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Enhancement of anaerobic biodegradation of petroleum hydrocarbons in contaminated groundwater: laboratory mesocosm studies

by

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Abstract

This project was a part of a study to evaluate natural attenuation (NA) as a viable remedial option for petroleum hydrocarbon (PHC) contamination at upstream oil- and gas-contaminated sites in Alberta, Canada. Laboratory mesocosms were set up using groundwater and sediment materials collected from two PHC contaminated sites (Site 1 and Site 3) in Alberta to investigate the enhancement of anaerobic PHC biodegradation by amendment of terminal electraon acceptors (TEAs, nitrate or sulfate) and/or nutrients (ammonium and phosphate).

Multiple lines of evidence, including the removal of benzene, toluene, ethylbenzene and xylenes (BTEX) and CCME F1 fraction hydrocarbons (C_6 to C_{10}), rapid depletion of TEAs, the production of biogenic gases, and detection of the metabolites verified that anaerobic PHC biodegradation was occurring in both laboratory mesocosm studies. Selective biodegradation of PHCs under different reducing conditions was observed. However, there was no conclusive evidence that one reducing condition will universally favor the biodegradation of specific PHCs. In both studies, nutrient amendment showed no enhancement effects.

The calculated first-order biodegradation rates in Site 1 mesocosm study ranged from 0.0032 to 0.033 d^{-1} for benzene, 0 to 0.028 d^{-1} for ethylbenzene, 0.0021 to 0.036 d^{-1} for m-, p-xylenes, and 0.0006 to 0.0045 d^{-1} for $F1_{-BEX}$ (F1 hydrocarbons exclduding BEX) under the tested conditions. The laboratory first-order biodegradation rates of BEX were higher than the estimated field rates, indicating

the potential of enhanced anaerobic biodegradation *in situ*. However, when comparing the TEA amended mesocosms with the unamended controls (in which iron reduction might be the predominant process), the enhancement effects were less apparent and inconsistent.

The calculated first-order biodegradation rates in Site 3 mesocosm study ranged from 0 to 0.0009 d^{-1} for benzene, 0 to 0.011 d^{-1} for ethylbenzene, 0 to 0.0016 d^{-1} for m- and p-xylenes, and 0 to 0.15 d^{-1} for o-xylene. Sulfate amendment significantly stimulated biodegradation of all xylenes and CCME F1 hydrocarbons. However, there was no definitive evidence that nitrate or sulfate amendment could enhance benzene or ethylbenzene biodegradation.

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LIST OF SYMBOLS

BSS	benzylsuccinate synthase
BEX	benzene, ethylbenzene and xylenes
BSTFA	*N, O*-bis(trimethylsilyl)trifluoroacetimide
BTEX	benzene, toluene, ethylbenzene and xylenes
Ca^{2+}	calcium
CCME	Canadian Council of Ministers of the Environment
CH_4	methane
CO_2	carbon dioxide
CORONA	Consortium for Research on Natural Attenuation
Ctrl	unamended control mesocosms
Ctrl+NP	control mesocosms amended with nutrient
Ctrl(+NP)	Ctrl and Ctrl+NP mesocosms
CWS	Canada-Wide Standard
DO	dissolved oxygen
DOC	dissolved organic carbon
EA	enhanced attenuation
Eh	redox potential
4F1NA	4-fluoro-1-naphthoic acid
F1	C_6 to C_{10} fraction hydrocarbons measured by CCME method
Fe^{2+}	ferrous iron
Fe(III)	ferric iron
FID	flame ionization detector
g	gram
GC	gas chromatography
GC/MS	Gas Chromatography/Mass Spectrometry
H_2	hydrogen gas
HNO_3	nitric acid
H_2S	hydrogen sulfide
H_2SO_4	sulfric acid

