; WHO, 1997). It is needed in the manufacture of some pharmaceutical products, and is an essential gasoline additive, de-icing agent, cleaning agent for leather goods, glass and photographic film, as a flushing fluid for hydraulic systems, and an extractant in refining gasoline and removing impurities from animal and vegetable oils (CCOHS, 1986).

Methanol occurs as a natural volatile emission product of some plants and comes from biological decomposition of biological wastes, sewages and sludges (Howard, 1990b). Anthropogenic sources are largely from evaporation of the solvent. Photochemical reactions with hydroxyl radicals remove methanol from the atmosphere. Biodegradation

coupled with volatilization significantly reduces its concentration in water whereas biodegradation and leaching notably decompose it in soil.

Inhalation is the most likely route of exposure to humans although absorption through dermal contact and consumption of various food and waters cannot be disregarded (Howard, 1990b; WHO, 1997). Short-term exposure to methanol vapour can produce irritation of the eyes, nose and throat, headache, nausea, vomiting, dizziness, drunkenness and blurred vision (CCOHS, 1986). Massive exposure can cause blindness, unconsciousness and death. Long-term exposure causes headaches, giddiness, eye irritation, insomnia, abdominal pains, skin irritation, impaired vision and blindness (CCOHS, 1986).

Methanol is moderately toxic to test animals such as rats, mice or rodents (CCOHS, 1986). Toxicity data for animals obtained from their LD_{50} were placed at 6.2 to 13.0 g/kg for rats upon ingestion, 20.0 mL/kg for rabbit by absorption through the skin and LC_{50} 64,000 ppm for rat by inhalation for four hours.

For aquatic organisms, methanol is of low toxicity and effects due to environmental exposure are unlikely to be observed except in the case of a spill (WHO, 1997). The LC_{50} ranges from 1,300 to 15,900 mg/l for invertebrates (48-hour and 96-hour exposures), and 1,300 to 29,000 mg/l for fish (96-hour exposure) (WHO, 1997).

Reported toxicity threshold values for methanol in the cell multiplication inhibition test were 6,600 mg/l for the bacterium *Pseudomonas putida* (Bringmann and Kuhn, 1980).

1.2.5. Hexadecane

Hexadecane ($C_{16}H_{34}$) is an aliphatic straight chained hydrocarbon and a colourless liquid. This compound, also called cetane, is employed primarily as a reference compound for diesel fuels (Snell and Ettre, 1971). Generally, it is utilized for standardization of secondary reference fuels that are then used for quality control.

Like other alkanes, hexadecane occurs naturally in oil and gas deposits, and is discharged to the environment during spills and controlled emissions (Moore and Ramamoorthy, 1984). It is one of the volatile compounds isolated from heat-treated beef and cooked rice and it contributes to the typical flavor of meat (Dwivedi, 1971) and aroma of Basmati and Italian rice (Tara and Bocchi, 1999). Concentrations of hexadecane in soil and water environments are reduced by volatilization (Gidda *et al.*, 1999) and biodegradation (Bouchez-Naitali *et al.*, 1999; Erikson *et al.*, 1999; So and Young, 1999).

Exposure to high concentrations of hexadecane is extremely destructive to tissues of the mucous membranes and upper respiratory tract, eyes and skin.

1.3. *In situ* Bioremediation

The enormous impact of hydrocarbon contamination to the environment has spurred investigations to develop safe, effective and economically viable approaches to clean contaminated soils, surface and groundwater (NRC, 1993; Alexander, 1994; Baker and Herson, 1994; Crawford and Crawford, 1996; Tinari, 1997). One technique currently in use is *in situ* bioremediation. This method makes use of natural microbial processes to break down complex compounds in place into simpler, less harmless forms. For biodegradation of hydrocarbons to occur and for bioremediation to be successful, some basic requirements need to be considered. These include the presence of an appropriate microbial population, energy and carbon sources, electron acceptors, nutrients, and appropriate environmental conditions (Atlas, 1978; NRC, 1993; Bedient *et al.*, 1994).

Understanding the microbial degradation of any organic compound can be illustrated by the golden triangle (Fig. 1.2) adapted from Doelman (1995). It consists of knowledge of the microbial community, environmental conditions and knowledge of the structure and physico-chemical characteristics of the organic compound. The latter was discussed in the previous section.

The key players in bioremediation are the ubiquitous microscopic organisms. They are ideally suited to the task of contaminant destruction because they possess enzymes that allow them to use environmental contaminants as food and because they are so small that

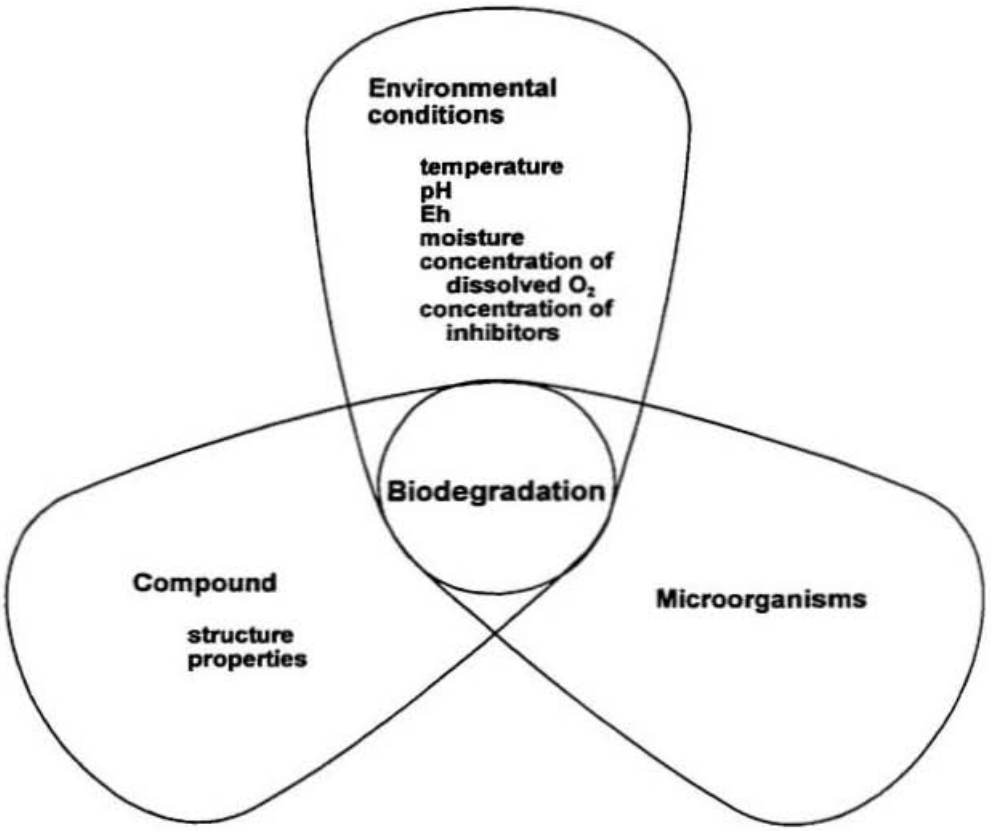

Fig. 1.2. The golden triangle for microbial degradation (after Doelman, 1995).

they are able to contact contaminants easily (NRC, 1993). Transformation of organic contaminants by microbial activity is a natural consequence of the microorganisms' ability to utilize organic material, such as contaminants, for their growth and reproduction. Organic contaminants such as hydrocarbons therefore become the source of carbon to the organism for production of new cell constituents. At the same time, they become a source of electrons from which the organisms obtain energy (NRC, 1993; Bedient *et al.*, 1994). Microbes break chemical bonds and transfer electrons from the contaminants to an electron acceptor. They then invest the energy along with some electrons and carbon from the contaminant to produce more cells (NRC, 1993).

The cellular components of microorganisms have a fixed elemental composition (NRC, 1993). Typically, a bacterial cell is 50% carbon, 14% nitrogen, 3% phosphorus, 2% potassium, 1% sulfur, 0.2% iron and 0.5% each of calcium, magnesium, and chloride (NRC, 1993). The lack of one of these elements will limit overall microbial growth and therefore retard contaminant removal. Thus, one important goal of bioremediation is to stimulate the microorganisms by supplying them with optimum levels of nutrients and other chemicals essential for their metabolism (NRC, 1993).

Aside from proper concentrations of nutrients, microbial activity is likewise dependent upon many environmental conditions. These include concentration of dissolved oxygen, temperature, pH, salinity, pressure, soil moisture, oxidation-reduction potential, concentration of pollutants, and presence of inhibitors (Atlas, 1978; Atlas, 1981; Cooney

et al., 1985; Wilson *et al.*, 1986; Leahy and Colwell, 1990; Daubaras and Chakrabarty, 1992; Riser-Roberts, 1992; Baker and Herson, 1994). Some of these parameters can be modified at the site to stimulate the biodegradative activities of indigenous microorganisms. Ideal conditions suited for efficient utilization of hydrocarbons by microbes described by Roberts-Riser (1992) include a temperature between 20 and 35°C, pH of 5 to 9 and a low population of predators, to name a few.

Site characteristics such as geological and chemical characteristics must also be assessed to determine the appropriate *in situ* bioremediation system to be implemented (NRC, 1993).

1.3.1. Microbial Processes

Processes by which microorganisms can break down various hydrocarbons can be classified according to electron acceptors utilized. Typical electron acceptors include oxygen, carbon dioxide, nitrate, sulfate and certain metals such as iron and manganese. Some organisms, known as aerobes, use only molecular oxygen as an electron acceptor to destroy organic compounds. In this process, known as aerobic respiration, oxygen oxidizes part of the carbon in the compound forming carbon dioxide while the remaining carbon is used for production of new cells. Oxygen, itself, is reduced producing water (NRC, 1993). Other microorganisms, referred to as anaerobes, exist without molecular oxygen utilizing a process called anaerobic respiration. In addition to new cell mass, the

by-products of anaerobic respiration may include nitrogen gas (N_2), hydrogen sulfide (H_2S), reduced forms of metals, and methane (CH_4), depending on the electron acceptor. Still other microorganisms use a variation of aerobic respiration. Cometabolism is one such process in which microbes transform a contaminant but the contaminant cannot serve as the primary energy source for the organisms (NRC, 1993). Under these circumstances, microbes require other compounds that can support their growth.

1.3.2. Microorganisms

A diverse group of microorganisms has been extensively reported on, utilizing a wide range of hydrocarbon compounds (Zobel, 1946; Walker *et al.*, 1975; Walker *et al.*, 1976; Austin *et al.*, 1977; Atlas, 1978; Ribbons and Eaton, 1982; Cerniglia, 1984; Gibson, 1984; Cerniglia, 1992; Muller, 1992; Atlas and Cerniglia, 1995; Hall *et al.*, 1999). Low molecular weight hydrocarbons are particularly susceptible. Bacterial species that use the aerobic process of degradation are described below.

Toluene and ethylbenzene are some of the most aerobically biodegradable petroleum hydrocarbons found in the subsurface environments. According to Gibson and Subramanian (1984) experiments done with the isolates of *Bacillus hexacarbovorum* showed that this type of microorganism could grow with toluene (and xylene). They also report that two organisms, *Bacterium benzoli a* and *b*, were capable of growth with toluene and other monoaromatic compounds such as benzene and xylene, and that a strain

of *Pseudomonas putida* could grow with ethylbenzene as the sole source of carbon and energy. Strains of *Pseudomonas* isolates were also shown to variably grow in toluene (Zylstra *et al.*, 1988; Chang *et al.*, 1993; Alvarez and Vogel, 1991). Hutchins (1991) worked on aquifer microorganisms that could degrade benzene, toluene and xylene by using different electron acceptors. Under aerobic conditions, he found out that these compounds degraded to concentrations below 5 µg/l within 7 days whereas only toluene and xylene were degraded when either nitrate or nitrous oxide was used.

Experiments done by Cox and Goldsmith (1979) showed that using hexadecane as the sole source of carbon aided in the conversion of ethylbenzene by a culture of *Nocardia tartaricans* ATCC 31190 into two metabolites of 1-phenethanol and acetophenone. Removal of hexadecane with *Pseudomonas aeruginosa* ATCC 15442 loaded to sterile sand columns was also studied by Herman *et al.* (1997). Species of *Cornybacterium*, *Micrococcus* and unidentified Gram-negative rods (Jones and Edington, 1968) as well as strains of *Alcaligenez* and *Rhodococcus* (Bouchez-Naitali *et al.*, (1999) were also found growing on hexadecane.

A wide variety of bacteria have the ability to oxidize polycyclic hydrocarbons including naphthalene (Walker *et al.*, 1976; Cerniglia, 1992). A considerable amount of research has been conducted on strains of *Pseudomonas putida* which are capable of metabolizing naphthalene (Davies and Evans, 1964; Patel and Barnsley, 1980; Cerniglia, 1984; Tagger *et al.*, 1990). The involvement of plasmids in the degradation of naphthalene has also been

reported (Dunn and Gunsalus, 1973). Mineralization half-lives of this aromatic hydrocarbon in microcosms obtained from sediment and water samples collected from three ecosystems ranged from 2.4 weeks in sediment chronically exposed to petroleum hydrocarbons to 4.4 weeks in sediment from a pristine environment (Heitkamp *et al.*, 1987).

Aerobic biodegradation has posed some problems particularly in terrestrial subsurface environments where oxygen concentration is initially low. Due to low solubility of oxygen in water, and its low rate of transport through saturated porous matrices such as soil and sediments, removal of hydrocarbon compounds such as BTEX and PAHs from such contaminated sites is inhibited (Fries *et al.*, 1994). Recent studies have therefore focused on the anaerobic biotransformation of these compounds by microorganisms under denitrifying, sulfate-reducing, and iron-reducing conditions (Evans, 1977; Vogel and Grbic-Galic, 1986; Zeyer *et al.*, 1986; Evans and Fuchs, 1988; Mihelcic and Luthy, 1988; Kuhn *et al.*, 1988; Grbic-Galic, 1989; Grbic-Galic, 1990; Lovley and Lonergan, 1990; Acton and Barker, 1992; Barbaro *et al.*, 1992; Edwards *et al.*, 1992; Evans *et al.*, 1992; Schinck *et al.*, 1992; Frazer *et al.*, 1993; Rabus *et al.*, 1993; Cozzarelli *et al.*, 1995; Rabus and Widdel, 1995; Ball *et al.* 1996; Biegert *et al.*, 1996; Krumholz *et al.*, 1996; Beller and Spormann, 1997; Gieg *et al.*, 1999).

1.4. Monitoring of *In situ* Bioremediation

Demonstrating that *in situ* bioremediation is working requires evidence not only that contaminant concentrations have decreased but that microorganisms caused the decrease. A considerable amount of work has focussed on the capability of aerobic microorganisms collected at hydrocarbon-contaminated sites to mineralize hydrocarbons to CO_2 and water under laboratory conditions (Austin *et al.*, 1977; Zylstra *et al.*, 1988; Stehmeir *et al.*, 1996; Herman *et al.*, 1997). Other research has concentrated on parameters such as bacterial number, metabolic by-products, inorganic carbon isotope ratios and electron acceptor concentration to show evidences of bioremediation in the field (NRC, 1993). These approaches, however, do not attribute contaminant loss to microbial activity unequivocally. Furthermore, experiments involving isolation of individual hydrocarbon degraders using conventional methods (e.g. plate count method) have encountered some difficulties as they are limited in the culturability of the targeted bacterial populations. Wilson and Lindow (1992) showed that up to 75% of the viable bacterial populations could be underestimated.

1.4.1. .Stable Carbon Isotope Analysis

One technique that has gained much attention to verify natural attenuation is the determination of stable carbon isotope signatures. This method involves the measurement of isotopic ratios ($\delta^{13}C$) of carbon dioxide in soil gas and/or dissolved inorganic carbon

(DIC) (NRC, 1993; Suchomel *et al.*, 1990; Aggarwal and Hinchee, 1991; Trust *et al.*, 1995; Van de Velde *et al.*, 1995; Jackson *et al.*, 1996; Kelley *et al.*, 1997; Landmeyer *et al.*, 1996; Aggarwal *et al.*, 1997; Conrad, 1997; Conrad *et al.*, 1997). While such isotopic measurements render invaluable information on microbial degradation of hydrocarbons, other sources and sinks of CO_2 contribute to changes in $\delta^{13}C$ values (Suchomel *et al.*, 1990). Furthermore, significant overlap that exists between $\delta^{13}C$ values of CO_2 derived from biodegradation of hydrocarbon contaminants and those resulting from indigenous respiration (e.g. root respiration or degradation of endogenous soil organic matter) can produce ambiguous results.

The recent development of compound specific isotope analysis (CSIA) using gas chromatography isotope ratio mass spectrometry (GC-IRMS) interfaced with a combustion furnace, has provided a more efficient way to assess *in situ* bioremediation by enabling direct isotopic analysis of the contaminants themselves.

1.5. Carbon Stable Isotope Biogeochemistry

Carbon appears in nature as one of several isotopes, two of which are considered stable isotopes and important in the present study: ^{12}C with a natural abundance of 98.89% and ^{13}C with a natural abundance of 1.11% (Hoefs, 1987). The enrichment or depletion of the less abundant isotope in the products of chemical reaction is referred to as isotopic

fractionation (Lajtha and Michener, 1994) and is caused by two main processes: isotope exchange reactions and kinetic processes.

1.5.1. Isotope Exchange

Isotope exchange involves the equilibrium distribution of isotopes between different compounds, phases and molecules (Hoefs, 1987) expressed as:

$$aA_1 + bB_2 = aA_2 + bB_1$$

where A and B are different chemical species and the subscripts 1 and 2 indicate whether they contain the lighter or heavier isotopes, respectively. For such reaction, the equilibrium constant will be equal to:

$$K = \frac{\left(\frac{A_2}{A_1}\right)^a}{\left(\frac{B_2}{B_1}\right)^b}$$

The equilibrium constant (K) is related to the fractionation factor α which is defined as:

$$\alpha = \frac{R_A}{R_B}$$

where R_A and R_B are the ratios of the heavy and light isotopes (A_2/A_1, B_2/B_1) in chemical compounds A and B, respectively. In some cases where only one atom is exchanged, the equilibrium constant is identical with the fractionation factor, $K=\alpha$.

Stable isotope ratios are measured usually by utilizing isotope ratio mass spectrometer that measures the ratio of the heavy and light isotopes in a sample (R_{sample}) and compares this to that of a standard ($R_{standard}$). Therefore, the isotopic composition of a compound is generally defined as the differences in isotope ratios of a compound and the standard, and is calculated in 'del' (δ) notation and expressed in units per mil (‰):

$$\delta = [(R_{sample}/R_{standard} - 1)] \times 10^3$$

The primary standard for carbon is a marine limestone fossil, the Peedee Belemnite (PDB) (Hoefs, 1987). For the stable isotopes of carbon, the ratio is $^{13}C/\ ^{12}C$ and the isotopic composition is written as $\delta^{13}C$.

1.5.2. Kinetic Effects

Kinetic isotope effects occur during unidirectional or incomplete reactions (Hoefs, 1987). Galimov (1985) pointed out that kinetic effects are produced by differences in the reaction rates of compounds resulting from mass differences between isotopes. Faure (1986) and Hoefs (1987) further explained this based on dissociation energies of the isotopes.

Because of mass differences, isotopes have different dissociation energies, and the bonds that are formed by the light isotope are weaker than those involving the heavy isotope, and thus are more easily broken. During a reaction, compounds bearing the light isotope will, in general, react slightly more readily than with the heavy isotope (Hoefs, 1987). This then implies that during unidirectional chemical reactions, there is preferential enrichment of the lighter isotope in the reaction products.

In biological systems, living organisms, particularly plants, discriminate against ^{13}C in their uptake of CO_2 during photosynthesis such that the organic molecules produced from such process are enriched in ^{12}C relative to ^{13}C (Abelson and Hoering, 1961; Broecker and Oversby, 1971; O'Leary, 1988). This fractionation stems from the differences in activation energies of these isotopes. Because ^{13}C is heavier than ^{12}C and forms slightly stronger chemical bonds, higher activation energies need to be overcome to break the bonds formed by a heavier isotope than that the same bonds formed by a lighter isotope. Hence, molecules having the light isotope will generally react at a slightly faster rate, causing the residual molecules to become heavier. In a similar manner, in microbially mediated processes, e.g., *in vitro* biodegradation of an organic contaminant, isotopic fractionation due to preferential microbial metabolism of isotopically light isotopes of a substrate can also occur and lead to progressive enrichment of the heavy isotopes in the residual substrate.

1.5.3. Rayleigh Distillation

Since differences in the vapor pressures of isotopic compounds can also lead to fractionations, evaporation-condensation processes are significant in the study of carbon isotope systems (Hoefs, 1987). This isotopic separation process can be approached theoretically in terms of fractional distillation or condensation under equilibrium conditions and this can be expressed by a Rayleigh equation. As most of the compounds selected in this study are volatile, only the distillation process will be given attention to.

For a distillation process, the instantaneous isotope ratios of the remaining liquid and the vapor leaving the liquid are different and given by:

$$\frac{R_l}{R_{lo}} = f^{(1/\alpha - 1)}$$

and

$$\frac{R_v}{R_{lo}} = \frac{1}{\alpha} f^{(1/\alpha - 1)}$$

where R_{lo} is the isotope ratio of the initial bulk composition; R_l is the instantaneous ratio of the remaining liquid; R_v is the instantaneous ratio of the vapor leaving the liquid; and f is the fraction of the residual liquid. In this process, either one isotope will preferentially

fractionate to the vapor phase or vice versa. As the process progresses, the remaining liquid will become progressively enriched with respect to the heavy isotope (Hoefs, 1987).

1.6. Compound Specific Isotope Analysis (CSIA)

As previously mentioned, CSIA has the potential to be used to trace the source of a compound (Galimov *et al.*, 1983; O'Malley, 1994; O'Malley *et al.*, 1994; O'Malley *et al.*, 1996). The technique basically makes use of the compound's distinct isotopic compositions with regards to the known stable isotopes of carbon, nitrogen, sulfur, oxygen and hydrogen. For analysis of carbon stable isotopes, it involves on-line chromatographic separation and micro-combustion of organic compounds, purification of produced CO_2, and real time measurement of $^{13}C/^{12}C$ ratios (Abrajano *et al.*, 1992). This method has potential in the determination of biosynthetic pathways used in the formation, diagenesis, or indigeneity of a material (Macko and Estep, 1984; Macko *et al.*, 1987; Hayes *et al.*, 1989; Freeman *et al.*, 1990; Hayes, 1993; Macko, 1994; Boschker *et al.*, 1998). Several biotic and abiotic processes occurring in the natural environment can also change the isotopic composition of compounds of interest. If such compositions can be investigated, the CSIA can also be utilized to study the mechanisms involved in the transformations of compounds being studied. Dayan *et al.* (1999) suggested that abiotic transformations of pollutants in the environment maybe predicted using information on the direction and magnitude of the change in isotopic composition from their experiments on reductive dehalogenation of chlorinated ethenes. In the same manner, biological processes

in which a compound is involved can likewise be studied and isotopic data generated can then be used in the monitoring of *in situ* bioremediation.

To date, only a few studies have been found that used CSIA in demonstrating in situ bioremediation in the field as well as in the laboratory. Dempster *et al.* (1997) developed a pentane extraction technique that can remove dissolved BTEX compounds, at very low concentrations, from groundwater prior to isotopic analysis. In conjunction with the GC/IRMS method, application of such a technique enables the accurate determination of the $\delta^{13}C$ composition of dissolved BTEX at concentrations significant to contaminated settings and with great spatial and temporal precision. In addition, its suitability in field conditions has significant implications with respect to both tracing the source of a particular contaminant and identifying the processes affecting saturated zone behavior. On the other hand, Stehmeier *et al.* (1999) and Sherwood Lollar *et al.*, 1999 have actually applied compound specific studies to determine any isotopic fractionation accompanying aerobic degradation of some low-molecular weight hydrocarbons such as benzene and styrene, and toluene, respectively. Sherwood Lollar *et al.* (1999) also studied changes in isotopic compositions of a chlorinated compound undergoing anaerobic degradation.

Chapter 2

Experimental Procedures

Bench scale biodegradation experiments and taxonomic identification were undertaken using the facilities of the Department of Earth Sciences Isotope Biogeochemistry Laboratory and the Department of Biology Applied Microbiology and Biotechnology Laboratory. Sterilization of all glassware and media needed in the experiment was initially done with an autoclave maintained for one hour at 121°C and 15 psi. This was done to ensure that only the desired microorganisms were being cultured. During experiments, proper aseptic procedures were employed to prevent external contamination.

2.1. Materials

Hydrocarbon compounds used in the experiments were obtained from different chemical companies. Toluene was purchased from Sigma-Aldrich Chemical Companies, St. Louis, MO and Milwaukee, WI, U.S.A. while ethylbenzene came from Aldrich Chemical Company, Inc., Milwaukee, WI, U.S.A. Methanol was acquired from Fischer Scientific, Nepean Ontario, Canada and crystals of naphthalene were purchased from Supelco, Inc., Bellefonte, PA, U.S.A. All chemical reagents and solvents were of highest available purity.