IC	Ion Chromatography
ICP-MS	inductively coupled plasma-mass spectrometry
IRB	iron reducing bacteria
ITRC	Interstate Technology & Regulatory Council
K^+	potassium
L	litre
M	mole per litre
mg	milligram
Mg^{2+}	magnesium
mL	millilitre
mM	millimolar
MNA	monitored natural attenuation
MPN	most probable number
N_2	nitrogen
Na^+	sodium
NA	natural attenuation
NAPL	non-aqueous phase liquid
NH_4^+	ammonium
nM	nanomole
N_2O	nitrous oxide
NO_2^-	nitrite
NO_3^-	nitrate
NO3	mesocosms amended with nitrate
NO3+NP	mesocosms amended with nitrate and nutrient
NO3(+NP)	NO3 and NO3+NP mesocosms
NRB	nitrate reducing bacteria
NRSOB	nitrate reducing - sulfide oxidizing bacteria
O_2	oxygen
PHC	petroleum hydrocarbon
PO_4^{3-}	phosphate
RIC	reconstructed ion chromatogram

R.T.	retention time
S^{2-}	sulfide
SC	sterile control mesocosms
SIS	selective ion storage (MS data acquisition mode)
SO_4^{2-}	sulfate
SO4	mesocosms amended with sulfate
SO4+NP	mesocosms amended with sulfate and nutrient
SO4(+NP)	SO4 and SO4+NP mesocosms
SRB	sulfate-reducing bacteria
TEA	terminal electron acceptor
TEAP	terminal electron accepting process
TIC	total ion chromatogram
μg	microgram
USEPA	United States Environmental Protection Agency

Chapter 1. General Introduction

1.1 Introduction

1.1.1 Petroleum Hydrocarbon (PHC) Contamination

The manufacture, transportation, and distribution of petroleum and chemical products in the last century have resulted in hydrocarbon contamination becoming an ongoing environmental problem (Atlas and Cerniglia 1995). Hydrocarbon contaminated soils are found where crude oil or its derivative fuels have been spilled during exploration, production, refining, transport, or storage. Gasoline, diesel and jet fuel may be accidentally released into the environment during transportation and storage. Reported gasoline spillage between 1984 and 1995 was 19,730 tonnes in Canada (Environment Canada 1998). Gasoline leaks from underground storage tanks are a major source of groundwater contamination. As of September 2008, over 479,000 releases from underground storage tanks had been confirmed in the United States (USEPA 2008). After being released into subsurface environment by accidental spills or leaks, hydrocarbons are present as non-aqueous phase liquid (NAPL) or partition into different environmental media, such as sorb to soil particles, evaporate into soil airs, or dissolve into soil water or groundwater.

PHCs released into the environment may cause a wide variety of issues as a result of their toxicity, mobility and persistence. These issues include fire and explosion hazards, human and environmental toxicity, odour, and impairment of soil processes such as water retention and nutrient cycling (CCME 2008a). Contamination of soil and groundwater by PHCs poses potential risks to human health and the environment. Humans may be exposed to PHCs via inhalation of contaminated air, ingestion of contaminated food and water, and dermal contact with contaminated water or soil (CCME 2008b). Occupational exposure to PHCs may also occur at higher hydrocarbon concentrations. The health effects of exposure to specific hydrocarbons can be classified as non-carcinogenic, such as

skin or eye irritation or damage to human organs, or carcinogenic. For example, benzene, a six-carbon (C_6) aromatic hydrocarbon, is classified as a known human carcinogen for all routes of exposure based upon convincing human evidence as well as supporting evidence from animal studies (USEPA 1998; ATSDR 1997).

PHCs are one of the most widespread soil and groundwater contaminants in Canada. About 60% of Canada's contaminated sites involve PHC contamination (CCME 2008a). Contaminated sites should be properly assessed and remediated to prevent and minimize impacts on human health and environment. According to CCME (1997), remediation of a contaminated site "involves the development and application of a planned approach that removes, destroys, contains or otherwise reduces availability of contaminants to receptors of concern". Sites contaminated with PHCs vary widely in complexity, physical and chemical characteristics, and the potential risk they may pose to human health and the environment. CCME endorsed the PHC Canada-Wide Standard (CWS) in 2001 to provide a consistent approach to managing PHC contaminated sites across Canada (CCME 2008a). The risk-based CWS sets out generic remediation objectives, as well as the process for developing site-specific remediation objectives and related remedial options which are protective of both human and environmental health.

1.1.2 Bioremediation and Natural Attenuation

The persistence of PHCs at contaminated sites depends on the quantity and properties of the hydrocarbon mixture as well as on the properties of the affected ecosystem. PHCs may persist in one environment for years, but be biodegraded rapidly under different conditions. Studies on marine oil spills have revealed that hydrocarbon-degrading microorganisms are ubiquitously distributed in soil and aquatic environments, but the rates of natural degradation are typically low and limited by environmental factors (Atlas 1995). However, given sufficient time PHCs are biodegraded (Atlas and Cerniglia 1995). These studies provide the basis for PHC bioremediation in which the rates of hydrocarbon biodegradation are accelerated by overcoming rate-limiting environmental factors, by adding

nutrients (Atlas 1995) or amendments of terminal electron acceptors (TEAs) (Anderson and Lovley 2000).

Under favourable conditions, natural physical, chemical, and biological processes (collectively termed as natural attenuation (NA) processes) may act to reduce the mass, toxicity, mobility, volume or concentrations of contaminants in soil or groundwater and achieve remediation objectives within a reasonable time frame (Wiedemeier *et al.* 1995). NA occurs through a variety of processes including dispersion, dilution, sorption, volatilization and biodegradation by indigenous microorganisms (McAllister and Chiang 1994). Of these processes, biodegradation is the most important mechanism of NA and it can result in significant reduction of contaminant mass (Wiedemeier *et al.* 1998). After exposure to hydrocarbon contamination, extensive anaerobic zones will develop at the contaminated sites due to the rapid depletion of dissolved oxygen (DO) (Anderson and Lovley 1997; Christensen *et al.* 1994; Lovley 1997). Because of the low water solubility and slow diffusion of oxygen, the replenishment of oxygen from recharging groundwater and atmosphere is very slow. Therefore, anaerobic biodegradation of PHCs has important implications for bioremediation strategies of PHC contaminated sediments and soils.

1.1.3 Monitored Natural Attenuation (MNA) and Enhanced Attenuation (EA)

NA is an attractive remedial alternative because of the potential for effective remediation of subsurface contamination with minimal intervention and therefore low expense. Monitored natural attenuation (MNA) and enhanced attenuation (EA) are two environmental management strategies that rely on a variety of NA processes to degrade or immobilize contaminants. MNA refers to a remediation approach that is based on understanding and quantitatively documenting NA processes that can reduce contaminant concentration to levels that pose no risk to possible receptors. Rather than a “do-nothing” approach, MNA is considered a “knowledge-based remedy” (USEPA 2001). When properly employed and

combined with source control, MNA may meet site remedial requirements within a reasonable time frame (USEPA 2001).

The use of MNA for the remediation of contaminated sites, at which benzene, toluene, ethylbenzene and xylenes (BTEX) are the sole contaminants of concern, is maturing scientifically and has been accepted at certain sites as an adequate approach to address subsurface remediation concerns (USEPA 2001). In considering sites contaminated with other PHCs, great uncertainty still exists regarding the utilization of MNA as a remediation approach. Therefore, MNA of PHCs should be assessed for each specific site.

For a specific contaminated site, sufficient evidence should be obtained to determine the feasibility of MNA as the remedial option. Three types of evidence are described by USEPA (2000) as follows:

1) Historical data that demonstrate decreases in contaminant mass, concentration, and/or toxicity;
2) Hydrologic, geochemical, biological, or mineralogical data that demonstrate indirectly that specific types of NA processes are occurring at the site that will reduce contaminant concentrations to desired levels;
3) Data from field or laboratory microcosm studies that demonstrate directly the occurrence of a particular NA process and its extent to degrade the contaminants of concern (typically the biodegradation process).