2.2. Hydrocarbon Degrader Medium

The minimum salts medium or the hydrocarbon degrader medium (HDM) used in the experiments is described in Stehmeier *et al.* (1996). It contained per liter of distilled water: 1 g K_2HPO_4, 1 g KH_2PO_4, 2 g NH_4NO_3, 0.3g $MgSO_4.7H_2O$, 0.001 g $CaCl.2H_2O$, 0.001 g $FeSO_4.7H_2O$. One milliliter of micronutrients was also added to the solution. The micronutrients were prepared by dissolving the following in one liter of distilled water: 2.9 g H_3BO_3, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.2 g $ZnSO_4 \cdot 7H_2O$, 0.4 g $Na_2MoO_4 \cdot 2H_2O$, 0.08 g $CuSO_4 \cdot 5H_2O$ and 0.05 g $Co(NO_3) \cdot 6H_2O$.

2.3. Source of the Microcosm

The source of hydrocarbon degraders was a mixed microbial culture obtained from a monitoring well at a petrochemical site (Stehmeier, pers. comm., 1998). The mixed culture was originally enriched in minimum salts medium containing pyrolysis gas as a carbon source. Pyrolysis gas is a mixed hydrocarbon liquid obtained during the cracking of ethane to make ethylene (Francis *et al.*, 1997). From this consortium, specific hydrocarbon-degrading cultures were enriched and maintained by subculturing every month utilizing one of the hydrocarbons of interest as the only substrate (toluene, ethylbenzene, hexadecane, naphthalene and methanol).

2.4. Microbial Degradation Experiments

The biodegradation protocol undertaken in this study was adapted and modified from an earlier microbial and isotopic study of benzene and styrene (Stehmeier *et al.*, 1999). Microbial cultures were grown aerobically at room temperature in sidearm flasks having an average volume of 274 ml and equipped with Teflon miniert valves for ease of sampling. Each flask contained 35 ml of hydrocarbon degrader medium (HDM) augmented with 2 µl of a particular hydrocarbon, inoculated with 5 ml of microbial culture, and shaken at about 150 rpm on a Gyratory shaker at room temperature (22 ° ± 1 °C). The pH of the starting and final culture suspensions of each experiment were recorded to determine whether acid was produced during microbial growth.

It should be noted that the amount of hydrocarbons (2 µl) used in the experiments when added to the medium (40 ml) resulted in concentrations that are comparable to concentrations found in groundwater affected by actual spills. This concentration is close to the solubility of the hydrocarbons in water (Table 1.1).

The previous method cited above used duplicate vials, one for optical density measurements and the other for headspace analysis (Stehmeier *et al.*,1999). In the present study, only one flask of modified design was used for each experiment to prevent opening of the bottle that would lead to inevitable loss of hydrocarbon in the headspace.

Furthermore, the design of the flask allows easy measurement of optical density by tilting the bottle and inserting the side arm into the spectrophotometer (Fig. 2.1).

To determine the growth of microorganisms, optical density measurements were done utilizing a Bausch and Lomb spectrophotometer set at 600 nm. Measurements were taken at the beginning, at hourly intervals (or whenever necessary) and at the end of each experiment.

Hydrocarbon isotope analyses were done at approximately the same intervals as the optical density measurements. The Isochron II Series gas chromatograph continuous flow isotope ratio mass spectrometer (GC-IRMS) (Fig.2.2) of the Department of Earth Sciences, Memorial University was used. The GC was a Hewlett Packard HP5890 Series II equipped with a Restek RTX 502.2 column having a length of 105 m, an internal diameter of 0.53 mm and a 3-μm crossband phenylmethyl polysiloxane film (Chromatographic Specialties, Inc.). A specified concentration of hydrocarbon in the headspace being analyzed (listed in Table 2.1) was injected into the GC, and was then carried by helium gas (12 psi) to a furnace that combusted the organic compound at 300 °C. Table 2.2 shows the different chromatographic parameters used in setting a temperature program for the analysis of toluene and ethylbenzene. The resulting carbon dioxide then passed into a VG Optima dual inlet triple collector gas source mass spectrometer. Data acquisition and processing was done by PC-based software supplied by OPTIMA. Carbon stable isotope compositions are expressed as:

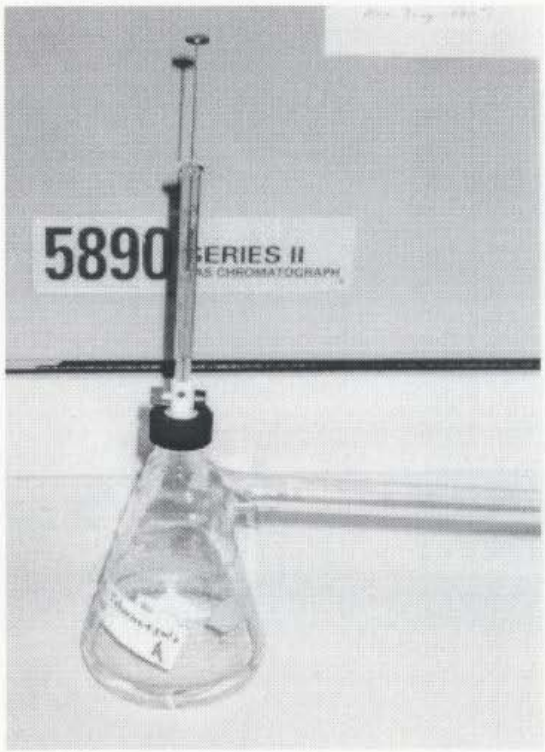

Fig. 2.1. Side-arm flask used in laboratory degradation experiments.

Fig. 2.2. Gas chromatography continuous flow isotope ratio mass spectrometer (GC-IRMS) of the Isotope Biogeochemistry Laboratory, Department of Earth Sciences, Memorial University.

Table 2.1. Headspace concentration.

Compound Used	Concentration (µl)
Toluene, 2 µl	30
Blank	30
Toluene, 10 µl	10
Blank	8
Ethylbenzene, 2 µl	20
Blank	20

Table 2.2. Chromatographic conditions for toluene and ethylbenzene.

Compound	Toluene	Ethylbenzene
Injector	250°C	250°C
Hold	1 minute	1 minute
Initial temperature	35°C	35°C
Rate	25°C/minute	25°C/minute
final temperature	250°C	265°C
hold	12 minutes	12 minutes
Total	21.6 minutes	22.2 minutes

$$\delta^{13}C\ (‰) = [R_{sample}/R_{reference})-1] \times 10^3$$

where $\delta^{13}C$ is per mil (‰) difference of $^{13}C/^{12}C$ of the sample and reference standard PDB (Peedee Belemnite).

The initial concentrations of the hydrocarbons in the headspace were calculated using Henry's law constant from data listed in Table1.1. The corresponding concentrations (C_t) at a given time t were determined by using the peak areas in the chromatograms from the GC-IRMS:

$$C_t = \left(\frac{A_t}{A_0}\right) C_0$$

where A_t is the peak area at time = t and A_0 the peak area at time = 0.

Isotopic analysis and optical density measurement of blanks containing 40-ml HDM and 2 µl (or 10 µl) of the selected hydrocarbon were also undertaken to act as controls, to document hydrocarbon loss due to volatilization and to ensure that the HDM used was totally devoid of unwanted microorganisms.

In each experimental run, three replicates were done but inoculation was not simultaneous. Based on initial experiments involving optical density measurements, it

was found that the log phase of microbial growth was 15 to 30 hours and 4 to 7 days for toluene and ethylbenzene, respectively. For toluene, because of the short log phase duration, it was deemed necessary to separate each inoculation by a period of 24 hours in order to have as many isotopic measurements as possible, especially during the exponential rise in each replicate.

Another set of experiments using 10 µl of toluene was also conducted to obtain more isotopic measurements and to determine if there were differences in ^{13}C values with increases in substrate concentration. The same procedures and analytical conditions mentioned above were duplicated except that the injected headspace concentration of individual experiments and blank solutions were different (Table 2.1).

Initial experiments conducted with methanol and naphthalene as substrates showed that degradation of these compounds was occurring as indicated by the increase in microbial biomass (optical density measurements increased over time). Headspace analyses carried out during these experiments were deemed inconclusive. For methanol, it was difficult to measure isotopic signatures as no methanol was likely detected in the headspace. This was probably due to the high miscibility of methanol in water (Howard, 1990b), in this case water in the hydrocarbon degrader medium (HDM). Similar difficulty in headspace measurement was also observed for naphthalene. Since the naphthalene used was in crystal form, its low solubility in water prevented it from going into solution and interacting with the headspace. This resulted in determination of unreliable isotopic

measurements. Small peaks recorded in blank measurements could not be resolved and this led to differences in $\delta^{13}C$ values. For hexadecane, by the time isotopic work was scheduled, the GC-IRMS started to experience downtime which eventually led to a situation where no isotopic data were obtained.

2.5. Taxonomic Identification of Microcosm

2.5.1. Isolation of Pure Colonies

Because accurate identification of the bacteria depends significantly on obtaining a pure culture, isolation of pure strains was the first step undertaken. This was initially done by using a combination of dilution processes known as the spread-plate and streak-plate methods. The aim of these techniques was to deposit individual cells far apart on the plate so that each cell could grow into isolated colonies (Morris, 1998) (Fig. 2.3). The spread-plate method was accomplished by initially mixing 1 ml of hydrocarbon culture to 10 ml of HDM. From this mixture, several dilutions were prepared and then 0.1 ml from each dilution was aseptically removed and directly plated on a trypticase soy agar. The streak plate was then employed when growth occurred after 24 to 72 hours at an incubation temperature of 25 °C. Isolated colonies distinguished by notable differences in morphological characteristics were picked and streaked on fresh plates. Further streaking

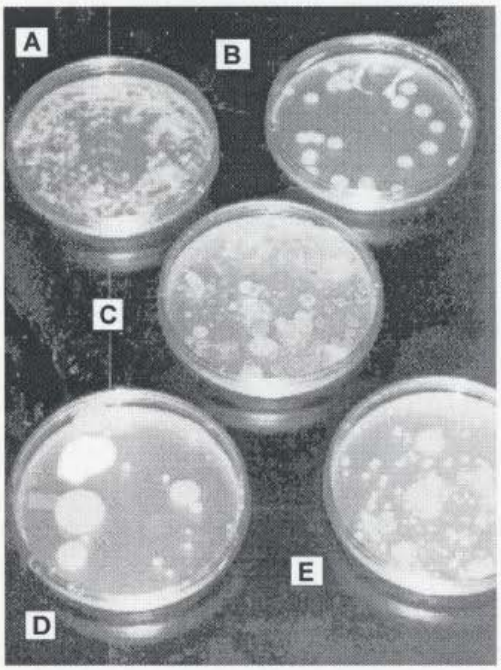

Fig. 2.3. Different colonies of microbial species found in each specific hydrocarbon culture: naphthalene (A), toluene (B), hexadecane (C), methanol (D) and ethylbenzene (E).

was done to confirm the purity of the cultures. The colony morphological characteristics such as shape, margin (edge), elevation, color, texture and pigmentation of these pure isolates were also noted. Once this was accomplished, pure cultures were maintained in trypticase soy agar plates and stored at a refrigeration temperature of about 5 °C.

2.5.2. Testing Procedures

The next step required physiological and biochemical tests described by several microbiology manuals (Blazevic and Ederer, 1975; Ballows *et al.*, 1991; Leboffe and Pierce, 1996; Morris, 1998). Bacterial cultures grown for 24 to 72 hours at 25°C in either tryticase soy broth or trypticase agar plates were employed to inoculate test media. Unless otherwise stated, all tests were examined every 24 hours for a period of five days.

Gram staining was done not only to differentiate between Gram-positive and Gram-negative cells but likewise to obtain information on the size, shape and arrangement of microbial cells using a light microscope. It was based on a four-step process in which a decolorization step occurred between the application of two basic stains. The first step involved staining a loopful of bacterial cells smeared and heat-fixed in a glass slide with basic dye crystal violet followed by treatment with iodine that functioned as a mordant to increase the interaction between cell and the dye forming a crystal violet-iodine complex (Leboffe and Pierce, 1996). The smear was then decolorized by flooding it with 95% ethanol. Finally, the smear was counterstained with another basic dye known as safranin

O. This dye caused Gram-positive cells to stain purple and Gram-negative cells to stain pink. The Gram staining also allowed the determination of the shapes of the microbial cells, whether they were cocci or rods, as well as their average dimension. The arrangement of cells, i.e., either occurring singly, in pairs or in chains, was also observed.

Aside from the Gram stain, other methods used to further verify whether the unknown microbial cells were Gram-positive or Gram-negative were the MacConkey agar and antibiotic sensitivity tests. Test plates of McConkey agar were inoculated with different microbial strains. As the MacConkey agar was a selective and differential medium containing nutrients, including lactose as well as bile salts, neutral red and crystal violet, the presence of bile salts and crystal violet inhibited growth of Gram-positive bacteria. For the antibiotic sensitivity test, Vancomycin-impregnated discs were placed on trypticase soy agar plates smeared with the cultures. Development of an inhibition zone around the discs was indicative that the microorganisms were affected by the antibiotic and thus were Gram positive cells.

The aerotolerance or the ability of the organisms to grow in the presence of oxygen was determined by several techniques. Such techniques included thioglycollate deep tubes, the anaerobic jar and oxidation-fermentation (OF) tests. In the first method, thioglycolate broth deep tubes were stabbed with an inoculating needle down to the bottom of the tubes and incubated for 24 and 48 hours. Diffusion of free oxygen was limited only to the top portion of the tubes as indicated by formation of a blue layer. Growth on top then

indicated the presence of aerobes (organisms that requires oxygen), while growth only in the lower portion indicated anaerobes (organisms that cannot live with the presence of oxygen). Growth throughout, but more in the aerobic zone, was indicative of facultative anaerobes (microbes that grow in both aerobic and anaerobic conditions).

The second method utilized an anaerobic jar into which inoculated culture media were placed and where the addition of 10 ml of water to a mixture of dry chemicals (i.e. sodium carbonate, iron powder and an inert extender) produced hydrogen gas and carbon dioxide. The jar was then sealed to prevent entry of atmospheric oxygen. The rest of free oxygen inside the jar reacted with the evolved hydrogen to form water. Occurrence of growth in the culture media indicated that the tested organisms were anaerobic, that is, they can live without oxygen.

The ability of the organisms being studied to oxidize or ferment a specific sugar was determined by the oxidation-fermentation (OF) test. This medium contained a high sugar to peptone ratio. Two tubes were stab-inoculated with the same organism being tested. After inoculation, one tube was covered with sterile mineral oil and the other was left unsealed. Because the oil excluded oxygen from the medium, this method was also used in determining the aerotolerance of the organisms. Oiled and unoiled tubes that turned yellow showed that the tested organisms could ferment and oxidize the sugar and were facultative anaerobes. Unsealed yellow medium and sealed green medium indicated that the organism could only oxidize the sugar and were basically obligate aerobes.

Motility was detected with motility test medium containing 2,3,5-triphenyltetrazolium chloride (TTC). Motility agar tubes were inoculated by stabbing with an inoculating needle and incubated for 24 to 72 hours. A positive result was indicated by diffuse growth outwards from the line of inoculation.

2.5.3. Characterization of Aerobic Organisms

Organisms that were identified as Gram-negative and Gram-positive aerobes required further investigations. For the former, an additional test was needed to ascertain whether they were non-enteric (GN-NENT) or not. The oxidase test was performed by moistening a piece of filter paper with a few drops of 1% tetramethyl-p-phenylenediame dihydrochloride and smearing it with a loopful of growth. Formation of a blue/violet color after 10 to 20 seconds was indicative of a positive result that in turn showed a GN-NENT organism.

Gram-positive isolates were further characterized by conducting the catalase test. The catalase reaction was determined by dropping 3% hydrogen peroxide solution on a trypticase agar slant containing growth of tested organisms. Immediate formation of gas bubbles indicated a positive catalase production.

2.5.4. Characterization Using the Micrologᵀᴹ Microbial Identification System

The Micrologᵀᴹ, or Biolog, is a computer based system for the identification of bacteria and other microbes (Solit, 1999). Using this system, a 95-well microplate is inoculated and incubated for an approximate length of time. Identifications are made based on metabolic profiles obtained from 95 substrates. A redox dye, tetrazolium-violet, is reduced to an insoluble violet product that can be read visually or by using an automated reader. For each plate the metabolic profile of the organism is recorded by the computer and cross-referenced with a bank of profiles of known organisms. The computer then gives an identification.

Before a microplate was inoculated, a bacterial suspension was made. Depending upon the characteristics of the bacterium, it was suspended in one of the suspending fluids (either GN/GP-IF suspending fluid or GN/GP-IF+T suspending fluid). The difference between the two fluids is that GN/GP-IF+T contains thioglycollate. The choice of suspending fluid was based on the preliminary results of Gram stain reaction, aerotolerance tests, the oxidase test and the catalase test. These tests, made on the basis of the morphological, cultural and biochemical characteristics of the unknown bacteria, were described earlier and are shown in the flowchart (Fig.2.4).

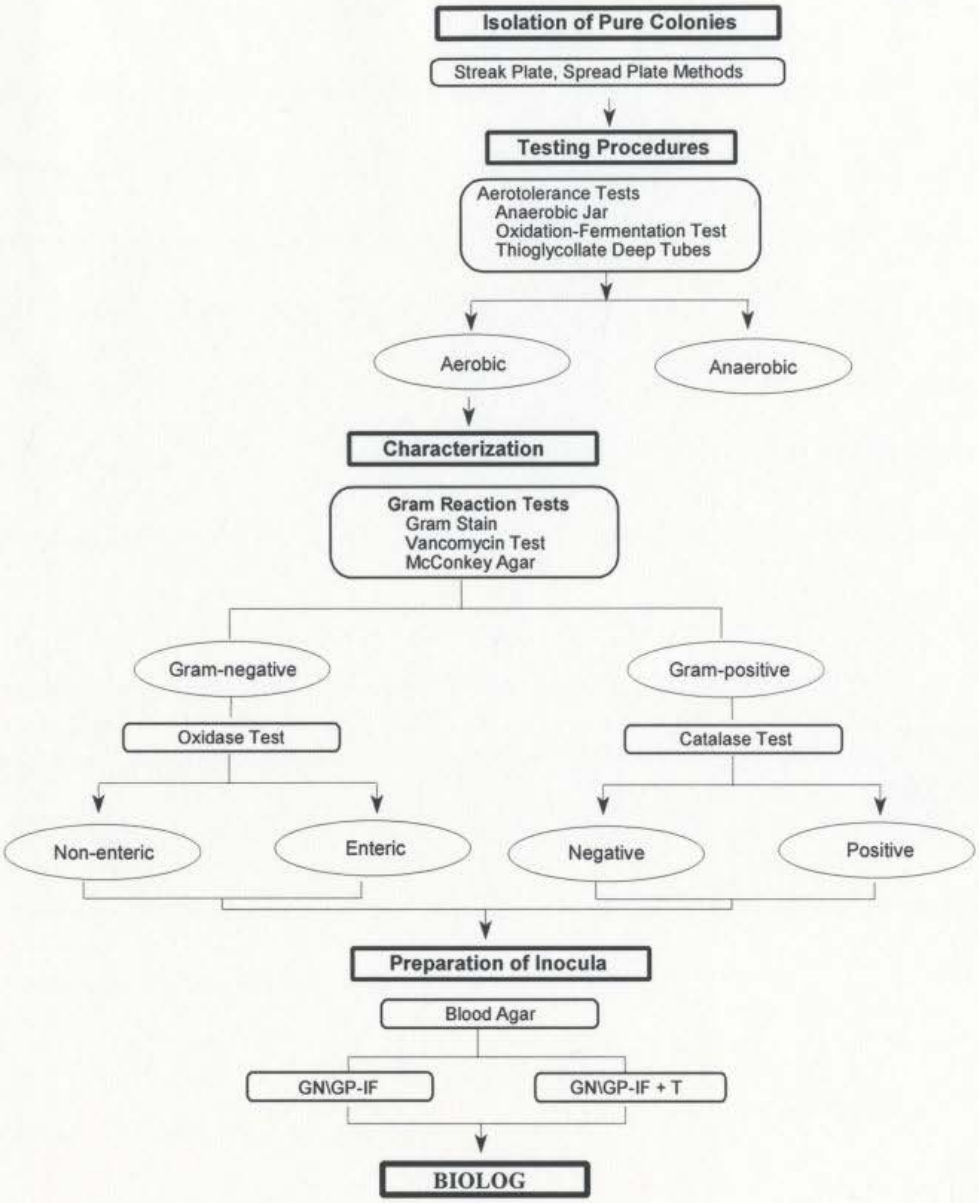

Fig. 2.4. Flowchart for taxonomic identification using MicroLog™ system.

2.5.4.1. Preparation of Liquid Inocula

The strains were inoculated onto blood agar and incubated for 24 hours or longer for slow growing species, and these in turn were suspended in special inoculating fluids. GN/GP-IF for Gram negatives and GN/GP-IF + T for Gram positives at a specified density. The GN/GP-IF was prepared by mixing 0.1 g Gellan Gum, 4 g of NaCl and 0.3 g Pluronic F-68 in 1000 ml of distilled water and was dispensed in tubes and autoclaved. The same preparation was carried out for GN/GP-IF + T, except for the addition of three drops of thioglycolate solution. The cell suspensions, about 150 µl, were inoculated into individual wells in microplates provided specifically for Gram negatives and Gram positives and were incubated at the same temperature used to culture the microorganisms.
As described earlier, the microplates contained 95 preselected carbon sources. Cells that used the carbon sources in certain wells respired, reducing the tetrazolium dye leading to formation of a characteristic pattern of purple wells which comprised the "metabolic fingerprint" of the capabilities of the inoculated organisms.

2.5.4.2. Reading of Microplates

The final step was the reading of the patterns produced after 24 to 48 hours of incubation using a MicroStation Reader (Fig.2.5). The fingerprint data were analyzed by the Biolog MicroLog software that automatically searched its extensive databases and provided an identification in seconds.

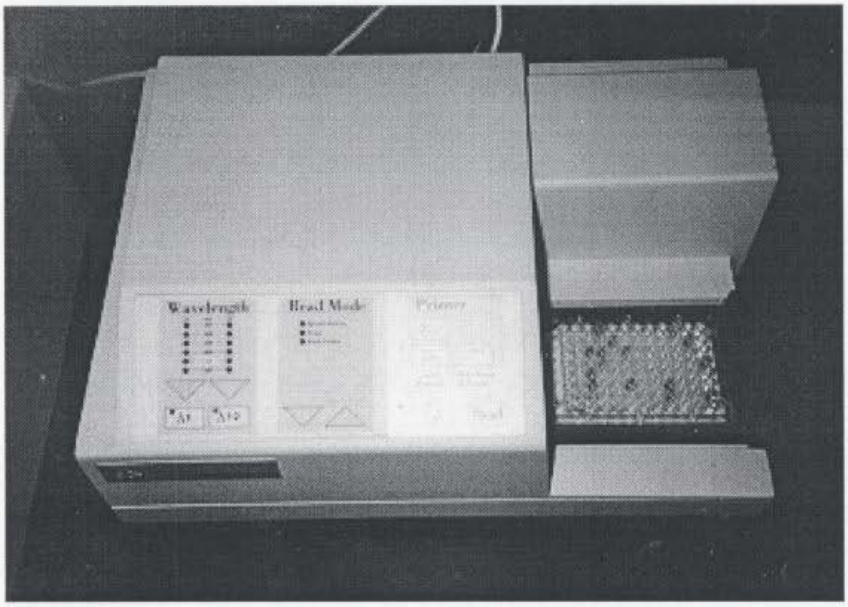

Fig. 2.5. MicroStation Reader for BIOLOG identification.