Field investigation and laboratory microcosm studies may be conducted to obtain evidence of (Bhupathiraju *et al.* 2002),

1) decreasing contaminant concentration;
2) TEA reduction and production of their reduced products;
3) production of metabolites; and
4) distinct elevation of biomass concentration and activities.

EA is another environmental management strategy that relies on NA processes. The basic premise of EA is that, for some contaminated sites, NA processes may not be sufficient to reduce contaminant concentrations to acceptable levels within a reasonable timeframe and thus MNA alone may not be a viable treatment option. Some type of intervention, i.e. some enhancement, must therefore be implemented to accelerate the NA processes and to meet remediation objectives. Generally, enhancements fall into two main categories (ITRC 2008):

1) Source strength reduction technologies, and
2) Attenuation capacity enhancement technologies.

Enhancements could also be classified according to the types of attenuation processes that are enhanced. Enhancements to physical attenuation processes may include hydraulic manipulation (such as interception and diversion of surface runoff or groundwater, cap or cover systems, and modifying the hydraulic gradient), source containment, and enhanced source removal by passive soil vapour extraction or some other technology. An example of enhancements to chemical attenuation processes is installation of a reactive barrier. Enhancements to biological attenuation processes may be achieved by (ITRC 2008):

1) Biostimulation, i.e. the addition of nutrients or TEAs that stimulates a naturally occurring consortium of bacteria to increase the rate of degradation or the overall extent of degradation,
2) Bioaugmentation, i.e. augmenting the natural consortia of microbes with additional species that can function in the plume environment and will increase the overall degradation of contaminants, and
3) Construction of a wetland at the groundwater-surface water interface that can take advantage of several attenuation mechanisms, including microbial degradation and plant-based degradation/extraction processes (phytoremediation), to increase attenuation in this region.

1.2 Scope of Work

In this thesis, laboratory mesocosm studies were conducted to evaluate the potential for EA of PHC contamination at two specific contaminated sites. The EA options being investigated were amendment of TEAs and/or nutrients to stimulate the anaerobic biodegradation of PHCs in groundwater. The objective of this project was to determine the enhancement effects of TEA and/or nutrient amendment on anaerobic biodegradation of target PHCs, measure the enhanced first-order anaerobic biodegradation rates, and identify the signature metabolites from the anaerobic biodegradation of the PHCs present at the contaminated sites.

This project was part of a larger study into NA of contaminants in the subsurface associated with the upstream oil and gas industry, referred to as the Consortium for Research on Natural Attenuation (CORONA). The objective of the CORONA research program was to evaluate NA as a viable remedial alternative for PHC contamination at upstream oil- and gas-contaminated sites. CORONA comprised three major components:

1) Detailed review of existing field data on NA at upstream sites and database development;
2) Detailed evaluation of NA at selected upstream sites;
3) Laboratory studies to improve the understanding of the variables and processes.

Three contaminated sites in Alberta, denoted as Sites 1, 2, and 3, were selected for detailed site characterization after evidence of NA was interpreted from the site monitoring data. Amongst them, Site 1 and Site 3 provided the best opportunity to study anaerobic PHC biodegradation in laboratory studies. Two mesocosm studies were conducted in the Department of Civil & Environmental Engineering at University of Alberta to investigate the enhancement of anaerobic biodegradation of BTEX and CCME F1 fraction hydrocarbons (C_6 to C_{10}) by TEA (namely nitrate or sulfate) and/or nutrient (ammonium and phosphate) amendment. The mesocosms were designed and custom made to facilitate sub-sampling of the

mesocosms and to maintain anaerobic conditions. The mesocosms contained groundwater and sediment samples collected from each site. The mesocosms were selectively amended and then incubated for about two years under laboratory-controlled conditions. The depletion of target PHCs and TEAs was monitored as a function of time. Changes in headspace gases were also measured at prescribed intervals to verify the anaerobic conditions and to measure the generation of biogenic gases. Microbial enumeration, metabolite analysis, and sediment characterization were also conducted.

1.3 Outline of Thesis

The relevant theoretical background and literature is presented in Chapter 2. The laboratory mesocosm studies are discussed in Chapters 3 and 4. For each mesocosm study, after presenting multiple lines of evidence for the occurrence of anaerobic biodegradation, estimated first-order biodegradation rates and enhancement effects are discussed. Metabolite analysis for both mesocosm studies is addressed in Chapter 5. A general discussion of the laboratory mesocosm studies and the enhancement effects of TEA and/or nutrient amendments on anaerobic PHC biodegradation is presented in Chapter 6. General conclusions and recommendations are presented in Chapter 7.

All supporting and supplementary information is compiled in the appendices. The details of all analytical methods and the results from the reproducibility tests are presented in Appendices A and B. Appendices C to F provide the laboratory data, statistical analyses, and sample calculations.

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Chapter 2. Literature Review

2.1 Petroleum Hydrocarbons (PHCs)

Hydrocarbons are organic compounds containing only carbon and hydrogen. They can be classified as aliphatic and aromatic hydrocarbons (Silberberg 2006). Aliphatic hydrocarbons can be cyclic or acyclic; and can also be characterized as saturated compounds (alkanes) and unsaturated compounds, containing C-C double bonds (alkenes) or C-C triple bonds (alkynes) (IUPAC 1997). Aromatic hydrocarbons, also known as arenes, may be mono- or polycyclic (IUPAC 1997). Due to the lack of functional groups, hydrocarbons are generally non-polar and have low chemical reactivity at ambient temperature. Hydrocarbons are either of natural origin, formed biologically or geochemically, or chemically produced from natural hydrocarbons (Widdel and Rabus 2001).

Petroleum is a naturally occurring mixture of hydrocarbons, generally in a liquid state, which may also include compounds of sulfur, nitrogen, oxygen, metals, and other elements (ASTM 2005). A variety of processing steps is required to convert petroleum from its raw state to fractions that have commercial values as bulk products, such as liquid fuels, lubricating oils, waxes, and asphalt. Petroleum-derived products are also mixtures, but have well-defined properties (Speight 2007). Different petroleum products are characterized by a different hydrocarbon composition, thus showing various physical and chemical properties. Light crude oils generally contain more mono-aromatics and fewer heterocyclic compounds than heavy viscous crude oils (Speight 2007). Gasoline consists primarily of normal and iso-alkanes and monoaromatics with carbon numbers ranging from C_5 to C_{11}, while diesel fuel contains mainly normal and cycloalkanes and polyaromatics with carbon numbers ranging from C_8 to C_{21} (Salanitro 2001).

Additives, usually complex chemical mixtures, are commonly added to petroleum products to enhance their natural properties, improve their performance, and

extend their life (Speight 2007). For example, oxygenates such as methyl tertiary-butyl ether (MTBE) and methanol are often added to gasoline to increase the octane number, promoting better combustion and thus reducing air emissions. Fuel M85, a mixture of 85% methanol and 15% gasoline can reduce hydrocarbon emissions by 30 to 40 percent as compared with gasoline (USEPA 2005).

The environmental fate and transport of PHCs is regulated by the contaminant's properties and the site conditions. NA processes are also influenced by the physical and chemical properties of the contaminant, such as water solubility, vapour pressure, and the partition coefficients (K_{ow} and Henry's law constant for example), as well as the biodegradability of the contaminant.

BTEX components are considered amongst the most prevalent groundwater pollutants (Anderson and Lovley 1997). It is estimated that two million underground gasoline storage tanks in North America have resulted in gasoline contamination of soil or groundwater, and BTEX compounds may comprise more than 60% (by mass) of the gasoline introduced into groundwater (Barbaro *et al.* 1992). Because of their relatively high water solubility and toxicity, BTEX contamination represents a significant health risk. Some major physical and chemical properties of BTEX are listed in Table 2-1.

Table 2-1. Chemical and Physical Properties of BTEX at 25°C (ATSDR 2000, 2007a, 2007b, 2007c; Zogorski *et al.* 1997).