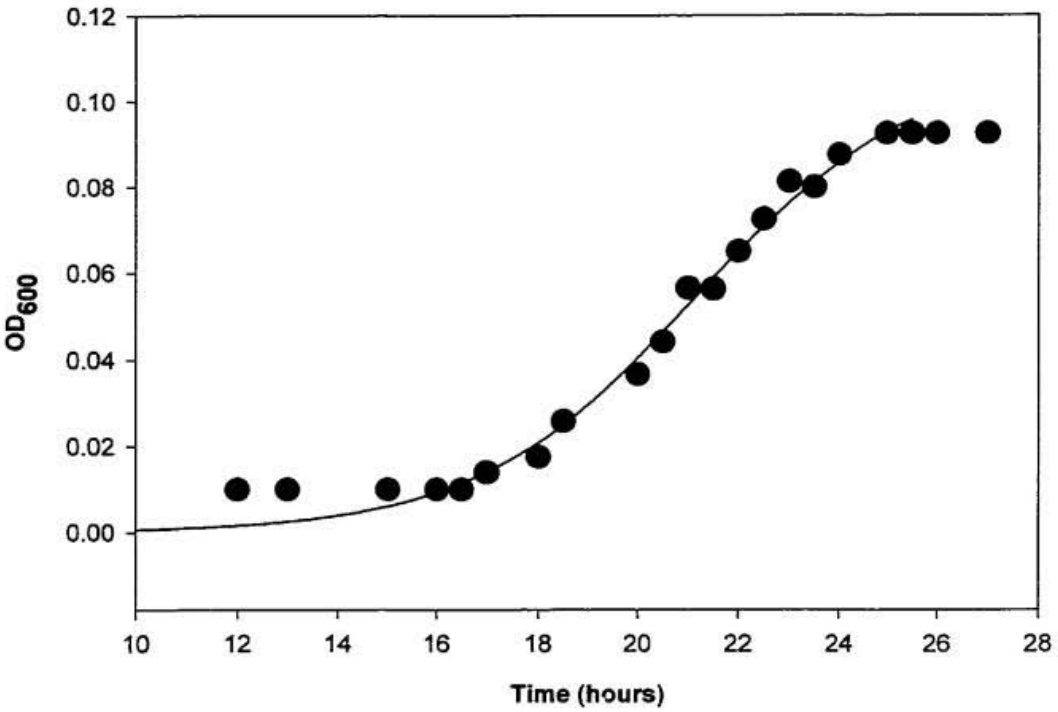

Fig. 3.1. Plot of optical density (OD) readings against incubation time using 2μl of toluene. Solid line indicates growth curve of the degrading microcosm.

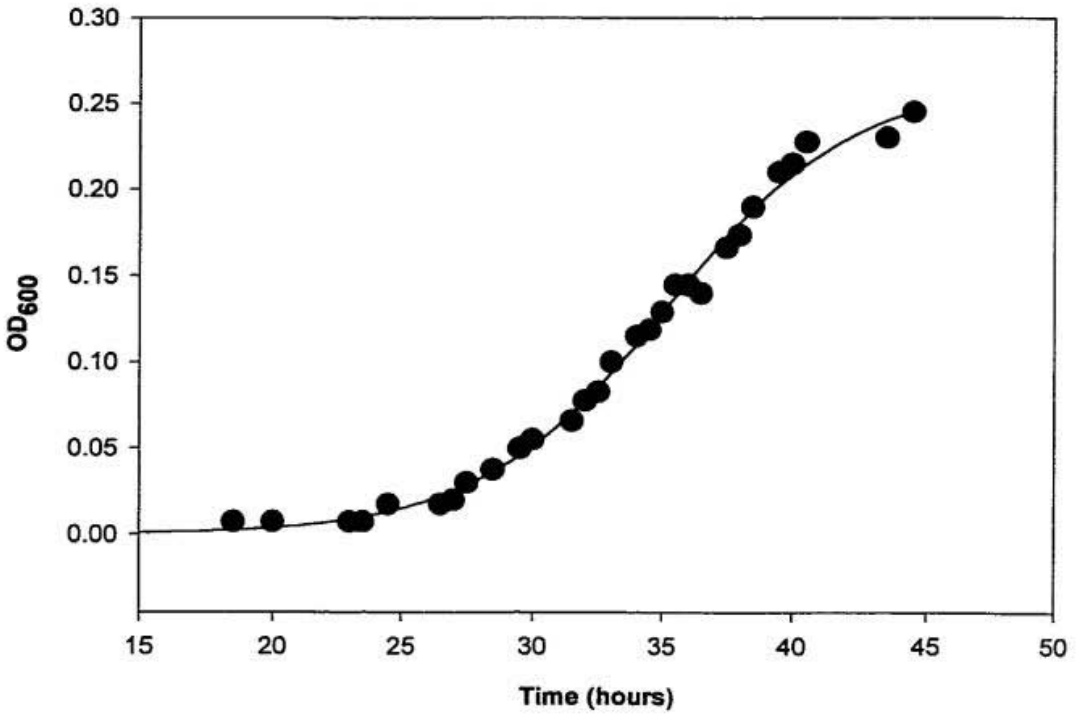

Fig. 3.2. Plot of optical density (OD) readings against incubation time using 10μl of toluene. Solid line indicates growth curve of the degrading microcosm.

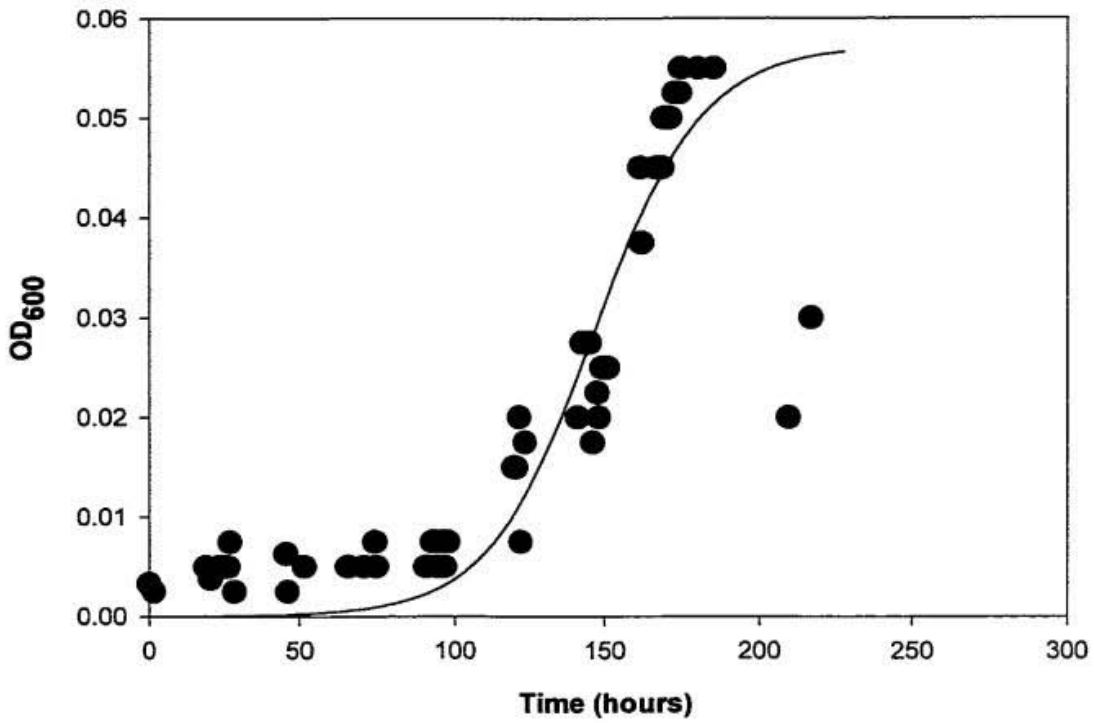

Fig. 3.3. Plot of optical density (OD) readings against incubation time using 2μl of ethylbenzene. Solid line indicates growth curve of the degrading microcosm.

0.0075 and reached up to 0.245 occurred within 25 to 45 hours from the time of inoculation (Fig. 3.2). Likewise, an increase in optical density measurements was also noted for ethylbenzene. The exponential growth of the consortium utilizing this compound was initiated only after 3 to 7 days of incubation (Fig. 3.3). Initial and final OD readings were about .0025 and 0.08, respectively.

Using the relationship described in Section 2.4, concentrations of residual hydrocarbons in the headspace were calculated from the peak area measurements from the GC-IRMS. These concentrations are shown in Figs. 3.4 to 3.6. Comparing Fig. 3.1 to Fig. 3.4, it is noted that an inverse relationship exists between optical density and concentration of residual hydrocarbons. A similar relationship is discernible when Fig. 3.2 is compared to Fig. 3.5 for 10 μl toluene and Fig. 3.3 to Fig. 3.6 for ethylbenzene. With an increase in biomass as indicated by the increase in optical density, a corresponding decrease in concentration was observed indicating microbial removal of the hydrocarbon.

Measurements of hydrocarbon concentration in the control solutions remained relatively consistent over time for the two sets of experiments for toluene as well as for ethylbenzene. The plots for the blank solutions are also shown in Figs. 3.4 to 3.6. Any observed deviations in the blank solution measurements were likely caused by fluctuations in the performance of the GC-IRMS or in other sources of analytical error. All in all, peak areas in the control solutions can be said to have remained unchanged.

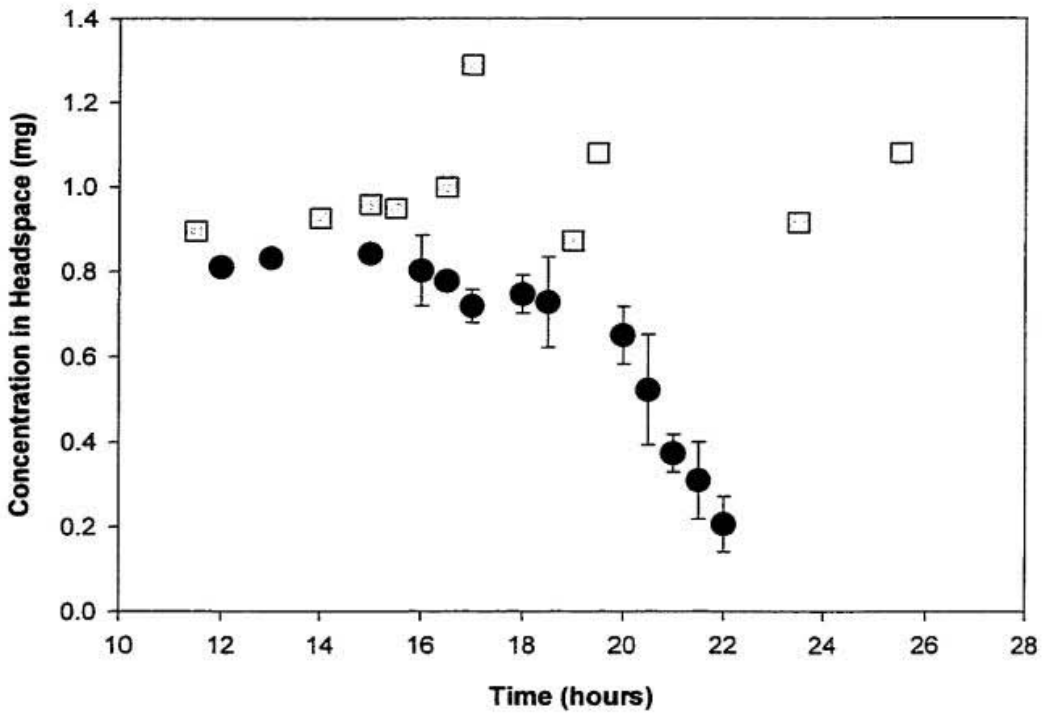

Fig. 3.4. Concentrations of toluene (2μl) in the headspace over time during biodegradation experiments. (●) represents data from experimental flasks and (□) from the control flasks. Error bars represent $\pm 1\sigma$.

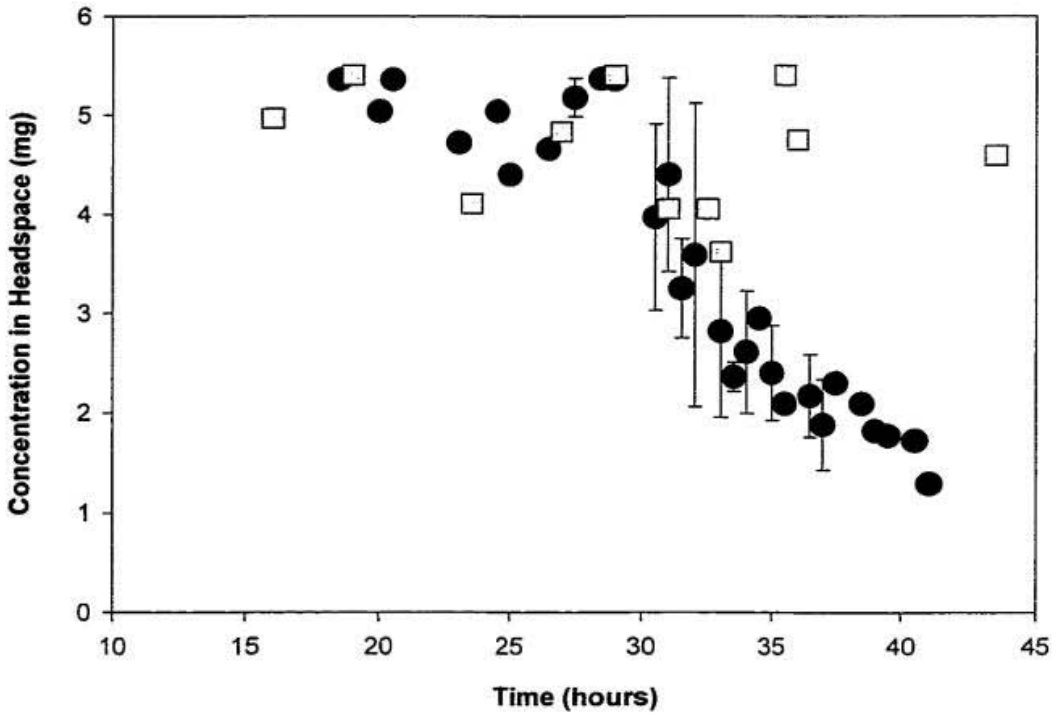

Fig. 3.5. Concentrations of toluene (10μl) in the headspace over time during biodegradation experiments. (●) represents data from experimental flasks and (□) from the control flasks. Error bars represent ±1σ.

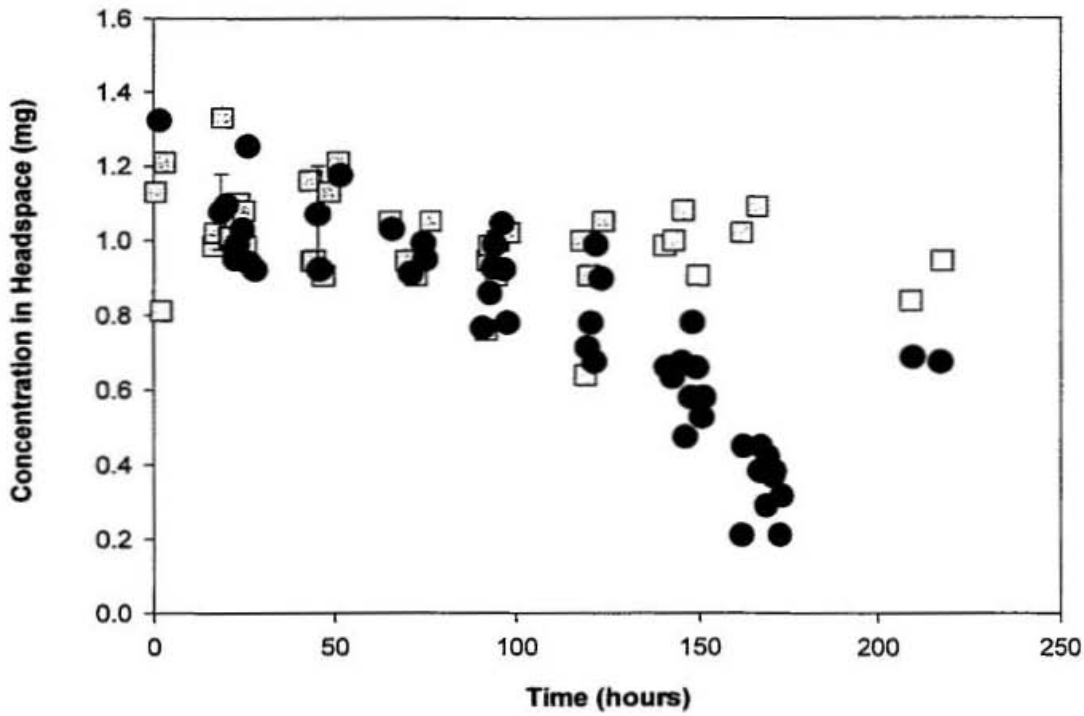

Fig. 3.6. Concentrations of ethylbenzene (2μl) in the headspace over time during biodegradation experiments. (●) represents data from experimental flasks and (□) from the control flasks. Error bars represent ± 1σ.

Figures 3.7 to 3.9 give the $\delta^{13}C$ values of each experiment with respect to time. As the hydrocarbon concentration was depleted and microbial biomass increased during the course of experiments, the $\delta^{13}C$ values did not show any significant changes. The values were still within analytical uncertainty of the initial $\delta^{13}C$.

In Figure 3.7, with 2 μl of toluene as the substrate, the last measurement has significantly high $\delta^{13}C$ compared with the starting value of approximately -27 ‰. This last observation, however, has a large standard deviation of 3.17 ‰ associated with it that could be attributed to increased analytical variability with decreasing concentration of residual hydrocarbon. The isotopic measurement of the solution in the control flasks averages about -27 ‰ with standard deviation of 0.65 ‰ but this includes the last measurement of -28.8 ‰ seemingly anomalous compared to other values. Without the last one, the standard deviation is only 0.37 ‰ which is close to analytical variability. The uncertainty of the last measurement could be due to performance of the machine or other sources of analytical errors.

For the experiments using a higher substrate concentration (10 μl of toluene) which was used to determine whether there would be isotopic effects induced by increased concentration (Fig. 3.8), the isotopic compositions were considerably constant at about -27.3 ‰ ± 0.16 ‰. At the same time, the blanks gave very similar values (mean of -27.3 ‰ ± 0.2 ‰). It is very clear that variations of both samples and blanks are within

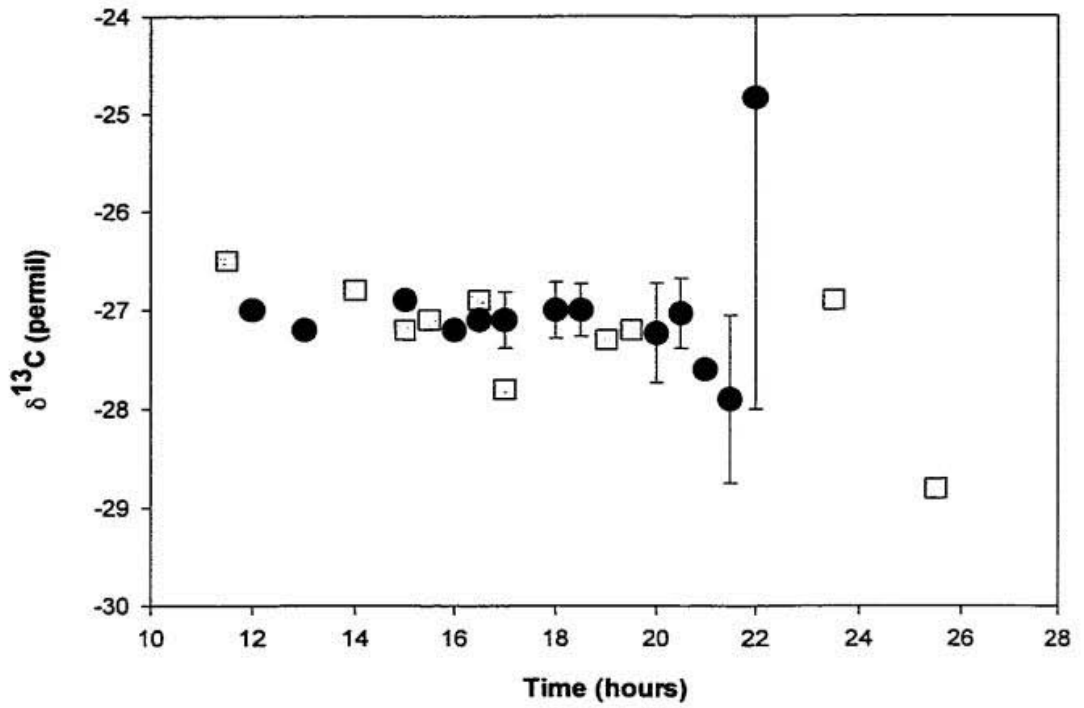

Fig. 3.7. $\delta^{13}C$ values of toluene (2μl) over time. (●) represents data from experimental flasks and (□) from the control flasks. Error bars represent $\pm$ 1σ.

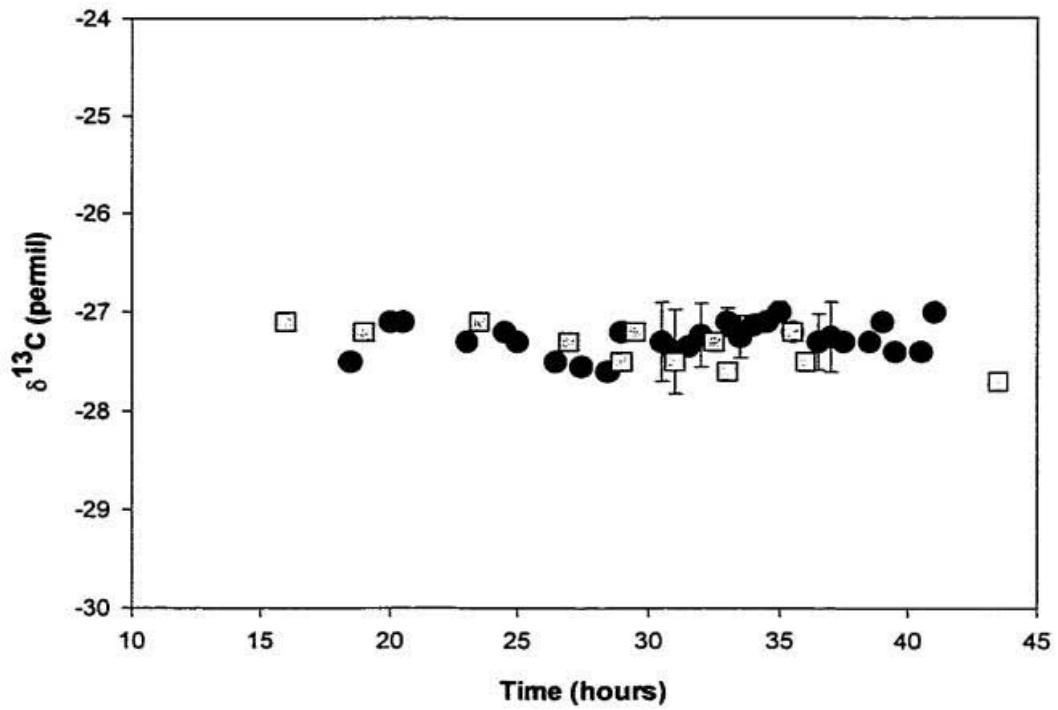

Fig. 3.8. $\delta^{13}C$ values of toluene (10μl) over time. (●) represents data from experimental flasks and (□) from the control flasks. Error bars represent $\pm 1\sigma$.

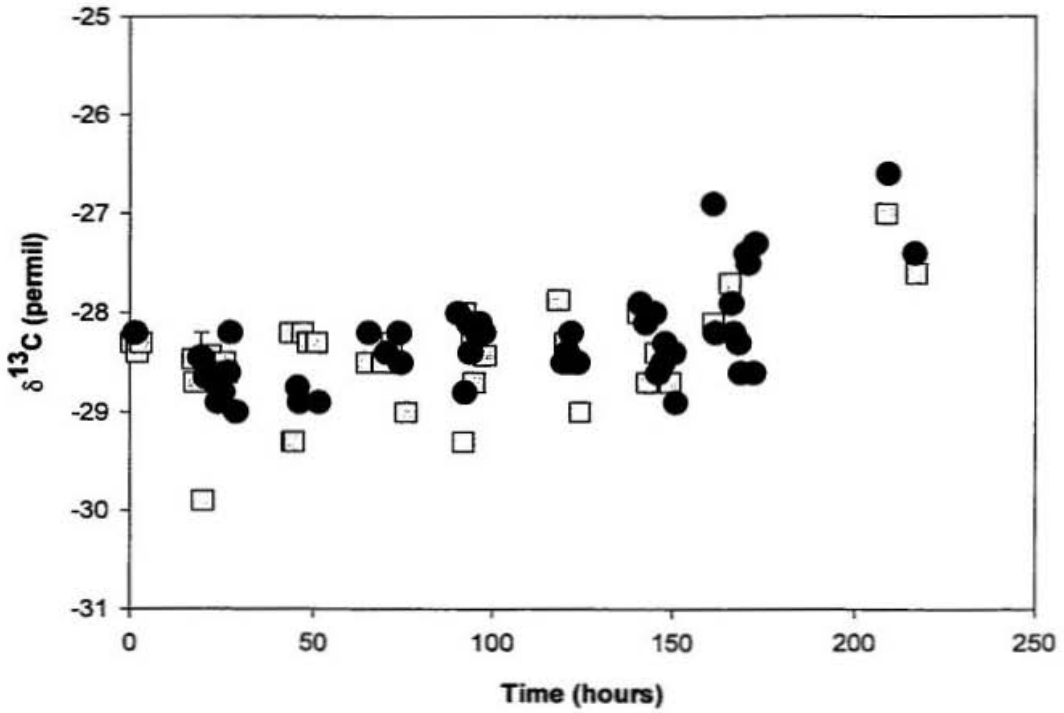

Fig. 3.9. $\delta^{13}C$ values of ethylbenzene (2μl) over time. (●) represents data from experimental flasks and (□) from the control flasks. Error bars represent $\pm 1\sigma$.

analytical variation.