	Benzene	Toluene	Ethylbenzene	m-Xylene	o-Xylene	p-Xylene
Molecular Weight (g/mol)	78.1	92.1	106.2	106.2	106.2	106.2
Specific Gravity	0.88	0.87	0.87	0.88	0.88	0.86
Boiling Point (°C)	80.1	110.6	136.2	139.1	144.4	138.4
Water Solubility (mg/L)	1780	535	161	146	175	156
Vapour Pressure (mm/Hg)	75 (at 20°C)	28.4	9.5	8.3	6.6	8.7
Log K_{ow}	2.1	2.7	3.2	3.2	3.1	3.2
Log K_{oc}	1.8-1.9	1.6-2.3	2.2* 2.4†	2.2	2.1	2.3
Henry's Law Constant (unitless)	0.22	0.24	0.35	0.31	0.021	0.31

*: data from Chiou *et al.* (1983), as cited in ATSDR 2007b
†: data from Hodson and Williams (1988) and Vowles and Mantoura (1987), as cited in ASTDR 2007b

2.2 Anaerobic PHC Biodegradation

Most PHCs are readily biodegraded under aerobic conditions (Gibson and Subramanian 1984). Prior to 1980s, it was commonly accepted that anaerobic biodegradation of hydrocarbons was negligible (Atlas 1981). It has long been considered that the initial attack of hydrocarbons always required molecular O_2 as a co-substrate. Monooxygenases are the key enzymes in the aerobic metabolic pathways of alkanes, while aromatic hydrocarbons are attacked by either monooxygenases or dioxygenases (Cerniglia 1984; Gibson and Parales 2000; Gibson *et al.* 1970). Therefore, metabolism of hydrocarbons appeared to be a strictly oxygen-dependent process.

Recently, it has been demonstrated that PHCs can be biodegraded under a variety of anaerobic conditions with nitrate (NO_3^-) (Burland and Edwards 1999; Coates *et al.* 2001; Ehrenreich *et al.* 2000; Mihelcic and Luthy 1988; Evans *et al.* 1991a), sulfate (SO_4^{2-}) (Coates *et al.* 1996a; Coates *et al.* 1996b; Edwards *et al.* 1992; Kazumi *et al.* 1997; Kropp *et al.* 2000; Lovley *et al.* 1995; Phelps *et al.* 1996;

Weiner *et al.* 1998), manganese (Mn) and iron (Fe) (Anderson *et al.* 1998; Lovley and Lonergan 1990b; Lovley *et al.* 1994; Lovley *et al.* 1996), and carbon dioxide (CO_2) (Kazumi *et al.* 1997; Grbic-Galic and Vogel 1987; Vogel and Grbic-Galic 1986; Weiner and Lovley 1998) as terminal electron acceptors (TEAs), but at a lower degradation rate (Landmeye *et al.* 1996; Wiedemeier *et al.* 1999). Hydrocarbons that can be degraded anaerobically include aliphatic alkenes and alkanes (with chain lengths of C_6 to C_{20}), monocyclic alkylbenzenes, as well as benzene and some PAHs including naphthalene, 2-methylnaphthalene and phenanthrene (Heider *et al.* 1998; Widdel and Rabus 2001; Young and Phelps 2005). Since 1990, diverse strains of anaerobic hydrocarbon degraders that are either nitrate reducing bacteria (NRB), iron reducing bacteria (IRB), or sulfate reducing bacteria (SRB) have been isolated. These anaerobic bacteria are summarized in Table 2-2. These bacteria belong to the β- and δ-subclasses of the Proteobacteria. Many pure cultures of anaerobic microorganisms that can degrade hydrocarbons have been described and novel catabolic pathways have been elucidated, as reviewed in Heider *et al.* (1998), Spormann and Widdel (2000), and Widdel and Rabus (2001).

Table 2-2. Isolated pure cultures capable of anaerobic biodegradation of PHCs (Spormann and Widdel, 2000; Widdel and Rabus, 2001; Chakraborty and Coates, 2004).