For the ethylbenzene set up, the measurements toward the end were isotopically shifted slightly by about 2 ‰ from the value of nearly -29 ‰ at the start of the experiment (Fig. 3.9). Isotopic compositions of controls exhibited the same shift that could mean an artificially induced error by the IRMS rather than an actual change in the isotopic composition of the residual hydrocarbon.

The initial and final pHs of each culture flasks were also recorded. The initial pH of the hydrocarbon degrader medium was 6.34 to 6.5. To prevent hydrocarbon loss and introduction of unwanted organisms, the initial pH of the solution blanks and the culture flasks was not measured. It was assumed that their pH was almost the same as that of the hydrocarbon degrader medium due to the small amount of hydrocarbon added (2 μl and 10 μl) and that microbial activity had not yet started at that early stage of the experiments. However, final pH for each culture flask was obtained at the end of each experiment. For 2 μl of toluene, 10 μl of toluene and 2ul of ethylbenzene, the average final pH was slightly acidic with values of 6.31, 6.03 and 6.37, respectively. This suggests that some acidic metabolic products might have been produced by the consortium after degradation of each compound.

3.2. Taxonomic Identification of Microcosm

A series of continuous transfers gave taxonomic diversity to the hydrocarbon-degrading bacterial community used in the present study. Although the consortium was taken from an aquifer contaminated with various hydrocarbons, the degraders were initally acclimatized by supplementing them with specific hydrocarbon compounds as the only substrates. This gave rise to morphologically distinct colonies isolated from cultures specifically adapted to that compound, e.g., toluene culture, ethylbenzene culture. Though isotopic studies for the three selected hydrocarbons were not successful, identification of the different species that make up the naphthalene, methanol and hexadecane cultures was carried out for future work.

From the five different cultures, about 26 pure strains were identified according to their morphological characteristics as listed in Tables 3.1a, b and c. Some strains isolated from one culture were found to have characteristics similar to those in other cultures.

Results of the thioglycollate deep tubes, anaerobic jar and OF tests showed that all are aerobic bacteria. In all three tests, growth was affected by the absence or presence of free oxygen. Growth was limited to the upper portion of the deep tubes where free oxygen existed while TSA plates streaked with the organisms and placed in an anaerobic jar and the inoculated OF test tubes covered with oil showed no growth. The OF test also

Table 3.1a. Morphological characteristics of microbial strains isolated from toluene (EDT) and ethylbenzene (EDE) cultures.

Organism		Colony Description	Gram Reaction	Shape	Arrangement/ Size	Motility Test
EDT	1	brown, circular, convex, entire, smooth ave size 1.0mm	-	rods	single, pair, chain, rounded ends, stubby ave size 1.9 x 0.68 μm	motile
	2	yellowish, circular, convex, entire, smooth ave size 1.3 mm	-	rods	single, pair, chain, thin, stubby ave size 1.58 x 0.6 μm	motile
	3	beige, circular, convex, entire, smooth ave size 1.1 mm	-	rods	single, chain, thin ave size 2.35 x 0.58 μm	motile
	4	dark brown, circular, raised, entire, smooth, glistening ave size 1.6 mm	-	rods	single, pair, chain, almost square ends ave size 1.7 x 0.7 μm	motile
EDE	1	light brown, circular, raised (?), entire, smooth ave size 0.25 mm	-	rods	single, pair, chain ave size 1.6 x 0.35 μm	motile
	2	yellow, circular, raised, entire, smooth, glistening ave size 0.6 mm (?)	-	rods	single, pair, some are stubby ave size ? μm	motile
	3	light brown, circular, convex, entire, smooth ave size 0.75m (?)	-	rods	single, thin, long ave size ?μm	motile
	4	light brown, entire, punctiform, very small to measure	-	rods	single, very thin ave size 1.75 x 0.55μm	motile
	5a	white, circular, convex, entire, smooth, dull ave size 0.35 mm	+	cocci	single, pair, clusters, chains ave size 1.0 μm	nonmotile
	5b	light beige, circular, raised (almost flat), entire, smooth, dull ave size 0.45 mm	+	cocci	single, pair, more oval in shape ave size 0.8 μm	nonmotile
	7	dark brown, circular, raised, entire, smooth ave size 1.2 mm (?)	-	rods	single, long, stubby ave size ? μm	motile
	8	light brown, circular, convex, entire, smooth, dull, punctiform, very small to measure	-	rods	single, stubby, palisade ave size ?μm	motile

Table 3.1b. Morphological characteristics of microbial strains isolated from naphthalene (EDN) and methanol (EDM) cultures.

Organism		Colony Description	Gram Reaction	Shape	Arrangement/ Size	Motility Test
EDN	1	light brown (darker in the middle), circular, raised, entire, smooth ave size 1.3 mm	-	rods	single, pair, stubby, palisade ave size 1.05 x 0.5 μm	motile
	2b	yellowish, circular, raised (almost convex), entire, smooth ave size 1.1 mm	-	rods	single, pair, squarish ends ave size 1.45 x 0.5 μm	motile
	3	whitish brown, circular, convex, entire, smooth ave size 2.0 mm	-	rods	single, pair, palisade ave size 2.05 x 0.75 μm	motile
EDM	1	dark brown, circular, raised, entire, smooth, punctiform ave size 0.65 mm	-	rods	single, pair, chain, thin, stubby ave size 2.31 x 0.56 μm	motile
	3	beige, circular, almost flat, entire, dull, punctiform ave size 0.5 m m	-	rods	single, pair, stubby ave size 1.85 x 1.0 μm	motile
	4a	beige (dark brown), circular, convex, entire, smooth ave size 1.5 mm	-	Rods	single, pair, palisade, rounded ends ave size 1.65 x 0.65 μm	motile
	4b	dark brown, circular, raised, entire, smooth, punctiform, very small to measure	+	cocci	single, pair, tetrad ave size 0.5 μm	nonmotile
	5	whitish (cream), circular, convex, entire, smooth, punctiform ave size 0.5 mm	-	rods	Single ave size 1.35 x 0.5 μm	motile

Table 3.1c. Morphological characteristics of microbial strains isolated from hexadecane (EDH) culture.

Organism		Colony Description	Gram Reaction	Shape	Arrangement/ Size	Motility Test
EDH	2	light brown, circular, almost flat(?), entire, smooth ave size 0.25 mm	-	rods	single, palisade ave size 1.8 x 0.5 μm	motile
	3	dark brown, circular, convex, entire (irregular?), smooth ave size 1.0 mm	-	rods	single, chain, palisade ave size 1.4 x 0.75 μm	motile
	4a	salmon, circular, convex, entire, smooth, dull ave size 0.25 mm	+	cocci	single, tetrad ave size 0.95 μm	nonmotile
	4b	beige, circular, convex, entire, smooth, dull ave size 0.85mm	+	cocci	tetrads, chain	nonmotile
	6	mustard yellow, circular, convex, entire, smooth, dull ave size 0.65 mm	+	cocci	pair, chain (streptococci) chain, tetrad ave size 1.0 μm	nonmotile
	7	mustard yellow, circular, convex, entire, smooth, dull ave size 0.55 mm	+	cocci	pair, tetrad, chain (staphylococci) ave size 1.0 μm	nonmotile

indicated that they were oxidative organisms.

Based on the outcome of the Gram stain and antibiotic tests, all bacterial strains were Gram-negative rods except for two (2), one (1), and four (4) species from ethylbenzene, methanol and hexadecane, respectively, which were Gram-positive cocci. Motility tests using the TTC reagent indicated that the Gram-negative rods were motile while the Gram-positive cocci were non-motile.

The oxidase test further verified that the Gram-negative organisms were non-enteric as they exhibited positive results indicated by formation of a blue color when a loopful of bacteria was smeared on a filter paper moistened with tetramethyl-p-phenylenediame dihydrocloride. Gram-positive cocci, on the other hand, gave positive reactions with the catalase test where production of frothing or bubbling was observed.

Biolog identifications of the microbial strains are listed in Tables 3.2a and b. Gram-negative bacteria were mostly strains of the genera *Pseudomonas*, *Stenotrophomonas*, *Oligella, Bordetella* and *Acidovorax*. On the other hand, Gram-positives were identified as belonging to the genera of *Micrococcus*, *Staphylococcus, Dermacoccus* and *Kokuria* (or *Erythromyxa*).

Table 3.2a. Identification of the different microbial strains from toluene (EDT), ethylbenzene (EDE) and naphthalene (EDN) cultures.

Organism		Gram Reaction	Morphology	Identification
EDT	1	-	rods	*Pseudomonas fluorescens*
	2	-	rods	*Stenotrophomonas maltophilia*
	3	-	rods	*Pseudomonas fluorescens*
	4	-	rods	*Pseudomonas fluorescens/ Pseudomonas marginalis/ Pseudomonas fluorescens biotype F*
EDE	1	-	rods	*Pseudomonas pseudoalcaligenes/ Bordetella trematum (Bordetella hinzii)*
	2	-	rods	*Stenotrophomonas maltophilia*
	3	-	rods	*Oligella ureolytica (Bordetella hinzii)*
	4	-	rods	*Acidovorax facilis (Pseudomonas fluorescens/ Myroides odoratus/ Pseudomonas synxantha)*
	5a	+	cocci	*Staphylococcus warneri*
	5b	+	cocci	*Staphylococcus warneri*
	7	-	rods	*Pseudomonas fluorescens biotype G*
	8	-	rods	*Oligella ureolytica/ Bordetella trematum (Bordetella bronchiseptica)*
EDN	1	-	rods	*Pseudomonas fluorescens biotype G*
	2b	-	rods	*Stenotrophomonas maltophilia*
	3	-	rods	*Pseudomonas fluorescens*

Table 3.2b. Identification of the different microbial strains from methanol (EDM) and hexadecane (EDH) cultures.

Organism		Gram Reaction	Morphology	Identification
EDM	1	-	rods	*Bordetella hinzii*/ *Bordetella-like species* (*Oligella ureolytica*)
	3	-	rods	*Acidovorax facilis* (*Alcaligenes xylosoxydans*/ *Comomonas acidovorans*)
	4a	-	rods	*Pseudomonas putida biotype B*/ *Pseudomonas fluorescens biotype G*
	4b	+	cocci	*Dermacoccus nishinomiyaensis*
	5	-	rods	*Pseudomonas citronellolis*/ *Pseudomonas fluorescens*
EDH	2	-	rods	*Acidovorax facilis* (*Pseudomonas synxantha*/ *Pseudomonas fluorescens biotype C*)
	3	-	rods	*Pseudomonas fluorescens*/ *Pseudomonas fluorencens biotype G*
	4a	+	cocci	*Kocuria rosea*/ *Erythromyxa*
	4b	+	cocci	*Kocuria rosea*/*Erythromyxa*/ *Micrococcus diversus*
	6	+	cocci	*Micrococcus luteus*
	7	+	cocci	*Micrococcus luteus*

Chapter 4

Discussion

4.1. Laboratory Biodegradation Studies

4.1.1. Microbial Degradation

The toluene culture was dominated by two isolates belonging to the genera of *Pseudomonas* and *Stenotrophomonas* (Table 3.2a). These organisms are aerobic, Gram-negative rods. *Oligella*, *Acidovorax*, *Bordetella* and *Staphylococcus* species were also found in the ethylbenzene culture. The first three are Gram-negative rods but the latter is Gram- positive coccus.

Based on a number of published reports, *Pseudomonas* species are ubiquitous, and known to degrade wide classes of hydrocarbons in marine and soil environments (Gibson, 1984; Vanderbergh and Kunka, 1988; Swanson, 1992; Caldini *et al.*, 1995; Whitman *et al.*, 1998). On the other hand, *Stenotrophomonas maltophilia*, commonly found in soil environments, has only been reported to degrade high molecular weight hydrocarbons (Boonchan *et al.*, 1998). The role of the other identified species in biodegradation of hydrocarbons has not been previously described.

The present work was not able to determine the metabolic pathways and the associated metabolic products as well as the enzyme systems used by the above-mentioned aerobic microorganisms. However, the general degradative pathways established in previous works are described. These pathways could have been used by the said organisms to degrade the aromatic hydrocarbons (e.g. toluene, ethylbenzene, etc.) but future work would benefit from determining the exact pathways that these organisms used and the by-products that influence the overall degradation of the compounds.

Several studies have demonstrated the capability of microorganisms to transform hydrocarbon compounds utilizing a wide array of chemical reactions or metabolic pathways. However, most aerobic bacteria use three types of initial reactions to transform the compounds into products that are structurally similar to chemicals that microorganisms are used to metabolizing (Schwarzenbach *et al.*, 1993). With only one or a few initial transformations, the resulting chemical products can be included in the more common degradation pathways and be fully degraded. These reactions are often mediated by a variety of enzymes that function as catalysts, hence increasing the rate of chemical reactions in the bacterial cell.

Oxidation by species of *Pseudomonas* is frequently accomplished using an electrophilic form of oxygen to actively mineralize aromatic hydrocarbons in the environment (Atlas, 1978; Gibson, 1984). The oxidation of monoaromatic hydrocarbons such as the BTEX compounds, for instance, may be initiated by two functionally distinct classes of

oxygenase enzyme systems extensively described in the literature (Gibson and Subramanian, 1984; Schwarzenbach *et al.*, 1993). These include the monooxygenase and dioxygenase enzyme systems. Generally, the aromatic compounds are first transformed into catechol or its derivatives by these systems and subsequently metabolized through common metabolic pathways (Fig. 4.1) (Fewson, 1981; Ribbons *et al.*, 1982; Gibson, 1984; Cerniglia, 1984; Pitter and Chudoba, 1990; Muller, 1992; Baker and Herson, 1994; Hall *et al.*, 1999). Benzene is initially oxidized by the introduction of two hydroxyl groups from a two-component enzyme system forming *cis*-hydrodiols, which in turn are dehydrogenated to yield catechol (Fig. 4.2) (Gibson and Subramanian, 1984). Toluene has many separate biodegradative pathways, some of which include 3-methylcatechol as an intermediate product (see succeeding section). Many separate pathways also exist for ethylbenzene, which can be degraded to 3-ethylcatechol. In each of these cases, the aromatic ring of the substituted catechol is later cleaved by dioxygenase enzymes.

After catechol formation, the aromatic nucleus in these compounds is broken through one of two pathways: the ortho-cleavage or the meta-cleavage pathway. The ortho pathway involves cleavage of the carbon bonds between the hydroxyl groups (Fig. 4.3) (Baker and Herson, 1994). This leads to the formation of the respective muconates and muconolactones, which are further metabolized to 4-oxoadipate enol-lactone and then to 3-oxoadipate (β-ketoadipate). Metabolism finally proceeds to intermediates acetyl-CoA and succinate of the trunk pathway called the Krebs cycle. These intermediates are metabolized by trunk pathway enzymes and used as growth substrates.

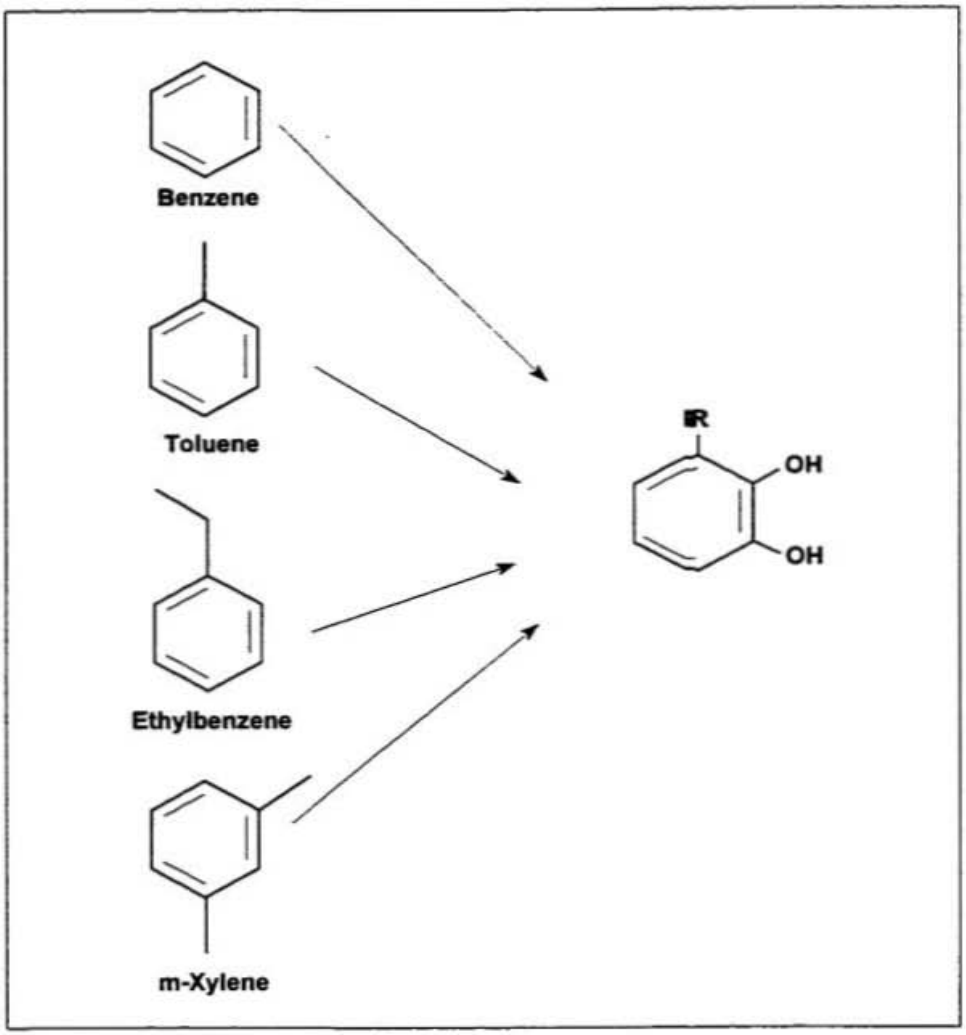

Fig. 4.1. Aerobic degradation of the BTEX compounds. Benzene: R = H; Toluene: R = CH_3; Ethylbenzene: R = CH_2CH_3; m-Xylene: R = CH_3.

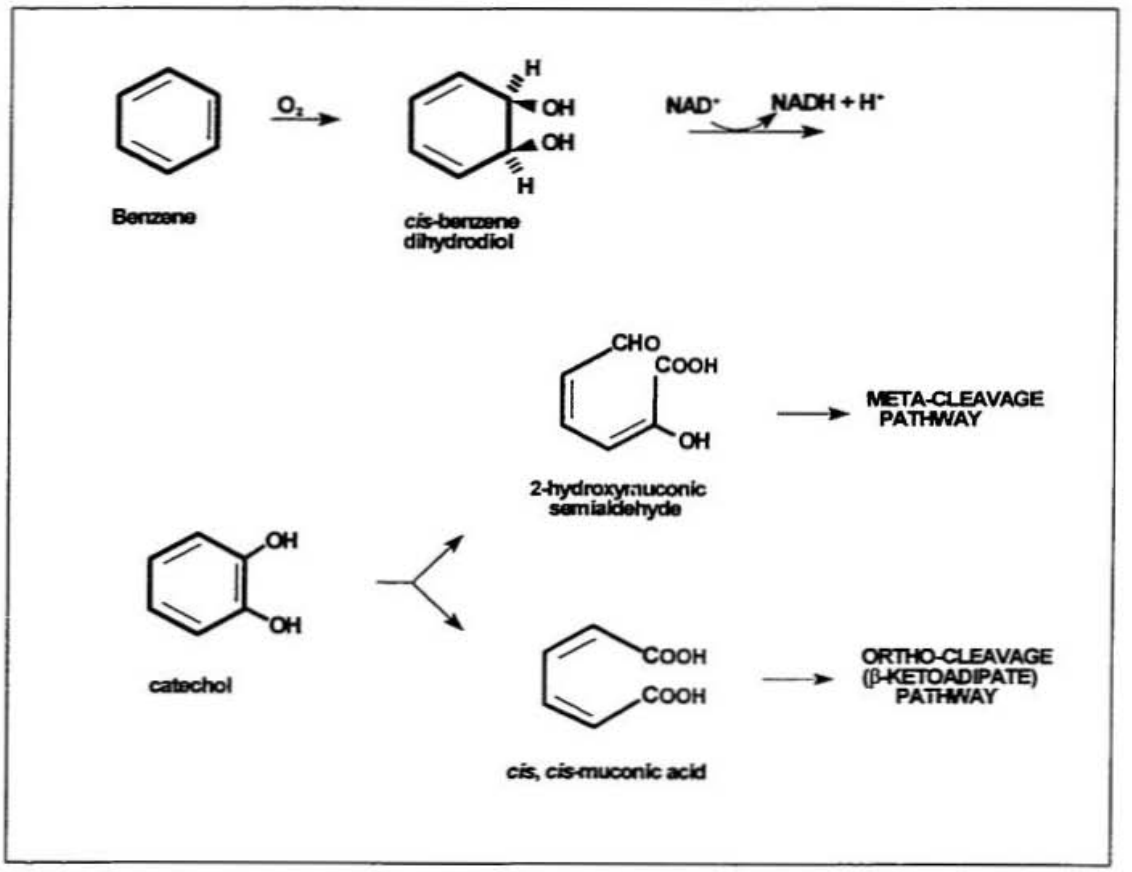

Fig. 4.2. Initial reactions utilized by bacteria to oxidize benzene (after Gibson and Subramanian, 1984).

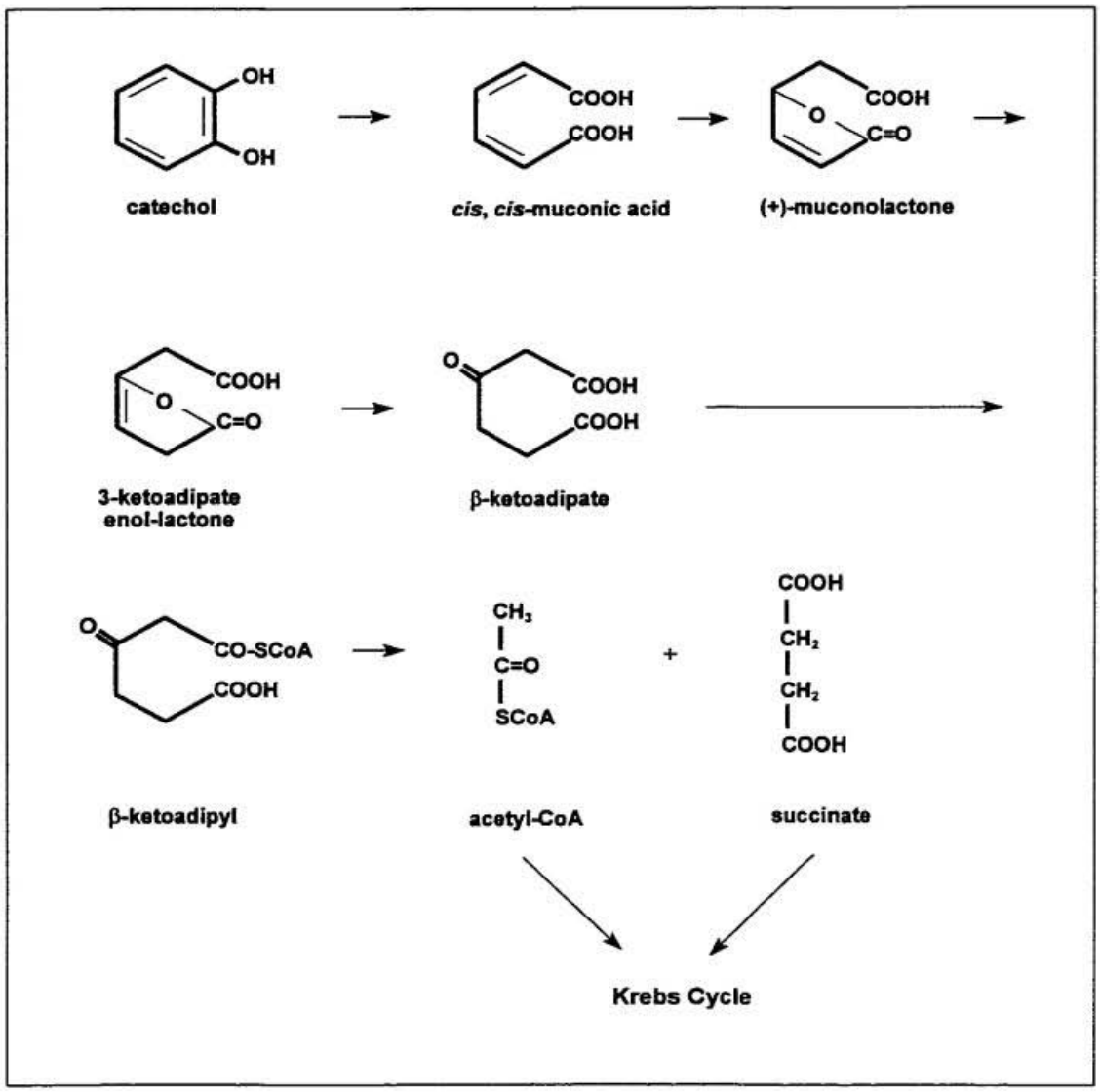

Fig. 4.3. The ortho-cleavage pathway (after Baker and Herson, 1994).

In meta cleavage, ring cleavage occurs between a carbon atom with a hydroxyl group and the adjacent unsubstituted carbon atom, forming a 2-hydroxy-muconic semialdehyde (Fig. 4.4) (Baker and Herson, 1994). Subsequent metabolism results in the formation of final products such as pyruvate, formate and acetaldehyde, which are further oxidized via the Krebs cycle.

Naphthalene, the simplest polycyclic aromatic molecule, is degraded by initial attack of a dihydrogenase, forming a *cis*-dihydrodiol, which is consequently dehydrogenated to 1,2-dihydroxynaphthalene (Figure 4.5) (Gibson and Subramanian, 1984). The aromatic ring is then cleaved oxidatively. The side chain, from the resulting molecule, is subsequently removed forming salicylate. Salicylate is oxidized to catechol whose oxidation has been described above.

4.1.2. Isotopic Fractionation

The molecular and stable isotopic compositions of hydrocarbons and other organic contaminants in surface and groundwater reflect the combined effects of the (1) nature of contaminant sources, (2) biotic and abiotic transformation during transport, (3) dynamics of source mixing, and (4) post-accumulation diagenetic reactions. This multiplicity of possible sources and processes affecting organic contaminants requires a complete understanding of the impact of specific processes or sources on the molecular and

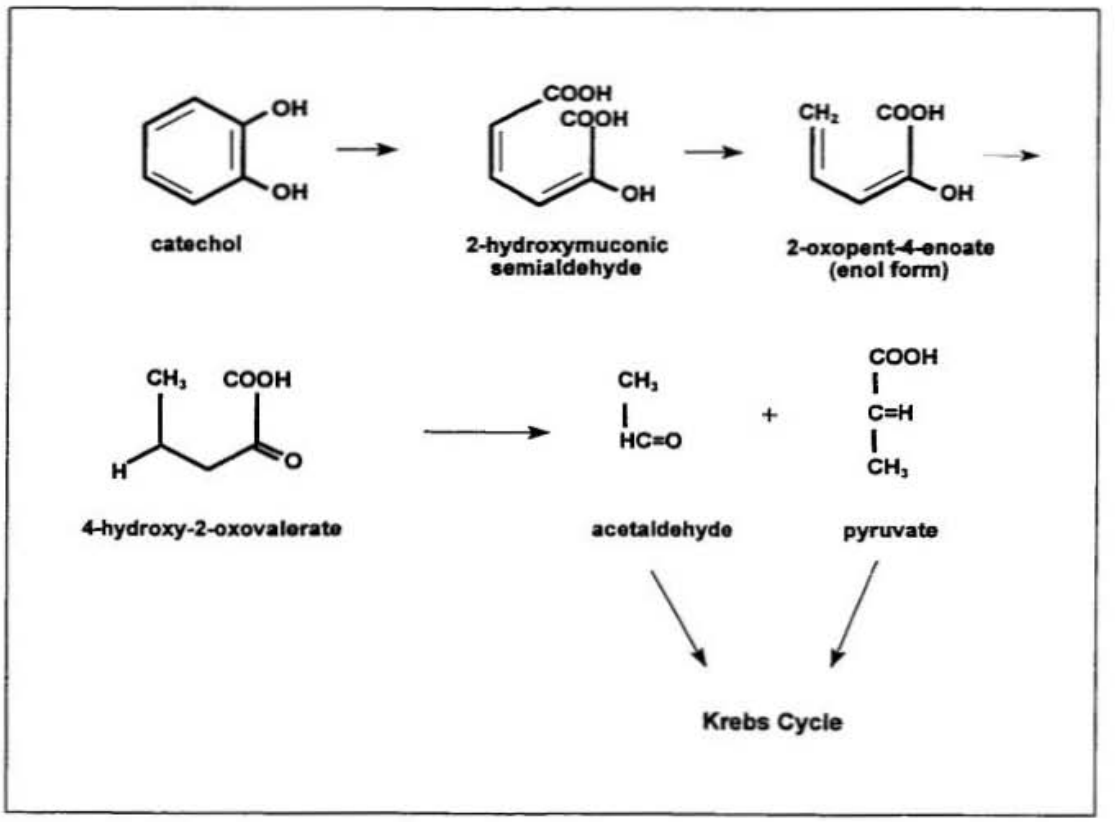

Fig. 4.4. The meta-cleavage pathway (after Baker and Herson, 1994).

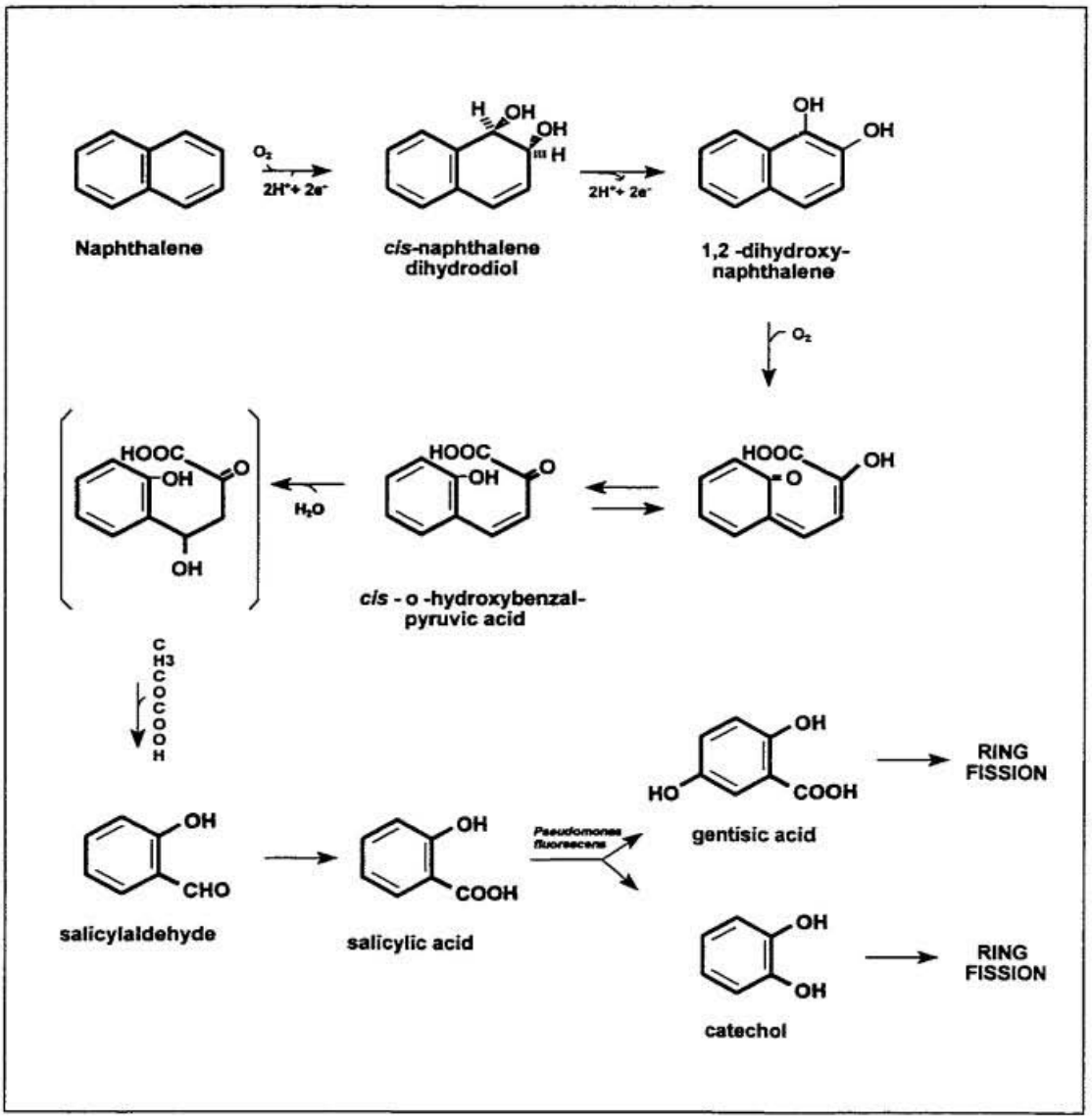

Fig. 4.5. Aerobic degradation pathway of naphthalene (after Gibson and Subramanian, 1984).

isotopic chemistry of the contaminant. This in turn requires the availability of multiple geochemical tools that are able to uniquely identify or seriously constrain specific contaminant sources and/or pathways. One of the goals of the present study is to examine the possible utility of carbon isotopic compositions as a means of quantifying biodegradation. If carbon isotope fractionation accompanies biodegradation, it was our secondary goal to examine the systematics of such fractionation.

Large carbon isotopic fractionation has been shown to accompany a variety of microbial processes (e.g., Abrajano and Sherwood Lollar, 1999). It has been observed during sulfate reduction (Jones and Starkey, 1957; Nakai and Jensen, 1964; Rees, 1973), denitrification (Mariotti *et al.*, 1988; Aravena and Robertson, 1998), and methanogenesis (Barker and Fritz, 1981; Coleman *et al.*, 1981; Krzycki *et al.*, 1987; Botz *et al.*, 1996). However, the magnitude of isotopic fractionation exhibited by microbial degradation of organic contaminants differs depending on their composition and structure. For instance, a large carbon isotopic fractionation was associated with microbial dechlorination of chlorinated ethenes (Hunkeler *et al.*, 1999; Huang *et al.*, 1999) and aerobic degradation of a chlorinated aliphatic compound, dichloromethane, (Heraty *et al.*, 1999) but no significant fractionation occurred during microbial degradation of aromatic hydrocarbons (O'Malley *et al.*, 1994; Trust *et al*, 1995).

The result obtained in previous studies on hydrocarbons (O'Malley *et al*, 1994) was confirmed by the present study in which laboratory experiments were performed to

determine carbon isotopic variations during aerobic degradation of low molecular weight hydrocarbons (toluene and ethylbenzene). As shown in Figs. 3.1 to 3.6, biodegradation was manifested by increases in microbial biomass and decreases in hydrocarbon concentrations. In Fig. 4.6, as the fraction of remaining toluene with initial concentration of 2 μl decreases (and disregarding the last measurement as quantity of residual toluene was not sufficient at this point to perform a reliable isotopic measurement), the $^{13}C/^{12}C$ remains relatively constant indicating no isotopic fractionation occurring. With a greater initial substrate concentration of toluene (10 μl), the same observation can be seen in Fig. 4.7. The $\delta^{13}C$ values stay relatively uniform though the amount of residual toluene was reduced to about 20%. This similar result for 2 μl and 10 μl toluene suggests that concentration is not a limiting parameter for isotopic fractionation to occur. Likewise, no significant change in $\delta^{13}C$ was noted for ethylbenzene as the residual concentration of the compound was diminished to as low as 20% of the original concentration (Fig. 4.8).

Recent batch vial experiments carried out by Sherwood Lollar *et al.* (1999) on biodegradation of toluene under aerobic conditions also showed results identical to that of the present study.

In contrast to the aforementioned results, a substantial isotopic fractionation (6-10‰) was obtained by Meckenstock *et al.* (1999) associated with aerobic and anaerobic

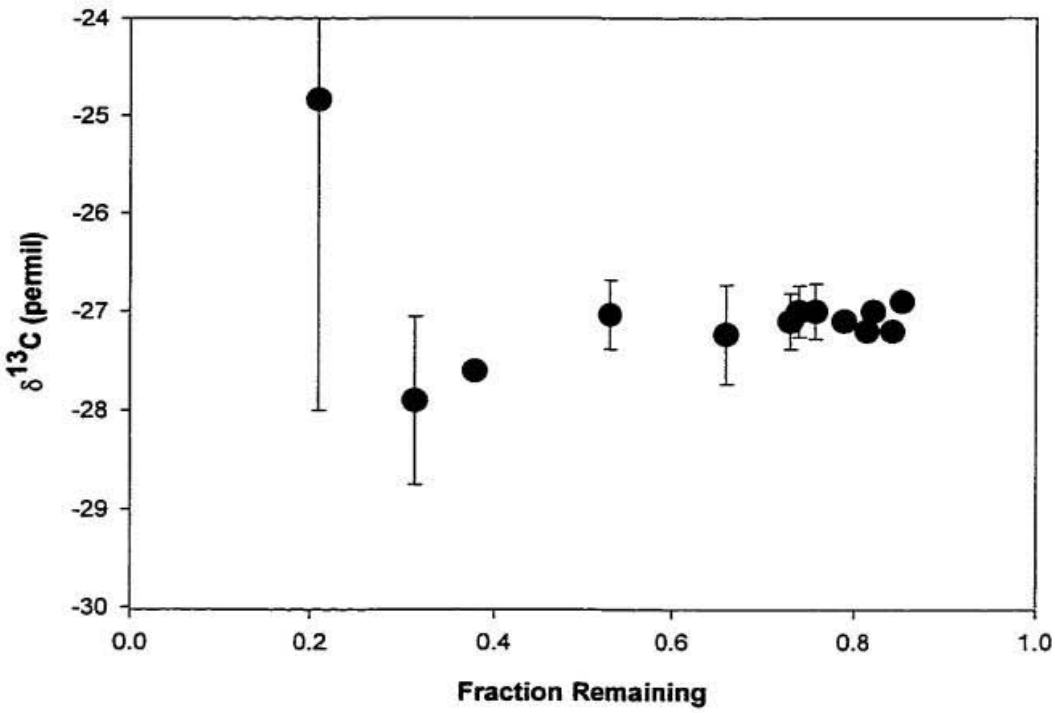

Fig. 4.6. Isotopic compositions versus fraction of residual toluene (2μl). Error bars represent $\pm \sigma 1$. The fraction of toluene remaining is calculated by assuming the concentration in each of the sample vials at t=0 is equal to that of the control flask.

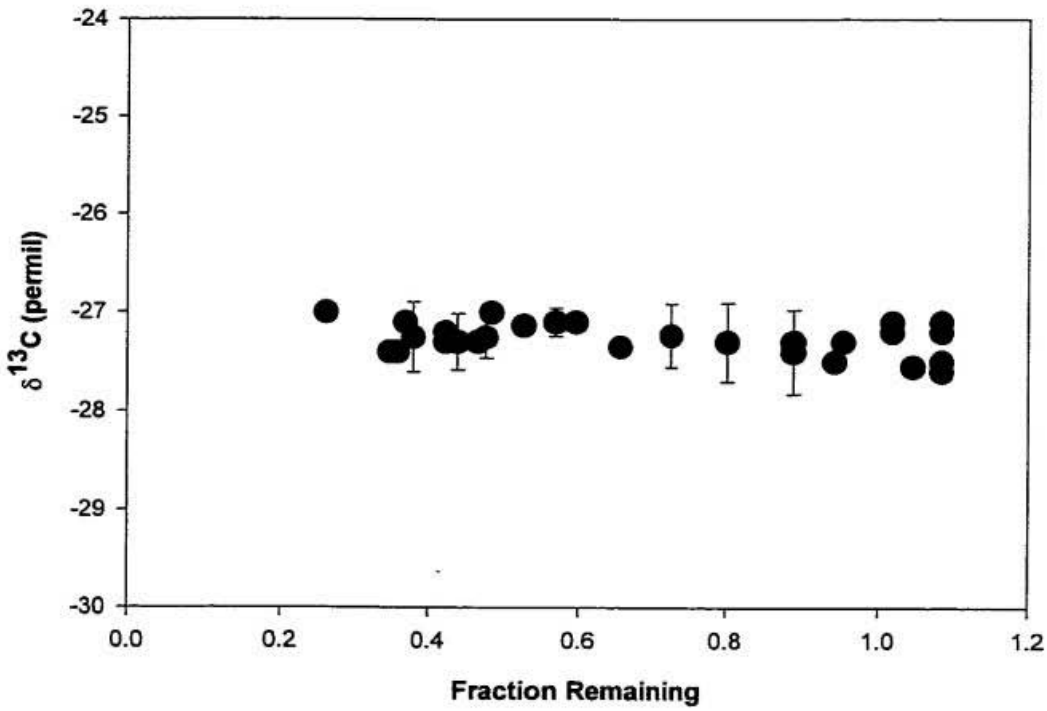

Fig. 4.7. Isotopic compositions versus fraction of residual toluene (10μl). Error bars represent $\pm \sigma 1$. The fraction of toluene remaining is calculated by assuming the concentration in each of the sample vials at t=0 is equal to that of the control flask.

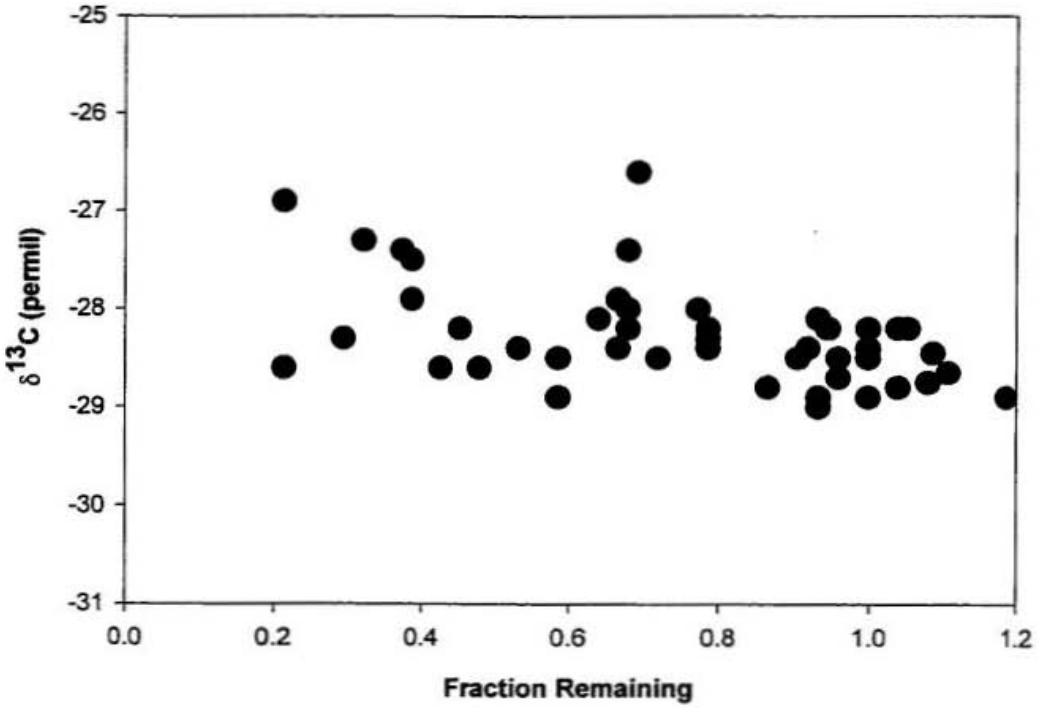

Fig. 4.8. Isotopic compositions versus fraction of residual ethylbenzene (2μl). Error bars represent $\pm \sigma 1$. The fraction of ethylbenzene remaining is calculated by assuming the concentration in each of the sample vials at t=0 is equal to that of the control flask.

biodegradation of the same compound (toluene) used both by Sherwood Lollar *et al.* (1999) and the current study. The contrasting results observed by Meckenstock *et al.* (1999) and the present experiment as well as that of Sherwood Lollar *et al.* (1999) suggest that different microbial communities and environmental conditions could control isotopic fractionation (Abrajano and Sherwood Lollar, 1999). It is also possible that differences in experimental design could have affected the results since these experiments were conducted under different conditions (e.g., temperature, initial concentrations, pH). However, this is not the case with Sherwood Lollar *et al.* (1999) and the present study. Although they employed a different experimental design and microcosm, the outcome revealed that aerobic biodegradation of toluene involves no significant fractionation in its carbon isotopic composition. The same observation was made by Sherwood Lollar *et al.* (1999) when a different consortium containing toluene degraders was utilized.

One major difference between the studies of Sherwood Lollar *et al.* (1999), Meckenstock *et al.* (1999) and the present study was the species employed in the respective experiments. The kinetic isotopic fractionation obtained by Meckenstock *et al.* (1999) reflects only the effect of the degradation of toluene by a single strain of bacterium (aerobic bacterium *Pseudomonas putida* strain mt-2). The present study as well as that of Sherwood Lollar *et al.* (1999) reflects the overall effect of degradation by mixed populations of microbial species. It is a common observation that the rate of biodegradation of a particular compound is faster with microbial communities compared to pure cultures (Slater and Lovatt, 1984). This is due to the interactions between species

making up the microbial community where competition between bacterial strains vying for the same substrate can lead to a faster degradation rate.

It is unclear why carbon isotopic fractionation would be influenced by the presence of a competitive microbial consortia, given that kinetic isotope effect, if present, will likely result in similar heavy isotope discrimination (i.e., ^{13}C-enriched residual hydrocarbon). It is therefore tempting to speculate that the difference between the Meckenstock *et al.* (1999) experiments on the one hand, and the present experiments and those of Sherwood Lollar *et al.* (1999) on the other is that the microbial degradative pathways are different (Abrajano and Sherwood Lollar, 1999). This would likewise imply that the bacterial species dominantly responsible for the hydrocarbon degradation in our competitive consortia experiments is not the *Pseudomonas putida* strain utilized by Meckenstock *et al.* (1999).

During aerobic microbial degradation, toluene has two likely sites for oxidative metabolic attack: the aromatic ring itself (Zylstra *et al.*, 1988) and the methyl group (Kitagawa, 1956; Nakazawa and Yokota, 1973). Some microorganisms such as the *Pseudomonas putida* F1 (PpF1) of Zylstra *et al.* (1988) oxidize toluene by the incorporation of both atoms of molecular oxygen into the aromatic nucleus to form *cis*-toluene dihydrodiol (Fig. 4.9A). This reaction is facilitated by multi-component enzyme system designated as toluene dioxygenase. Further metabolism of *cis*-toluene dihydrodiol involves an NAD+-independent dehydrogenation reaction to form 3-methyl catechol. Initial oxidative attack

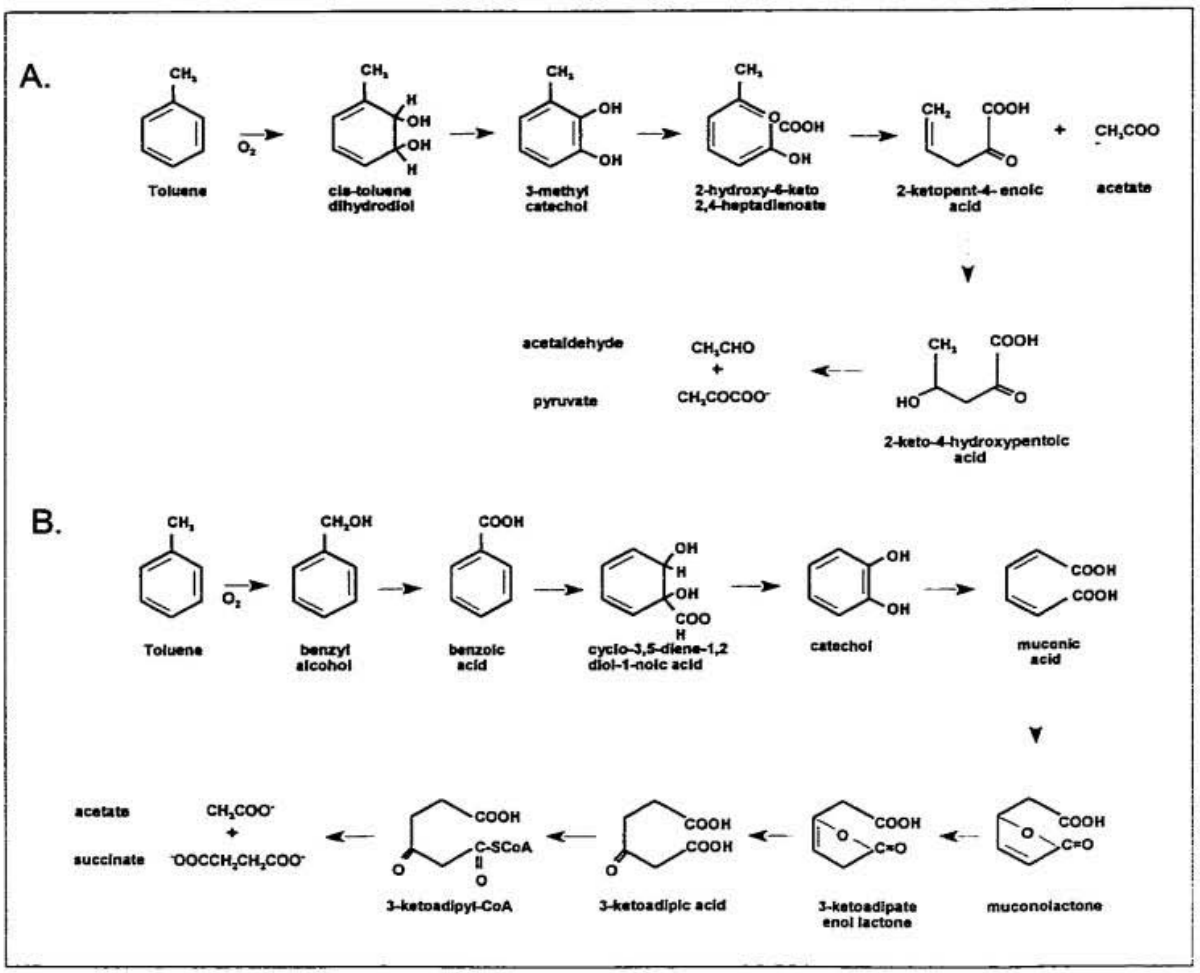

Fig. 4.9. Degradative pathways of toluene through oxidation of (A) the aromatic ring (Zylstra *et al.*, 1988) or (B) the methyl group (Kitagawa, 1956; Nakazawa and Yokota, 1973).

of toluene can also take place at the methyl constituent mediated by a monooxygenase enzyme system. This involves incorporation of one atom of oxygen into per dioxygen consumed. In this reaction, hydroxylation of toluene gives rise to the formation of benzyl alcohol and requires NADH and FAD (Nakazawa and Yokota, 1973; Gibson and Submaranian, 1984) (Fig. 4.9B). The benzyl alcohol is subsequently transformed to benzoic acid which is in turn converted to cyclo-3,5 diene-1,2 diol-1-noic acidate and finally to catechol.