Species and/or Strain	Hydrocarbons
NRBs	
Thauera aromatica K172	Toluene
Thauera aromatica T1	Toluene
Azoarcus sp. Strain T	Toluene, m-Xylene
Azoarcus tolulyticus Tol4	Toluene
Azoarcus tolulyticus Td15	Toluene, m-Xylene
Azoarcus tolulyticus ToN1	Toluene
Azoarcus tolulyticus EbN1	Ethylbenzene, Toluene
Azoarcus sp. Strain EB1	Ethylbenzene
Azoarcus sp. Strain PbN1	Ethylbenzene, propylbenzene
Strain mXyN1	Toluene, m-Xylene
Strain T3	Toluene
Strain M3	Toluene, m-Xylene
Strain pCyN1	p-Cymene, Toluene, p-Ethyltoluene
Strain pCyN2	p-Cymene
Strain HxN1	C_6-C_8 Alkanes
Strain OcN1	C_8-C_{12} Alkanes
Strain HdN1	C_{14}-C_{20} Alkanes
Dechloromonas strain RCB	Benzene, Toluene, Ethylbenzene, Xylenes
Dechloromonas strain JJ	Benzene
IRBs	
Geobacter metallireducens GS15	Toluene
SRBs	
Desulfobacula toluolica Tol2	Toluene
Strain PRTOL1	Toluene
Desulfobacterium cetonicum	Toluene
Strain oXyS1	Toluene, o-Xxylene, o-Ethyltoluene
Strain mXyS1	Toluene, m-Xylene, m-Ethyltoluene, m-Cymene
Strain NaphS2	Naphthalene
Strain Hxd3	C_{12}-C_{20} Alkanes, 1-hexadecene
Strain Pnd3	C_{14}-C_{17} Alkanes, 1-hexadecene
Strain TD3	C_6-C_{16} Alkanes
Strain AK-01	C_{13}-C_{18} Alkanes
Anoxygenic photoheterotropic bacterium	
Blastochloris sulfoviridis strain ToP1	Toluene

2.2.1 Terminal Electron Accepting Processes (TEAPs)

Since hydrocarbons are highly reduced organic molecules, the reducing equivalents generated during hydrocarbon oxidation must be transferred to a TEA with a more positive redox potential to allow energy conservation for growth. It is often assumed that TEAPs occur sequentially with higher energy-yielding electron acceptors consumed before lower energy-yielding ones (Stumm and Morgan 1996). The theoretically calculated free energy for the oxidation of toluene coupled with reduction of different TEAs in Table 2-3 illustrates the order in which the reactions are expected to occur from a thermodynamic point of view. Based on Table 2-3, NRBs will outcompete IRBs, SRBs, and methanogens, if NO_3^- is present. Depletion of NO_3^- will allow the use of TEAs yielding less energy, and result in redox zonation where a given TEAP predominates.

Table 2-3. Examples of stoichiometric equations of anaerobic toluene degradation (Spormann and Widdel 2000).

Bacteria	Reactions	$\Delta G^{o'}$ (kJ/mole)
NRB	$C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+ \rightarrow 7\ HCO_3^- + 3.6\ N_2 + 0.6\ H_2O$	-3554
IRB	$C_7H_8 + 94\ Fe(OH)_3 \rightarrow 7\ FeCO_3 + 29\ Fe_3O_4 + 145\ H_2O$	-3398
	$C_7H_8 + 108\ Fe(OH)_3 \rightarrow 36\ Fe_3O_4 + 7\ HCO_3^- + 7\ H^+ + 159\ H_2O$	-3174
	$C_7H_8 + 36\ Fe^{3+} + 21\ H_2O \rightarrow 36\ Fe^{2+} + 7\ HCO_3^- + 43\ H^+$	-3630
SRB	$C_7H_8 + 4.5\ SO_4^{2-} + 3\ H_2O \rightarrow 4.5\ HS^- + 7\ HCO_3^- + 2.5\ H^+$	-205
	$C_7H_8 + 4.5\ SO_4^{2-} + 2\ H^+ + 3\ H_2O \rightarrow 4.5\ H_2S + 7\ HCO_3^-$	-273
Methanogen	$C_7H_8 + 7.5\ H_2O \rightarrow 4.5\ CH_4 + 2.5\ HCO_3^- + 2.5\ H^+$	-131