It is probable that the initial oxidation of toluene utilized by *Pseudomonas putida* strain employed by Meckenstock *et al.* (1999) could be the same pathway as that of *Pseudomonas putida* F1 (PpF1) of Zylstra *et al.* (1988). It is possible then that isotopic fractionation observed by Meckenstock *et al.* (1999) might be related to this initial attack to the aromatic ring where two atoms of oxygen were added. The kinetic isotope effects associated with breaking one of the carbon-to-carbon bonds within the ring that led to the formation of the *cis*-toluene dihydrodiol likely caused this observed fractionation.

Benzene, being the simplest aromatic hydrocarbon, possesses only the basic aromatic ring and its degradation generally occurs with the oxidation of the aromatic ring itself where it is converted to *cis*-benzene dihydrodiol aided by the benzene dioxygenase multi-enzyme complex (Fig. 4.2) (Gibson and Subramanian, 1984). It is interesting to mention at this point that our previous aerobic microbial degradation experiments using benzene as substrate demonstrated isotope enrichment of ^{13}C in residual hydrocarbon (Stehmeier *et*

al., 1999; see Appendix). This was accompanied by a decrease in concentration as indicated by hydrocarbon loss of an average of 83% and by an increase in microbial culture absorbance as an indicator of microbial growth. Although small in magnitude, the isotope enrichment ranges between 2 to 7 times the analytical error of 0.3 ‰ (Fig. 4.10). Based on these observations, it seems probable that if the degradative pathway used by a single bacterial strain or by microbial consortia starts with the attack on the aromatic ring itself, notable isotopic fractionation could be observed, as shown by the results of Meckenstock *et al.* (1999) and our benzene experiments.

In contrast, the respective competitive consortia of the present study and Sherwood Lollar *et al.* (1999) could have initiated the degradation of toluene through oxygenation of its methyl group (Kitagawa, 1956; Nakazawa and Yokota, 1973). In this first reaction, only a carbon-hydrogen bond within the methyl substituent is broken to yield benzyl alcohol and this is apparently not associated with significant isotopic fractionation.

What was exhibited by the monoaromatic hydrocarbons, toluene and ethylbenzene, was likewise described for some polycyclic aromatic hydrocarbons mentioned earlier (e.g., O'Malley *et al.*, 1994). Studies on microbial degradation of polycyclic aromatic hydrocarbons made by O'Malley *et al.* (1994) showed no enrichment of ^{13}C in the residual hydrocarbons. Pure culture aerobic degradation experiments on naphthalene indicated that although rapid bacterial growth and up to 95% consumption was observed after 6-hour exposure (Fig. 4.11), no significant alteration in isotopic values was noted

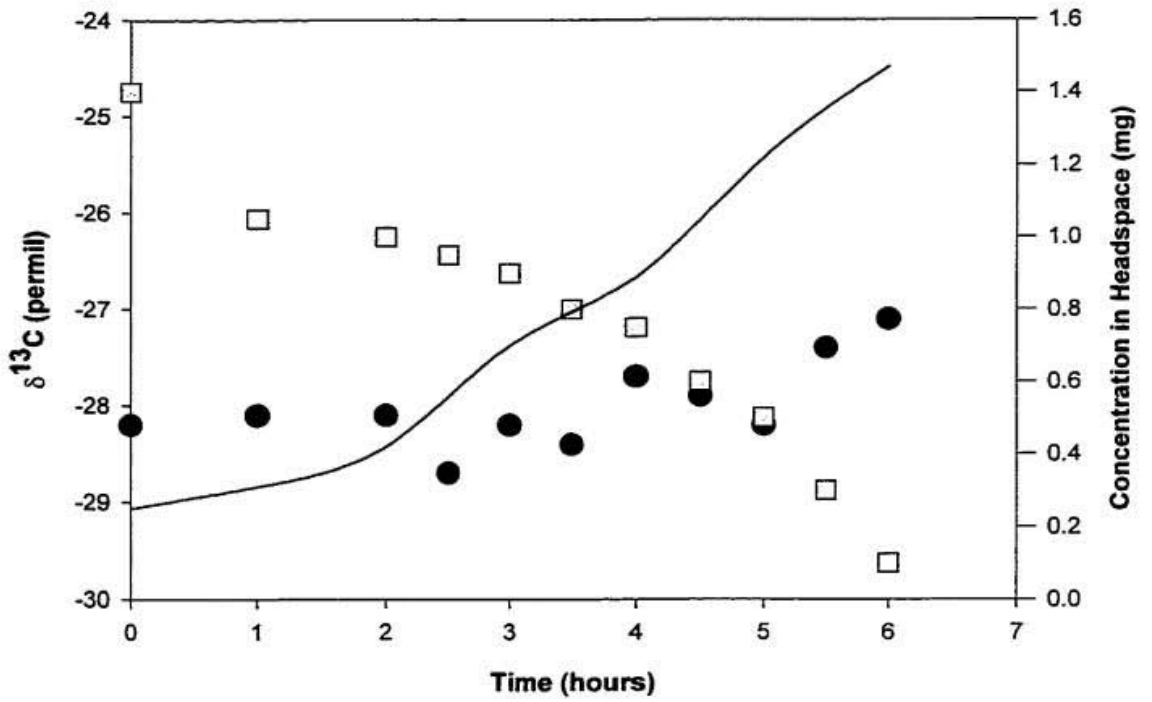

Fig. 4.10. Changes in isotopic composition (●) and concentration (□) over time during aerobic degradation of benzene (after Stehmeier *et al.*, 1999). Line represents growth curve of the degrading microcosm.

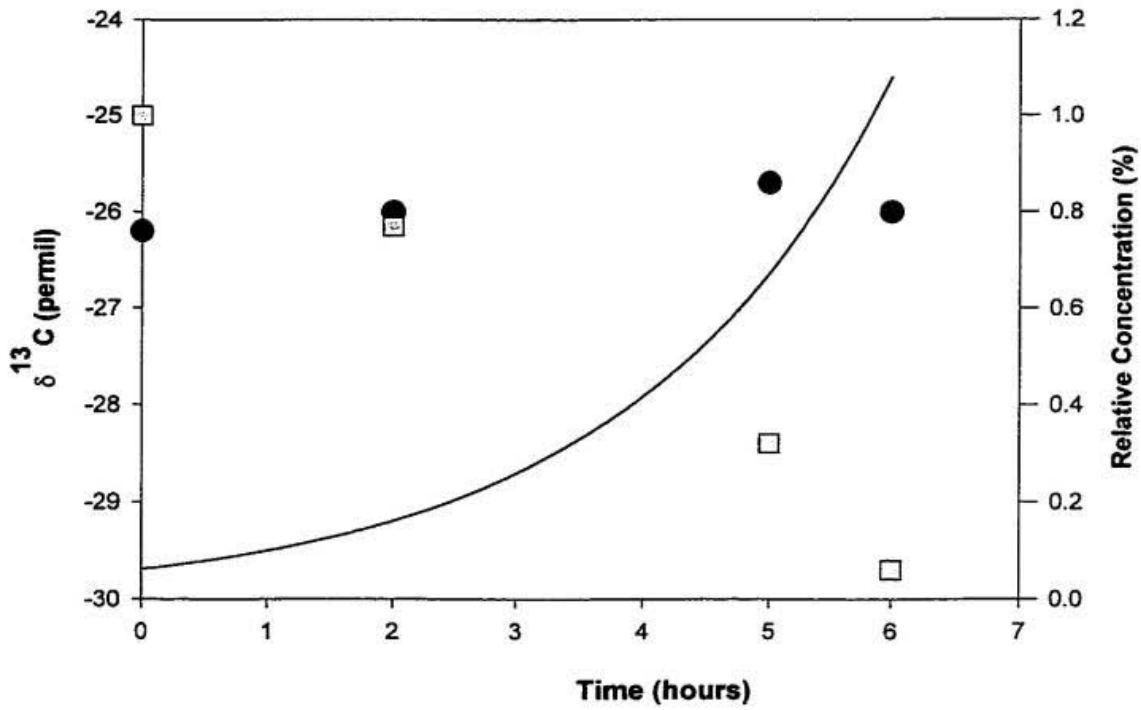

Fig. 4.11. Changes in isotopic composition (●) and concentration (□) over time during aerobic degradation of naphthalene (after O'Malley *et al.*, 1994). Line represents growth curve of the degrading *Pseudomonas putida* ATCC 17484.

when this compound was used as the sole source of carbon (O'Malley *et al.*, 1994). Similar results were recorded when a larger starting concentration was utilized.

The same holds true with that of the fluoranthene study conducted by O'Malley *et al.* (1994) in which the concentration of this hydrocarbon was reduced by 63% after 60 hours of exposure to an active bacterial population. Furthermore, there was no significant alteration in the isotopic values of this hydrocarbon (Fig. 4.12). Trust *et al.* (1995) also found no isotopic fractionation associated with the microbial degradation of this compound.

Biodegradation of polycyclic aromatic hydrocarbons (e.g., naphthalene) occurs with the initial attack on one of its aromatic rings (Fig. 4.5). Although one would expect isotopic fractionation to occur in such a case, no significant fractionation was observed by O'Malley *et al.* (1994) or Trust *et al.* (1995). Harrington *et al.* (1999) suggested that the lack of isotopic enrichment could be due to the mineralization rate of these compounds. The mineralization rate might have been so fast that the fractionation factor decreased with increasing degradation rates (Goldhaber and Kaplan, 1975). Another reason could be correlated with the molecular masses of the hydrocarbons. The isotopic fractionation factor will be effectively "diluted" over the number of carbon atoms in the molecule (Harrington *et al.*, 1999). It could also be possible that fractionation has occurred but this was not due to microbial degradation but to an abiotic process, e.g. dissolution process.

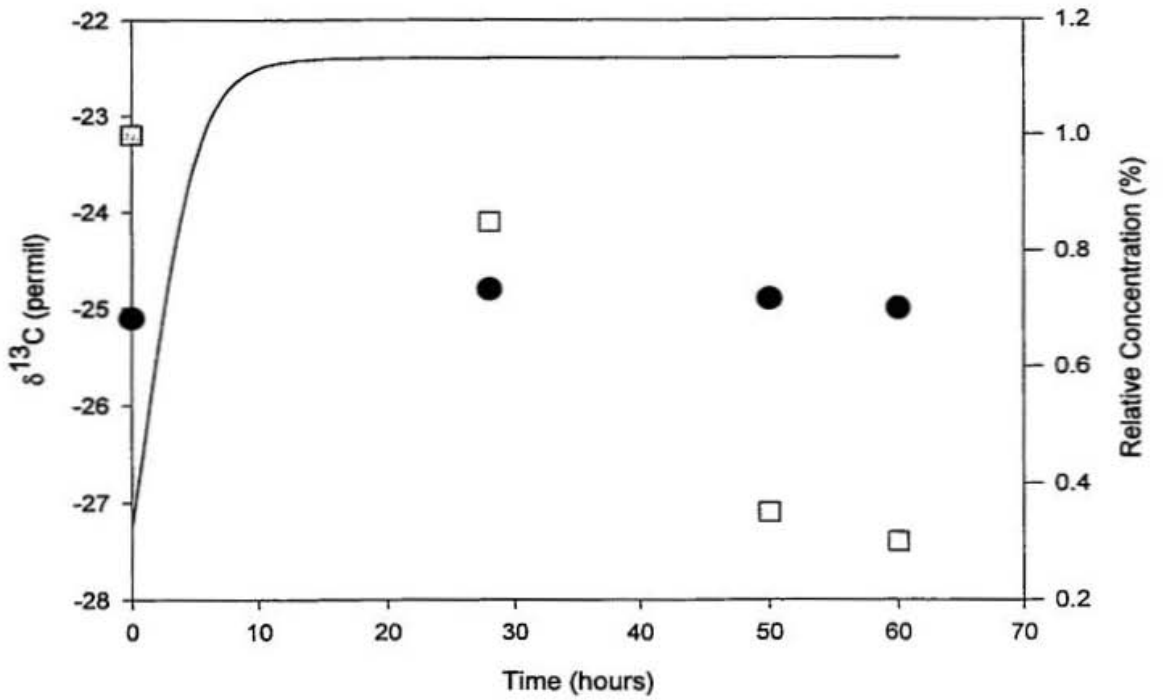

Fig. 4.12. Changes in isotopic composition (●) and concentration (□) over time during aerobic degradation of fluoranthene (after O'Malley *et al.*, 1994). Line represents growth curve of the degrading *Pseudomonas putida* ATCC 17484.

The hydrocarbons were in crystalline form and had to undergo a dissolution step prior to microbial degradation.

Further studies should still be conducted to elucidate the role of the different metabolic pathways and enzyme systems utilized by individual microorganisms as well as by mixed populations in their effects on the magnitude of isotopic fractionation.

4.2. Field Studies

By examining residual hydrocarbons in soil extracts and vapor samples collected from four different contaminated sites, Stehmeier *et al.* (1999; see Appendix) attempted to demonstrate the applicability of the isotope technique in the field. One of the sites (Site 2) is a biosparging operation in Alberta involving gasoline released from an underground storage tank. Field measurements from two monitoring wells within the site are illustrated in Fig. 4.13 to Fig. 4.14. About 28 hydrocarbon components were resolved in gas chromatography of samples from 2.2 m and 24 constituents from 4.3 m depth in monitoring well A (Fig. 4.13). Twenty-two of these compounds were found at both depths and therefore a comparison of their isotopic compositions can be made. Two compounds corresponding to retention times of 1275 and 1313 seconds have differences in $\delta^{13}C$ of less than 1‰. Eleven components increased in $\delta^{13}C$ values by more than 1 ‰ while 8 components increased by more than 2 ‰. In monitoring well B, 20 hydrocarbon constituents were resolved at 2.2 m depth and 18 constituents at 4.3 m (Fig. 4.14). Of the

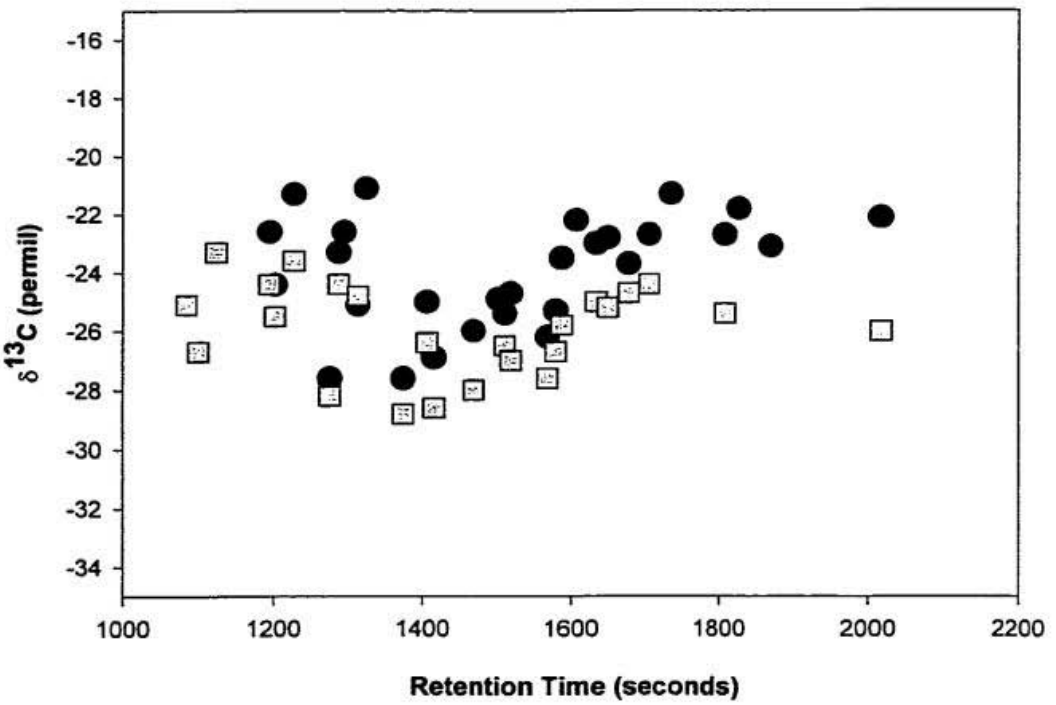

Fig. 4.13. Isotopic effects of gasoline contaminants from soil samples in Well A located in Site 2 (after Stehmeier *et al.*, 1999). (●) represents data from soil samples collected at 2.2 m depth and (⊡) from 4.3 m depth.

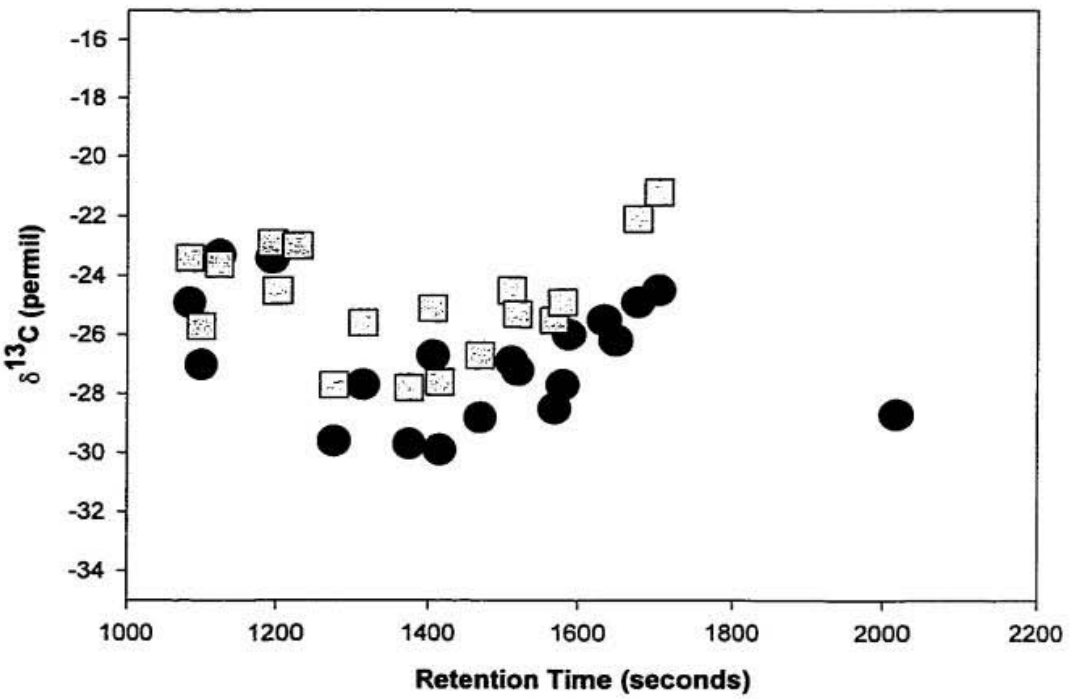

Fig. 4.14. Isotopic effects of gasoline contaminants from soil samples in Well B located in Site 2 (after Stehmeier *et al.*, 1999). (●) represents data from soil samples collected at 2.2 m depth and (□) from 4.3 m depth.

16 compounds found at both depths, only two components showed $\delta^{13}C$ differences of less than 1 ‰ with six and eight components having $\delta^{13}C$ shifts of greater than 1 ‰ and 2 ‰, respectively.

Based on laboratory degradation experiments carried out for benzene and styrene (Stehmeier *et al.*, 1999), increases in $\delta^{13}C$ of hydrocarbons in Well A seem to be due to biodegradation occurring at shallower depths. In contrast, enrichment of ^{13}C occurred at a deeper level in Well B (Fig. 4.14). Possible explanations for this disparity include substantially greater porosity at 2.2 m resulting in much higher concentrations of hydrocarbons at this depth which could have led to local reduction in E*h* that pre-empted aerobic biodegradation at the shallower depths. Another plausible explanation for the reversal in ^{13}C-enrichment pattern in Well B is the impact of toxicity of hydrocarbon at elevated levels.

The field results of Stehmeier *et al.* (1999), however, are inconsistent with the present study in which no significant isotopic fractionation was obtained with the degradation of low molecular weight aromatic compounds. Field experiments conducted on microbial degradation of BTEX compounds by Kelley *et al.* (1997) similarly showed that the isotopic composition of these compounds remained the same at different sampling periods. It is thus possible that the field observations (Stehmeier *et al.*, 1999) can be attributed to other processes involving the organic contaminants (Diegor *et al.*, 1999).

In natural environments, abiotic processes often play an important role in the transformation of organic contaminants. Only a few studies concerning isotopic fractionation effects associated with these processes were available in the literature. Whereas isotopic fractionation effects due to soil adsorption are likely small (e.g., Harrington *et al.* 1999), isotopic effects due to vaporization generally varies with respect to the variety of organic compounds. Unlike equilibrium isotope fractionation, where the heavier isotope fractionates into the liquid rather that the vapor, vaporization of organic compounds seem to exhibit what is referred to as an inverse isotopic effect (e.g., Huang *et al.*, 1999; Harrington *et al.*, 1999).

Balabane and Letolle (1985) found out that liquid fractions taken during distillation of benzene and toluene were enriched in the heavy isotope compared to the initial substrate and that the residual liquid was decreasingly depleted as distillation proceeded to completion, indicating a positive change in delta values. In addition, experiments on the BTEX compounds by Harrington *et al.* (1999) showed small positive isotopic effects. On the other hand, Huang *et al.* (1999) and Poulson and Drever (1999) pointed out that large isotopic fractionation was associated with vaporization studies on chlorinated aliphatic compounds and trichloroethylene, respectively.

The observations from the Alberta hydrocarbon spill can now be reconciled with the experimental observations. Note, in particular, the contrasting behaviour of the shallow

and deep samples from Wells A and B. In Well A, the hydrocarbon samples were shifted to isotopically heavier values, an observation consistent with aerobic biodegradation, if it is assumed that biodegradation pathways that promote preferential destruction of ^{12}C-involving bonds are involved. Preferential biodegradation at shallower levels is promoted by the greater access to oxygen at these depths. However, if it can be shown that the laboratory-cultured microcosm (i.e., those utilized in the present experiments) is responsible for biodegradation at the shallower levels, then the observed fractionation has to be due to processes other than biodegradation. Given that laboratory cultures inherently alter the microbial structure compared to what is present in the field, it is not surprising that carbon isotopic fractionation was observed in the field whereas none was observed in the microcosms.

Well B, however, exhibited shallow fractionation that favored enrichment of ^{12}C in the residual hydrocarbons, and opposite that observed in Well A. In the initial assessment of this fractionation pattern, it was speculated that carbon isotope fractionation preferentially occurred in the deeper samples (Stehmeier *et al.*, 1999). It was also suggested that anaerobic degradation, similar to those observed experimentally (Fig. 4.15), may have occurred. In light of the discussion noted above, it now appears more likely that the shallow hydrocarbon samples from Well B was affected by volatilization of hydrocarbons. The reverse carbon isotope fractionation observed is consistent with the reverse fractionation noted by Huang *et al.* (1999) and Harrington *et al.*, 1999). Indeed, the substantial carbon isotope shift shown by the shallow samples (2-3 ‰) from Well B

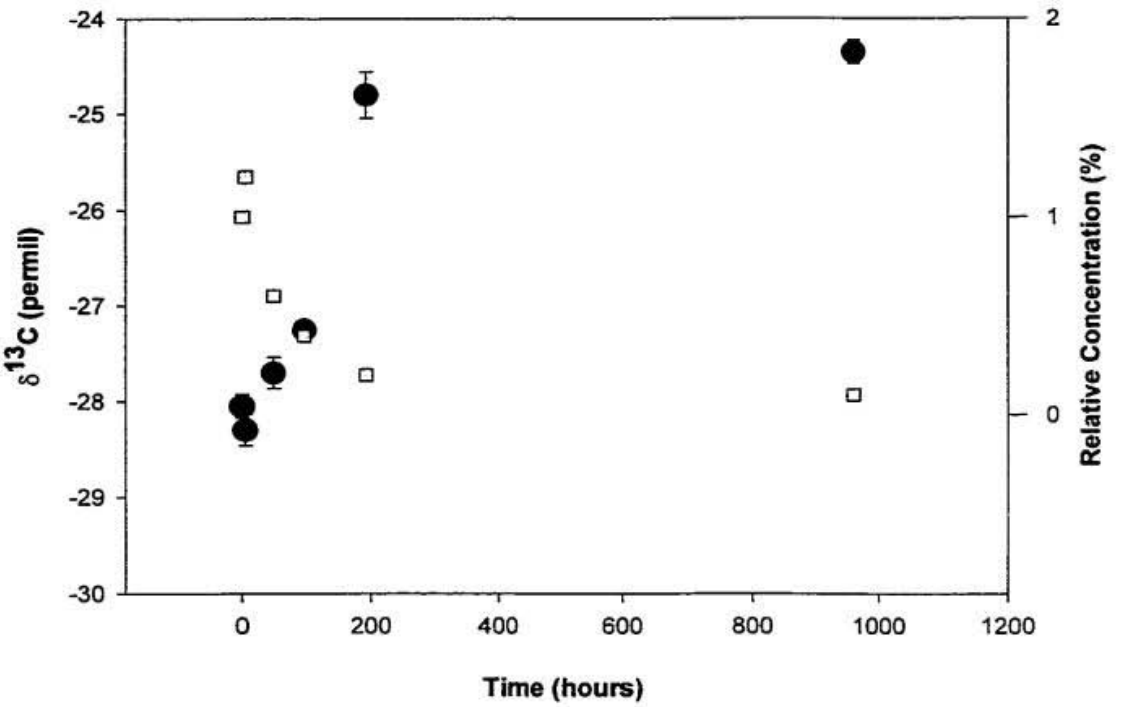

Fig. 4.15. Changes in isotopic composition (●) and concentration (□) over time during anaerobic degradation of toluene (Abrajano, pers. comm.,1999).

suggests very high degrees of volatilization. The problem with the previous suggestion of Stehmeier *et al.* (1999) is that the deeper samples for both Well A and Well B have very similar carbon isotope values. This would imply that if the deeper samples in Well A are relatively unaltered values, it becomes difficult to argue that the deeper hydrocarbons in Well B are residues of anaerobic degradation.

4.3. Application to *In situ* Bioremediation

In assessing the effectiveness of *in situ* bioremediation, the monitoring technique used must satisfy the criteria set by the National Research Council (NRC, 1993). These include documented loss of contaminants; laboratory assays showing that microorganisms from site samples have the potential to transform the contaminants under the expected site conditions; and one or more pieces of information showing that biodegradation potential is actually realized in the field.

Stable isotope analysis has already been used as a valuable technique in investigating the behavior of organic contaminants in the subsurface and to some extent in assessing the implementation of bioremediation. Biodegradation is of particular interest since it is often the only process that may result in complete transformation of contaminants to non-toxic product. With the application of this technique, it is shown that results of our bench scale experiments satisfied criteria 1 and 2 in which selected hydrocarbons have been microbially degraded under aerobic conditions. Evidence of such is manifested with

increase in biomass and decrease hydocarbon concentration. However, measurement of $\delta^{13}C$ in residual hydrocarbons such as toluene and ethylbenzene has shown no significant change in isotopic compositions of residual hydrocarbons. These observations still have to be demonstrated in the field, but if correct, the application of carbon isotope techniques to monitoring aerobic BTEX degradation seems suspect.

Sherwood Lollar *et al.* (1999) also suggested that in monitoring of *in situ* bioremediation by natural microbial communities using the stable carbon isotope analysis, field evidences should further show the following criteria. Systematic changes in $\delta^{13}C$ values, or fractionation must occur during biodegradation. Fractionation must be greater than analytical uncertainty, and under given set of conditions, the fractionation must be reproducible. Finally, the effects of isotopic fractionation during biodegradation must also be readily discernable from isotopic effects associated with other subsurface processes of mass attenuation such volatilization, dissolution, and sorption.

Site-specific studies are necessary to determine the presence of inherent microbial bacterial populations and to quantify the stable isotope fractionation occurring biologically. In conjunction with these studies, determination of the effects of the variety of environmental conditions such as temperature, pressure, pH, electron acceptors, geologic and hydrologic properties, on the magnitude of isotopic fractionation of organic contaminant should also be considered.

Chapter 5

Summary and Conclusions

The extensive occurrence of aromatic hydrocarbons through accidental spills and leakage of underground storage tanks, or through inadvertent releases during use, transport or disposal has caused tremendous contamination of surface and groundwater environments. Among the compounds of interest are low molecular weight hydrocarbons such as the monoaromatic BTEX compounds because of their toxic and carcinogenic potential.

Aerobic degradation catalyzed by inherent microbial populations is one of the mechanisms that could aid in the complete removal of aromatic hydrocarbons in the environment. Several approaches have been utilized to assess this process but their measurement of the changes over time (e.g., of hydrocarbon concentration, bacterial count, metabolites) may be affected not only by biodegradation but also by other chemical and physical processes.

Stable carbon isotope analysis is one technique that has been previously used to trace sources of organic pollutants. Compounds have characteristic carbon isotopic compositions that can be used to pinpoint their origins. Any process in which the compounds are involved with may likewise impart significant isotopic fractionation.

Microbial biodegradation experiments modified from an earlier protocol (Stehmeier *et al.*, 1999) were performed in replicates utilizing selected hydrocarbon compounds as the substrates. Microbial cultures acclimatized to the specific hydrocarbons were used and grown aerobically at room temperature in side-arm flasks. To establish microbial growth, measurement of optical density was undertaken. To determine changes in concentration and isotopic composition of residual hydrocarbons, hydrocarbon isotope analyses were performed by removing a specific headspace concentration and analyzing it by gas chromatography continuous flow isotope ratio mass spectrometry (GC-IRMS).

Biodegradation of toluene showed that microbial growth exhibited an overall increasing trend as indicated by increases in optical density. A corresponding decrease in hydrocarbon concentration with no significant changes in the $\delta^{13}C$ values was also noted. Similar observations were obtained using higher substrate concentration (10 µl of toluene). Experiments conducted on ethylbenzene as the substrate likewise demonstrated the same effects on microbial growth as well as in the concentration of residual hydrocarbon. Isotopic compositions also remained considerably constant.

Identification of the microcosm revealed various species that make up the different hydrocarbon-specific cultures. About 26 bacterial strains were identified that consisted of Gram negative rods as well as Gram positive cocci. Gram negatives included strains from the genera of *Pseudomonas*, *Stenotrophomonas*, *Oligella* and *Acidovorax* while Gram

positives belonged to *Micrococcus*, *Staphylococcus*, *Dermacoccus* and *Kokuria* (or *Erythromyxa*).

The present study revealed that no isotopic fractionation accompanied microbial degradation of toluene. A recent study employing two different competitive microcosms likewise exhibited the same outcome (Sherwood Lollar *et al.*, 1999). In contrast, another published work obtained a substantial fractionation associated with biodegradation of the same compound (Meckenstock *et al.*, 1999). These contrasting results indicate that the occurrence of isotopic fractionation depends on the degradative pathways utilized by the respective microbial consortia. Specifically, the nature of the initial metabolic step (e.g., attack on methyl group versus scission of aromatic ring) could control the extent of carbon isotope fractionation. The corresponding microcosms used in the present study and Sherwood Lollar *et al.* (1999) could have initiated the degradation of toluene through oxygenation of its methyl group (Kitagawa, 1956; Nakasawa and Yokota, 1973) in which a carbon-hydrogen bond was broken, and this was apparently not associated with isotopic fractionation. The *Pseudomonas* strain used by Meckenstock *et al.* (1999) might have initially attacked the aromatic ring (Zylstra *et al.*, 1988) in which the accompanying cleavage of one of the carbon-to-carbon bonds might have caused the fractionation.

Benzene is basically composed of an aromatic ring and thus its degradation occurs with the oxidation of the aromatic ring itself (Gibson and Subramanian, 1984). Earlier microbial degradation experiments done with benzene showed isotopic enrichment

ranges, though small in magnitude, from 2 to 7 times the analytical error of 0.3‰ (Stehmeier *et al.*, 1999). These observations, as well as the results obtained by Meckenstock *et al.* (1999), seem to point out that if the degradative pathway used by a single bacterial strain or microbial consortia occurs with the attack on the aromatic ring itself, notable fractionation could be observed.

The outcome exhibited by the monoaromatic hydrocarbons, toluene and ethylbenzene, was similar to that obtained from degradation studies of some polycyclic aromatic hydrocarbons (e.g. naphthalene and fluoranthene) (O'Malley *et al.* 1994; Trust *et al.*, 1995). Although initial microbial attack also occurs with one of the aromatic rings, the lack of isotopic fractionation could be attributed to several factors such as mineralization rate (Goldhaber and Kaplan, 1975), molecular masses (Harrington *et al.*, 1999) or to an abiotic process, e.g. dissolution.

Field results conducted on soil samples collected from an Alberta hydrocarbon-contaminated site (Stehmeier *et al.*, 1999) were inconsistent with the present study. The contrasting behaviour of shallow and deep samples from two monitoring wells suggested that other process e.g., volatilization (Harrington *et al.*, 1999; Huang *et al.*, 1999) might have affected the observed isotopic values.

Based on the results of the present study, application of stable carbon isotope analysis in aerobic degradation of aromatic hydrocarbons particularly the BTEX compounds do not

appear promising for assessment of natural or engineered *in situ* bioremediation. Future studies should look more closely into the different degradative pathways and enzyme systems used by individual microorganisms as well as mixed populations and their effects on the magnitude of isotopic fractionation. Site-specific studies are also necessary to determine the inherent presence of (these) microbial consortia and quantify the associated biological isotope fractionation. . In addition, the role of the various environmental conditions such as temperature, pressure, pH, electron acceptors should also be considered to determine their effects on the magnitude of isotopic fractionation accompanying biodegradation of organic contaminants.

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Appendix

A copy of the published paper entitled "Field and in vitro evidence for in-situ bioremediation using compound-specific $^{13}C/^{12}C$ ratio monitoring" by Stehmeier *et al.* (1999).

PERGAMON

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Field and in vitro evidence for in-situ bioremediation using compound-specific $^{13}C/^{12}C$ ratio monitoring

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Abstract

This work describes the use of $\delta^{13}C$ values of residual hydrocarbons as a method for demonstrating in-situ biodegradation. Microbial growth, hydrocarbon loss and increase in $\delta^{13}C$ values were demonstrated in vitro using benzene and styrene as carbon substrates. Isotope evidence of biodegradation were subsequently sought in four field sites contaminated with a wide variety of hydrocarbons. Analysis of residual hydrocarbons in the field indicated that an overall increase in the $\delta^{13}C$ generally accompanied loss of hydrocarbons, an observation consistent with in-situ biodegradation.

The field samples were analyzed using vapor or soil extracts, and the increases in $\delta^{13}C$ were observed using both types of samples. Vapor sampling is of practical interest because stable isotope ratio monitoring of soil vapor could dramatically reduce the number of wells required for monitoring of ongoing remediation efforts. Our preliminary studies of contaminated field sites allude to the potential of compound-specific isotopic monitoring techniques as a cost-effective measure of in-situ biodegradation.

Keywords: Aerobic microorganisms; Stable isotope fractionation; Dicyclopentadiene; Benzene; Styrene; Toluene; Residual hydrocarbon

1. Introduction

Natural attenuation of hydrocarbon contaminated soil and groundwater has received increased attention in the last few years because of a better understanding of risk-based approaches to remediation (Hinchee et al., 1995; Alleman and Leeson, 1997). The emphasis has changed from removing the contamination, at any cost, to protection of high-risk receptors (Davis et al., 1997). If regulators can be shown that: (1) biological degradation is occurring, (2) contaminant plumes are transported at rates that will not allow migration off-site, and (3) degradation products will not pose an environmental threat, then natural attenuation will be accepted as an option to expensive interventions.

Several processes contributing to natural attenuation or intrinsic remediation are volatilization, sorption and biological degradation (Cookson, 1995). The ultimate goal for remediation is the complete conversion of contaminants to benign end-products such as carbon dioxide and water. In the subsurface, this occurs almost

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exclusively through biological degradation. Proof of in-situ biological degradation can be difficult. A strategy proposed in the United States by the National Research Council (1993), and generally accepted for providing this proof, consists of:

1. Documenting loss of contaminants from the site
2. Laboratory assays showing that microorganisms from site samples have the potential for contaminant degradation at site conditions
3. Evidence showing that the biodegradation potential is actually realized in the field.

Criteria 1 and 2 have been amply demonstrated in many hydrocarbon contaminated sites. In contrast, proof of criteria 3 has remained fairly elusive.

Bacterial numbers and activity, metabolic by-products, redox conditions, inorganic carbon isotope ratios and electron acceptor concentration provide circumstantial proof of bioremediation. These types of analyses only indirectly suggest bioremediation because they could not specifically link contaminant loss to microbial activity. To make matters worse, many bacteria are difficult to isolate, and as much as 75% of the targeted organisms may be missed using conventional methods (Wilson and Lindow, 1992). Thus, no single method is presently available to pinpoint microbial removal of contamination definitively. The present work describes measuring the ratio of stable carbon isotopes in specific residual hydrocarbon compounds in vitro and in vivo as an attempt to define the extent of biodegradation. Such an analysis addresses the three criteria cited above (NRC, 1993): documenting the loss of hydrocarbons in vivo, showing the potential of microbial communities to degrade hydrocarbons in vitro, and finally linking the loss to biologically mediated isotope fractionation in vivo.

The first attempt to elucidate the environmental fate of organic molecules using compound-specific stable carbon isotope measurements are those of Abrajano et al. (1993), O'Malley (1994) and O'Malley et al. (1995). It has long been known that biological systems fractionate carbon isotopes (Stahl, 1980; Galimov, 1985; Blair et al., 1985; Zyakun, 1996). Galimov (1985) suggested that biological fractionation could occur by two mechanisms—enzymatic and mass transfer. Enzymatic fractionation and fractionation by mass transfer both lead to isotopic forms that correspond to the minimum free energy of the system. For biodegradation the system is assumed to be the boundaries of the microorganism. Kinetic effects of isotopes are produced by differences in the reaction rates of isotopic forms (Galimov, 1985). This means that in an ensemble of interacting atoms, the smaller masses have greater velocities, and molecules containing lighter isotopes are more mobile than those containing heavier isotopes. Second, chemical bonds formed by a heavy isotope are stronger than those formed by the light isotope resulting in higher activation energy for any reactions in which the heavier isotope participates.

In this work benzene and styrene were used as carbon substrates during in vitro studies to determine if isotopic fractionation of the residual hydrocarbon occurs during aerobic biodegradation. Four mixed hydrocarbon contaminated sites were then monitored for changes in their residual $\delta^{13}C$ values to assess the applicability of the technique to different field situations.

2. Methods

2.1. Laboratory degradation studies

Chemicals for laboratory degradation studies were purchased from Aldrich Chemicals, Milwaukee, WI. Changes in the carbon isotope ratio were measured as a function of hydrocarbon degradation for aerobic microbial cultures. The source of benzene and styrene degraders was a mixed microbial culture obtained from contaminated groundwater at an Alberta petrochemical site (Site 1). The groundwater sample was originally enriched with N and P nutrients and microscopically observed until microorganisms were abundant (approximately 10^7 per ml). The microbial culture was then maintained in minimal salts medium (1 g K_2HPO_4, 1 g KH_2PO_4, 2 g NH_4NO_3, 0.3 g $MgSO_4.7H_2O$, 0.001 g $CaCl_2.2H_2O$, 0.001 g $FeSO_4.7H_2O$ per liter of distilled water) containing pyrolysis gas as a carbon source (Stehmeier et al., 1997). Pyrolysis gas is a mixed hydrocarbon liquid obtained during the cooling step after ethane gas has been 'cracked' to ethylene. Maintenance of the degradative cultures was on benzene or styrene at concentrations of less than 100 mg/l. For biodegradation experiments, the microbial cultures were grown aerobically at room temperature in 118 ml serum vials, equipped with Teflon Mininert valves (Supelco Canada, Mississauga, ON) for ease of sampling. Each vial contained 40 ml of minimal salts medium augmented with between 50 and 250 mg/l of hydrocarbon and 5 ml of microbial inoculum from the respective degrading cultures. The cultures were incubated with intermittent shaking/stirring at 25°C.

Duplicate vials were prepared with one vial used for optical density measurements as an indicator of microbial growth and the other for headspace analysis of hydrocarbon. An abiotic control using minimal salts medium and respective hydrocarbons was also set up and sampled during the experiment to determine if isotope fractionation occurred during shaking and volatilization. Optical density measurements (OD_{600}) were taken throughout each experiment to provide a growth curve and an initial and final OD_{600} measurement on

Table 1
Summary of field sites assayed in the present study

	Contaminants	Remediation scenario	Sample medium	Monitoring duration
Site 1	Pyrolysis gas spill (C5+)	Stimulated in situ Bioremediation NH_4NO_3 and PO_4^{3-} augered in	Soil headspace	17 weeks
Site 2	Gasoline from a leaking underground storage tank	Patented biospurging system	Vapor swept from contaminated zones as well as discrete soil headspaces	6 weeks
Site 3	Mixed styrene contaminants	Biopile	Vapor swept from pile	6 weeks
Site 4	Unknown mixture of hydrocarbons ($>C_{10}$)	Natural attenuation in river sediments	Sediment extracted with dichloromethane	3 years

the headspace vial to ensure both vials were comparable. For hydrocarbon isotope analysis, a 30 μl headspace sample was analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC-C-IRMS) at the Isotope Biogeochemistry Facility, Memorial University of Newfoundland.

The conditions for the Hewlett Packard 5890 gas chromatograph have been described in O'Malley et al. (1996) with the following changes. The column was a Restek 502.2 (105 m length, 0.53 mm I.D.) with a 3 μm Crossbond® phenylmethyl polysiloxane film (Chromatographic Specialties, Brockville, ON). The chromatographic conditions were 35°C held for 1 min followed by a temperature ramp of 25°C/min to 250°C held for 15 min.

The carbon isotope compositions were expressed using the conventional delta notation:

$$\delta^{13}C = 1000(R_s/R_{PDB} - 1),$$

R represents the ratio $^{13}C/^{12}C$ and the subscripts s and PDB refer to sample and standard Pee Dee Belemite, respectively. The analytical reproducibility for $\delta^{13}C$ values during these tests was better than ±0.3‰.

2.2. Field sites

Soil and vapor samples from four different contaminated sites were obtained to determine if carbon isotope variations occur under field conditions, and if these variations occur in a manner consistent with biodegradation. Sites were chosen that could provide samples at different time intervals to determine if isotopic fractionation occurred. A summary of the sites is given in Table 1. Site 1 contained primarily volatile aromatic compounds generated at a petrochemical plant in Alberta. Site 2 is a biosparging operation in Alberta recovering in ground gasoline released from an underground storage tank. Site 3 is a biopile from Cambridge, ON, which contained mixed aromatics with the primary contaminant being styrene. Site 4 was a river sediment site (St Claire River, ON), contaminated with heavier petroleum compounds.

2.3. Field experiments

At Site 1, soil hydrocarbon concentrations were measured using a modification of EPA Method 3810 (EPA, 1986). The field soil samples (45 g) were placed in 118 ml crimp tops vials with 28 ml of deionized water and equilibrated at 25°C before a 30 μl headspace sample was analyzed by GC-C-IRMS (Isotope Biogeochemistry Facility, Memorial University). The hydrocarbon components were identified by injecting external standards and comparing retention times.

At Site 2, contaminated vapor samples were col-

lected in Tedlar bags (SKC Inc., Eighty Four, PA) and the gasoline vapor-phase hydrocarbons concentrated using solid-phase microextraction (SPME) following procedures outlined in the manufacturer's document (Supelco, 1994). The SPME fiber was injected directly into the GC-C-IRMS instrument. The first bag collected in each sampling period was discarded to ensure true subsurface vapor was being collected. In the present paper, the gasoline hydrocarbon components were identified only by their retention times, but external standards were injected and used to ensure retention times were comparable from sample to sample. Soil samples from three wells (A, B, and C) were analyzed using soil headspace technique previously described. Furthermore, samples from different depths were collected from two of the wells (A and B). Two wells (A and C) were drilled in approximately the same location and depth, but at different times, in order to evaluate the change in $\delta^{13}C$ over the time period (four months).

Site 3 contaminants were analyzed using the same method as Site 2 for soil vapor. Styrene was the only hydrocarbon identified using the injection of an external standard and comparing retention times. Hydrocarbon concentration was monitored for total hydrocarbons using a field monitor with a photoionization detector (PID) (Raymond, personal communication).

Site 4 contaminants were too heavy to be extracted with SPME and were solvent- extracted with *n*-pentane. Sediments (20 g) were placed in crimp-sealed vials and sonicated for 15 min in the presence of *n*-pentane using an ultrasonic bath. The extracts were transferred to new vials with a new Pasteur pipette and concentrated under nitrogen. The hydrocarbons were recovered again using pentane, and injected into the GC-C-IRMS instrument. No attempt was made to identify all the compounds present in these samples, but retention times were noted and external standards were used to ensure retention times were comparable between samples.

Microbial activity at Site 1 was measured using fluorescein diacetate (FDA) hydrolysis (Song, 1988). Soil (1 g) was added to 25 ml of sterile phosphate buffer (60 mM, pH 7.6) and 0.5 ml of 2 mg FDA per ml of acetone in a 50-ml Erlenmeyer flask. The reaction was stopped after 1 h with 25 ml of acetone; 5 ml of solution were filtered through a PTFE syringe filter (Chromatographic Specialties Inc., Brockville, ON) and the absorbance measured at 490 nm in a Turner Model 30 spectrophotometer.

3. Results

3.1. Laboratory studies

Biodegradation experiments using benzene as the substrate showed an inverse correlation between benzene concentration and optical density, indicating the depletion of benzene as the microbial culture increased in biomass (Fig. 1). The $\delta^{13}C$ for residual benzene also increased as benzene degraded, and microbial biomass

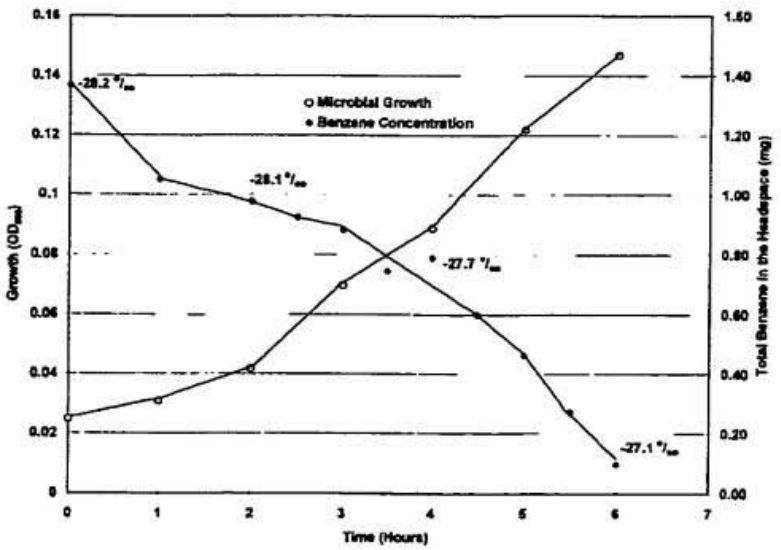

Fig. 1. Aerobic biodegradation of benzene as shown by an increase in turbidity and a shift in the $\delta^{13}C$ value. Benzene is measured as headspace concentration and increase in bacterial growth is measured at 600 nm. The $\delta^{13}C$ of residual benzene is shown at 2-h intervals as labels on the benzene concentration plot.

Table 2
Isotopic composition, hydrocarbon concentration and culture medium optical density before and after exposure to hydrocarbon degrading consortia under aerobic conditions[a]

HC initially present	Percent HC lost	Initial $\delta^{13}C$ (‰)	Final $\delta^{13}C$ (‰)	Change in $\delta^{13}C$ (‰)	Initial OD_{600}	Final OD_{600}
Benzene						
44 mg/l	80%	−28.2	−27.4	0.8	0.025	0.122
44 mg/l	86%	−28.2	−27.0	1.2	0.045	0.169
130 mg/l	90%	−28.2	−26.0	2.2	0.049	0.390
218 mg/l	83%	−28.2	−27.4	0.8	0.093	0.609
Styrene						
45 mg/l	77%	−27.9	−26.2	1.7	0.060	0.118

[a] OD_{600} = optical density at 600 nm; HC = hydrocarbon; ‰ = parts per thousand as defined in the text.

increased (Fig. 1). The initial concentration of benzene in this vial was 44 mg/l. The shift in $\delta^{13}C$ was not large but was significantly greater than the analytical reproducibility of carbon isotopic measurements. Additional experiments at different initial benzene concentrations showed similar magnitude of $\delta^{13}C$ shift from beginning to end of the experiment ranging from 80 to 90% benzene consumption (Table 2). Sterilized controls were run at the same time with benzene only. The average $\delta^{13}C$ value for 18 analyses was −28.3‰ with a standard deviation of 0.3‰. Also included in Table 2 are the results of an experiment where styrene was the substrate. Optical density increased while styrene decreased and the $\delta^{13}C$ for styrene was enriched from the initial to the final sampling point. In this experiment the enrichment of $\delta^{13}C$ for styrene was also substantially higher than the reproducibility of $\delta^{13}C$ measurements.

3.2. Field studies

3.2.1. Site 1

During the course of remediation, field-measured hydrocarbon loss of benzene, toluene and dicyclopentadiene (DCPD) were estimated at 99, 99 and 84% respectively at Site 1 (30 cm depth, Table 3). The corresponding $\delta^{13}C$ values of residual benzene, toluene and DCPD were enriched by 2.1, 5.9, and 2.3‰ respectively (Table 3). Microbial activity, measured by an increase in FDA hydrolysis, indicated that at 30 cm depth, there was a 400% increase (Table 3). This corresponds to the pattern of results obtained in the laboratory studies where $\delta^{13}C$ was enriched while the hydrocarbon concentrations decreased and indicators of microbial growth increased. Curiously, $\delta^{13}C$ increase observed for benzene was of a similar magnitude as that observed in vitro, specifically at the highest degrees of benzene degradation (cf Tables 2 and 3).

We note that the results for sampling at 90 cm depth (Table 3) seem to contradict the suggestion that biodegradation was occurring at this site. At this sampling depth, a significant amount of benzene appears to have been lost (99%), and FDA hydrolysis indicated microbial activity increased (by 156%) during the study period. Interestingly, the $\delta^{13}C$ composition of the residual benzene did not change significantly relative to our analytical $\delta^{13}C$ precision of 0.3‰.

Table 3
Isotopic composition, hydrocarbon concentration and FDA hydrolysis at Site 1 before and after stimulation by augering[a]

Depth and *Eh*	HC	Initial HC (mg/kg)	Final HC (mg/kg)	Initial $\delta^{13}C$ (‰)	Final $\delta^{13}C$ (‰)	Change in $\delta^{13}C$ (‰)	Initial FDA µg/h/g	Final FDA µg/h/g
30 cm							2.7[b]	10.8[b]
25 mV[b]	Benzene	39.6	0.01	−39.1	−37.0	2.1		
	Toluene	15.4	0.01	−39.9	−34.0	5.9		
	DCPD	32.4	5.0	−38.4	−36.1	2.3		
90 cm							1.6[b]	2.5[b]
−200 mV[b]	Benzene	28.8	0.01	−38.0	−37.7	0.3		
	Toluene	5.1	0.03	−39.2	−36.2	3.0		
	DCPD	10.1	30.4	−36.8	−36.9	−0.1		

[a] FDA = fluorescein diacetate, measured as µg hydrolyzed per h per g of soil or water; HC = hydrocarbon.
[b] Value at respective depth, 30 cm or 90 cm.

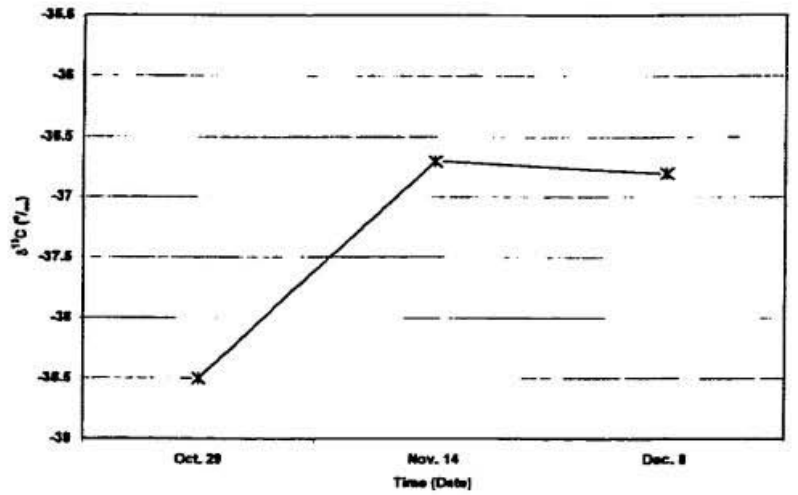

Fig. 2. The change in $\delta^{13}C$ value over time for an unknown contaminant peak at Site 2 with a retention time of 1314 s. Soil vapor was collected in a Tedlar bag from a manifold collecting air swept through gasoline contaminated soil.

3.2.2. Site 2

At Site 2, $\delta^{13}C$ values of gasoline components were determined from vapor and soil samples from a site actively being bioremediated. An increase in $\delta^{13}C$ values for gasoline components of the soil vapor occurred over approximately two months (Fig. 2). Soil samples from the site were also measured using the headspace technique described above (Tables 4, 5, and 6). In Well A, the hydrocarbon contamination ranged from 50 to 150 ppm over the two sampling depths with a uniform sandy clay soil (Table 4; Fontaine, personal communication). The $\delta^{13}C$ values were generally more enriched at 2.2 m than at 4.3 m (Table 4). Well B (Table 5) had a different subsurface lithological profile with a sand layer at 2.2 m with very high concentrations of hydrocarbon (3700 ppm) overlying a clay lens with substantially decreased contaminant (30 ppm) (Fontaine, personal communication). In this well

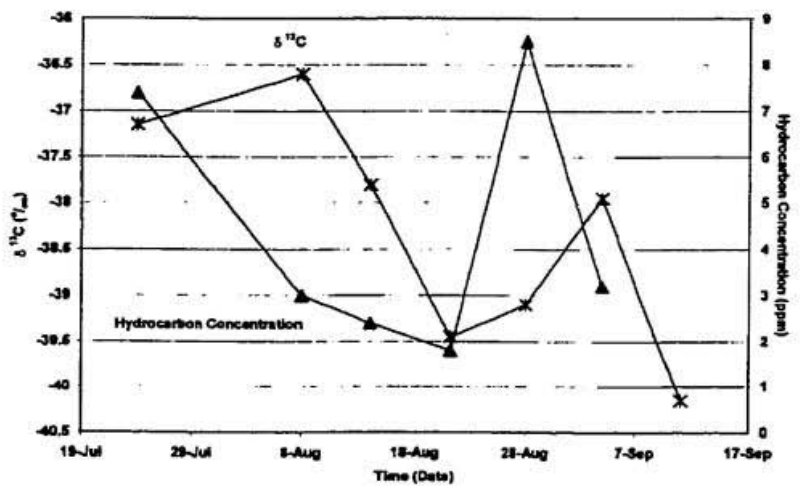

Fig. 3. Hydrocarbon analysis and $\delta^{13}C$ values of soil vapor samples withdrawn from a biopile at Cambridge, ON during remediation of styrene contaminated soil. Hydrocarbon analysis was a single sample analyzed with a field photoionization detector. The $\delta^{13}C$ values are an average of two 5-l Tedlar bag samples taken the same day.

Table 4
Stable isotope fractionation of gasoline contaminants from soil samples in a two meter interval at Well A. Hydrocarbons found at a greater depth (4.3 m) were isotopically lighter than those found nearer the surface (2.2 m)

Retention time (s)	Change in $\delta^{13}C$ from 2.2 m to 4.3 m	Monitoring Well A at 2.2 m depth	Monitoring Well A at 4.3 m depth
995.0			
1085.0			−25.1
1101.0			−26.7
1125.0			−23.3
1195.0	1.8	−22.6	−24.4
1202.0	1.1	−24.4	−25.5
1227.0	2.3	−21.3	−23.6
1275.0	0.6	−27.6	−28.2
1287.0	1.1	−23.3	−24.4
1294.0		−22.6	
1313.0	−0.3	−25.1	−24.8
1324.0		−21.1	
1374.0	1.2	−27.6	−28.8
1406.0	1.4	−25.0	−26.4
1415.0	1.7	−26.9	−28.6
1469.0	2.0	−26.0	−28.0
1503.0		−24.9	
1512.0	1.1	−25.4	−26.5
1520.0	2.3	−24.7	−27.0
1569.0	1.4	−26.2	−27.6
1580.0	1.4	−25.3	−26.7
1588.0	2.3	−23.5	−25.8
1607.0		−22.2	
1633.0	2.0	−23.0	−25.0
1648.0	2.4	−22.8	−25.2
1676.0	1.0	−23.7	−24.7
1704.0	1.7	−22.7	−24.4
1734.0		−21.3	
1808.0	2.7	−22.7	−25.4
1827.0		−21.8	
1870.0		−23.1	
2017.0	3.9	−22.1	−26.0
2088.0			

the $\delta^{13}C$ values were enriched more in the deeper clay layer (4.3 m) than in the sand layer (2.2 m, Table 5). The $\delta^{13}C$ values of the gasoline contaminants in Well C (same depth and soil as Well A) shifted over a four-month period and became enriched in ^{13}C (Table 6).

3.2.3. Site 3

Vapor from a styrene-contaminated soil bio-pile indicated some ^{13}C enrichment in the residual styrene but this cannot be simply related to the observed hydrocarbon concentration (Fig. 3). We note, nevertheless, that the $\delta^{13}C$ values shown in Fig. 3 are for styrene only but the hydrocarbon concentration reflects total hydrocarbon as measured via photoionization detector.

3.2.4. Site 4

Residual hydrocarbon from sediment samples in the river site that received discharge from petrochemical facilities was analyzed at an interval of three years. These results showed significant increase in $\delta^{13}C$ values during a three-year period for some components while a few other components seem to have been depleted in ^{13}C (Table 7).

4. Discussion

4.1. In vitro biodegradation

Enrichment of ^{13}C in residual hydrocarbon was demonstrated with benzene and styrene as substrates for aerobic microbial growth using an enrichment culture of soil organisms from Site 1 (Fig. 1 and Table 2). The enrichment of ^{13}C in laboratory experiments, while small, ranges between 2 and 7 times greater than

Table 5
Comparison of stable isotope fractionation of gasoline contaminants from soil samples in a heavily contaminated sand lens (Well B, 2.2 m) and the less contaminated underlying clay 2 m below (4.3 m). Compounds found at a greater depth were isotopically heavier than those found nearer the surface

Retention time (s)	Change in $\delta^{13}C$ from 4.3 m to 2.2 m	Monitoring Well B at 2.2 m depth	Monitoring Well B at 4.3 m depth
995.0			
1085.0	1.5	−24.9	−23.4
1101.0	1.3	−27.0	−25.7
1125.0	−0.3	−23.3	−23.6
1195.0	0.5	−23.4	−22.9
1202.0			−24.5
1227.0			−23.0
1275.0	1.9	−29.6	−27.7
1287.0			
1294.0			
1313.0	2.1	−27.7	−25.6
1324.0			
1374.0	1.9	−29.7	−27.8
1406.0	1.6	−26.7	−25.1
1415.0	2.3	−29.9	−27.6
1469.0	2.1	−28.8	−26.7
1503.0			
1512.0	2.4	−26.9	−24.5
1520.0	1.9	−27.2	−25.3
1569.0	3.0	−28.5	−25.5
1580.0	2.8	−27.7	−24.9
1588.0		−26.0	
1607.0			
1633.0		−25.5	
1648.0		−26.2	
1676.0	2.8	−24.9	−22.1
1704.0	3.3	−24.5	−21.2
1734.0			
1870.0			
2017.0		−28.7	
2088.0			

the analytical error of 0.3‰. Fig. 1 indicates that during incubation of a soil inoculum with benzene as the only carbon substrate, benzene concentration decreased, optical density increased and $\delta^{13}C$ values in the residual hydrocarbon became enriched. Four experiments with benzene were conducted, two with the same initial concentration and two others with increased hydrocarbon concentration (Table 2). While the extent of fractionation appeared to be dependent upon the amount of hydrocarbon degraded, our experiments are not sufficient to establish the nature of this dependence.

Other work in the literature suggested that ^{13}C fractionation of hydrocarbons did not occur during biodegradation (O'Malley, 1994; Trust et al., 1995). In these reports the carbon substrates were larger molecular weight polycyclic aromatic hydrocarbons. Previous work done in our lab using a mixed hydrocarbon substrate of primarily C5 alkanes, alkenes, and single ring aromatics found that the $\delta^{13}C$ of residual hydrocarbon was enriched by as much as 8‰ (standard deviation = 0.5‰) after biodegradation (Francis et al., 1997). Elsewhere in this volume, Heraty et al. (1999) also report that substantial ^{13}C fractionation occurred during aerobic biodegradation of dichloromethane. The magnitude of fractionation for styrene was comparable to the benzene results but fractionation in styrene occurred with less extensive degradation (Table 2).

4.2. In situ biodegradation

At Site 1, three compounds were identified and monitored for $^{13}C/^{12}C$ fractionation at two different depths (Table 3). Results for 30-cm depth suggest a shift in $\delta^{13}C$ of residual hydrocarbon that is comparable to that observed in the laboratory experiments. The

Table 6
Stable isotope fractionation of gasoline contaminants from soil samples during a four-month interval at Well C. Compounds found in September were isotopically lighter or the same as those found in January

Retention time (s)	Change in $\delta^{13}C$ from September to January	Well A January 1998	Well C September 1997
1085.0			−23.6
1101.0			−25.6
1125.0			−21.6
1195.0	0.9	−22.6	−23.5
1202.0	0.4	−24.4	−24.8
1227.0	1.6	−21.3	−22.9
1275.0	−0.7	−27.6	−26.9
1287.0	0.2	−23.3	−23.5
1294.0		−22.6	
1313.0		−25.1	
1324.0	0.7	−21.1	−21.8
1374.0	0.6	−27.6	−28.2
1406.0	0.7	−25.0	−25.7
1415.0	1.0	−26.9	−27.9
1469.0	1.2	−26.0	−27.2
1503.0		−24.9	
1512.0	0.3	−25.4	−25.7
1520.0	1.1	−24.7	−25.8
1569.0	0.8	−26.2	−27.0
1580.0	0.2	−25.3	−25.5
1588.0	0.7	−23.5	−24.2
1607.0		−22.2	
1633.0	1.2	−23.0	−24.2
1648.0	1.4	−22.8	−24.2
1676.0	0.9	−23.7	−24.6
1704.0	1.0	−22.7	−23.7
1734.0	0.2	−21.3	−21.5
1808.0	2.1	−22.7	−24.8
1827.0		−21.8	
1870.0		−23.1	
2017.0	2.9	−22.1	−25.0
2088.0			−23.6

extent of biodegradation for benzene and toluene in the field exceeded 99%, and the shift in $\delta^{13}C$ was greater in toluene than in benzene (but in the same direction). Thus, the observed isotopic shifts at 30-cm depth in Site 1 are consistent with aerobic biodegradation depleting both benzene and toluene. Note that another major process that could have occurred in the field, that of evaporation, would have had the opposite impact on $\delta^{13}C$ (i.e., deplete ^{13}C) of the residual hydrocarbons (cf. Harrington et al., 1999; Huang et al., 1999).

The shift in $\delta^{13}C$ of residual DCPD was similar to benzene but the extent of degradation was only 84%. Biodegradation of DCPD is difficult to measure in vitro because of its slow degradation rates and incomplete mineralization (Stehmeier, 1997). The relatively large fractionation observed for DCPD suggests stable isotope analysis may be a sensitive technique to determine when DCPD is being metabolized.

The results for the 90-cm sampling depth at Site 1 seem at odds with the observations at 30 cm. We note that the FDA hydrolysis indicated only a moderate increase of microbial activity at 90 cm compared to 30 cm. This observation is consistent with the dramatic difference observed in oxidation potential at the two sampling depths, with the 90 cm sampling depth showing quite reduced *Eh* values (−200 mV, Table 3). Hence one way of resolving the discrepancy is to suggest that the low *Eh* values dramatically limited the activities of aerobic organisms thereby also limiting the resulting $\delta^{13}C$ shift. If this is correct, however, we have to also conclude that the actual biodegradative loss is substantially less than what can be inferred from the total hydrocarbon loss.

Table 7
Stable isotope fractionation of heavier petroleum contaminants extracted from sediments below the St Claire River. The comparison is for a single site during a three-year interval

Retention time (s)	Change in $\delta^{13}C$ from June 1994 to June 1997	3D Nearshore 1994	3D Nearshore 1997
752		−30.3	
771		−31	
861			−34.7
899		−31.8	
948		−32.5	
1043		−32	
1088	1.3	−28.4	−27.1
1115	0.1	−31.4	−31.3
1126		−33	
1154			−35.8
1176		−32.1	
1237		−30.2	
1326	−0.3	−33	−33.3
1346	−2.2	−30.5	−32.7
1372			−32.3
1389	0.2	−31.9	−31.7
1432		−29.5	
1439		−28.5	
1503	−1	−29.5	−30.5
1516	−0.1	−29.4	−29.5
1526		−27.9	
1592		−28	
1643	1.6	−27.6	−26
1665	4.7	−27.5	−22.8
1720		−27.3	
1744		−27.4	
1777		−27.9	
1987			
2079		−31.5	

A more plausible explanation is that biodegradation indeed occurred at 90 cm depth, albeit, anaerobic degradation. Such possibility is consistent with the FDA measurement and hydrocarbon loss estimate, but it implies that anaerobic degradation may not result in similar $\delta^{13}C$ shift as that observed in the aerobic in vitro experiments. If correct, it may also be inferred that anaerobic metabolic pathways could exhibit different carbon isotope fractionation than aerobic degradation. Experiments comparing $\delta^{13}C$ fractionation for a given hydrocarbon in aerobic and anaerobic systems is currently in progress (Diegor, unpublished data).

The results of isotopic measurements at Site 2 offered even greater complexities than those observed in Site 1. In Well A, 24 hydrocarbon components were resolved at 4.3 m and 28 components at 2.2 m depth. Twenty-one components were comparable with only two having changes in $\delta^{13}C$ values of less than 1 (0.6‰ and −0.3‰). Eleven components increased in $\delta^{13}C$ by more than 1‰ and eight components increased by more than 2‰. The increase in $\delta^{13}C$ values suggests increased biodegradation occurred at the shallower depth compared to the 4.3 m depth in Well A. In Well B, again compared at 2.2 and 4.3 m depth, 20 hydrocarbon components were resolved at 2.2 m and 18 components at 4.3 m. Only two components had $\delta^{13}C$ differences of less than 1‰ (0.5‰ and −0.3‰), with six components showing $\delta^{13}C$ shift of greater than 1‰, and eight components showing shifts in excess of 2‰. In contrast to the observation in Well A, however, the ^{13}C enrichment occurred at the deeper (4.3 m) rather than at the shallower sampling point (2.2 m). At 2.2 m depth a sand lens existed with 100 times the concentration of hydrocarbon that existed at 4.3 m depth (Fontaine, personal communication). Our results are far from conclusive, although some possible explanations can be offered to reconcile the shift in $\delta^{13}C$ values in Well B. We note that the substantially greater porosity at 2.2 m did result in much higher concentrations of hydrocarbons at this depth. It is therefore possible that local reduction in *Eh* could have pre-empted aerobic biodegradation at these shallow depths, in contrast to the deeper less porous layer. The possibility that an

inverted *Eh* profile exists for this well is obviously testable, but these measurements were not available for these wells at the time of our sampling. An equally plausible explanation for this reversal in ^{13}C enrichment pattern in Well B is the impact of toxicity of hydrocarbon at elevated levels (Leahy and Colwell, 1990). In a hydrocarbon plume, the greatest level of degradative activity is at the periphery where concentration is less toxic and nutrients are more available (NRC, 1993).

The usefulness of the isotopic technique as a monitoring tool in a short time span was demonstrated at Wells C and A. Comparing Wells C and A at the same depth shows variable ^{13}C enrichment for the 22 comparable hydrocarbon components. Of these, two had differences greater than 2‰ and seven had differences exceeding 1‰. Hence, an overall $\delta^{13}C$ enrichment for the comparable hydrocarbon components was observed over the four-month period at the same time that the hydrocarbon concentration decreased.

One component in the soil vapor from Site 2 was also continuously monitored over approximately six weeks and indicated $\delta^{13}C$ enrichment of 1.7‰ (Fig. 2). One area of concern for monitoring in-situ biodegradation (using any technique) is the necessity of penetrating the ground surface to obtain samples. The results in Fig. 2 indicate that soil vapor can be used to determine isotopic shift on the substrate that is taking place in the subsurface. If substantiated by subsequent measurements, compound-specific carbon isotope monitoring of soil vapor could reduce the number of wells required for monitoring the progress of remediation. Slater et al. (1999) and Sherwood Lollar et al. (1999) have suggested that any isotopic effects associated with equilibrium volatilization, sorption and dissolution are less than 0.5‰. However, other papers in this volume (Huang et al., 1999; Harrington et al., 1999) point to the possibility of larger (>0.5‰) fractionation as a result of volatilization processes in the field.

At Site 3, vapor was also used to determine if $\delta^{13}C$ fractionation occurred during active bioremediation of styrene contaminated soil. In this instance the results were not conclusive, as seen in Fig. 3. We believe that the results shown in Fig. 3 could have been the result of mixing styrene of varying degrees of biodegradation through channeling or actual physical disturbance. In Fig. 3, two instances are observed where the $\delta^{13}C$ became heavier and then returned to a value of approximately −40‰. The hydrocarbon concentrations also showed periods of increase and decrease, though they did not correlate well with the decrease and increase of $\delta^{13}C$ values. When the biopile was dismantled, it was found that there were many pockets of hydrocarbon with high concentrations, and additional work was required before disposal was possible (Raymond, personal communication). This supports the idea that channeling occurred and that non-degraded hydrocarbons became admixed with more highly degraded counterparts.

The use of stable isotope ratios for monitoring the progress of historical surface spills was examined at Site 4 (Table 7). This site contained heavier petroleum contaminants released into the St Claire River approximately 10 years ago. Analysis of frozen samples taken three years apart found that in 1994, there were 25 resolvable components that decreased to 12 by 1997. These samples contained nine common compounds (based on RT) with only one compound enriched in ^{13}C by more than 2‰ (4.7‰) and two compounds enriched by more than 1‰. Indeed, two compounds showed substantial decrease in $\delta^{13}C$ (−2.2‰ and −1.0‰) whereas others were unchanged within the analytical error. Given the long duration of degradation that these sediments went through, it is likely that only the most recalcitrant hydrocarbons are left. Further molecular characterization is clearly required, but we note that O'Malley (1994) and Trust and coworkers (1995) have shown that the biodegradation of recalcitrant higher molecular weight compounds such as naphthalene and fluoranthene, did not result in isotopic fractionation. It is therefore possible for biodegradation to have left minimal imprint on the $\delta^{13}C$ of residual recalcitrant compounds. It should likewise be born in mind that the length of time that elapsed from initial hydrocarbon release could also have allowed a range of other 'weathering' reactions to affect the $\delta^{13}C$ values of residual compounds. The fact that both enrichment and depletion were observed indeed lend an indication that the residual hydrocarbons could have been exposed to a multiplicity of weathering reactions. Additional detailed molecular characterization is required to resolve alternative explanations for the carbon isotopic shifts in this case.

5. Conclusions

The objective of this work was to demonstrate the use of ^{13}C ratios in residual hydrocarbons for monitoring in vitro and in vivo biodegradation. The laboratory experiments with benzene and styrene indicated an enrichment in ^{13}C of the residual hydrocarbon with increasing degree of biodegradation. This shift was reproducible and correlated with the fraction of hydrocarbon degraded. The most significant shifts in $\delta^{13}C$ occurred when greater than approximately 75% of the hydrocarbon component had been degraded. Preliminary testing of the technique in the field also showed that isotopic measurement can be applied to both soil extracts and vapor samples, although only one sample type should be used for any given site. The

installation of wells for monitoring biodegradation could thus be substantially curtailed by analyzing vapor swept through the subsurface.

Whereas the same enrichment was generally observed in several field sites, the present study also provides examples of field situations where a single isotopic vector ($\delta^{13}C$, in this case) may not be able to resolve complex reaction histories.

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