Vroblesky and Chapelle (1994) found that the distribution of microbial TEAPs, such as methanogenesis, sulfate reduction, and iron reduction, at a PHC contaminated site is highly dynamic over both time and space. Lack of available sulfate could result in a shift from sulfate reduction to methanogenesis, while the addition of sulfate to methanogenic zones resulted in a TEAP shift from methanogenesis to sulfate reduction. Temporal shifts between sulfate reduction and iron reduction were also observed. Time lags associated with TEAP shifts ranged from less than 10 days to about 3.5 months. Westermann and Ahring

(1987) studied the dynamics of methanogenesis, sulfate-reduction, and denitrification in a permanently waterlogged alder swamp and suggested that the competition for common substrates between sulfate-reducing and methane-producing bacteria was a possible mechanism.

The composition and activity of microbial communities involved in hydrocarbon biodegradation may vary within and/or between TEAP zones (Zwolinski *et al.* 2000). Laboratory and field studies have shown that biodegradation of individual PHC compounds is strongly dependent on the TEAPs. For instance, ethylbenzene degradation occurs under aerobic and nitrate-reducing conditions, but degradation appears to be site specific under iron-reducing, sulfate-reducing, and methanogenic conditions (Schreiber *et al.* 2004). Kazumi *et al.* (1997) studied the anaerobic biodegradation of benzene in diverse anaerobic environments and found that benzene was biodegraded under iron-reducing, sulfate-reducing, and methanogenic conditions but not under nitrate-reducing conditions. Burland and Edwards (1999) confirmed anaerobic benzene biodegradation under iron-reducing and sulfate-reducing conditions, whereas their study demonstrated that benzene biodegradation could be linked to nitrate reduction.

There is also evidence that different anaerobic TEAPs affect the biodegradation rates of PHCs. Anaerobic BTEX biodegradation rates are consistently lower than rate constants estimated under aerobic conditions. Biodegradation rates of toluene and o- and m-xylenes were shown to be faster under nitrate-reducing conditions than under sulfate-reducing conditions (Hutchins 1991). Considering the dynamics of TEAPs and the relation between TEAP and PHC biodegradation, the biodegradation rate of PHCs vary temporally and spatially at a contaminated site (Vroblesky and Chapelle 1994). This concept has been very useful for understanding contaminant plumes and has been one basis for evaluating the extent of intrinsic biodegradation at PHC-contaminated sites (Borden *et al.* 1995; Wiedemeier *et al.* 1995). Furthermore, there is a potential that the addition of alternate TEAs with higher energy yield than indigenous TEAs can cause a shift

in the subsurface to a more efficient TEAP, potentially increasing the biodegradation efficiency of the PHCs. Therefore, it is important to identify and even modify the TEAPs at contaminated sites to understand and potentially enhance biodegradation of PHCs at a contaminated site.

Different methods have been applied to measure the redox conditions and characterize the TEAPs at contaminated sites, including measurement of electrochemical redox potentials (Eh), redox-sensitive parameters in groundwater, hydrogen (H_2) concentrations in groundwater, sediment characteristics, and microbial measurements, such as microbial enumeration, biomarker measurements, and TEAP bioassays. However, no standardized or general accepted approach exists (Christensen *et al.* 2000).

The conventional Eh measurement may not be applicable to identify TEAP processes in a groundwater system (Chapelle 2001). Measurement of a certain Eh indicates that a redox reaction is possible from a thermodynamic point of view, but not that the reaction actually occurs. Also, there are many examples showing that groundwater samples could not be at thermodynamic equilibrium due to the fact that redox reactions mediated by microorganisms are inherently kinetic processes (Chapelle 2001). Despite the problems mentioned above, Eh is easy to measure and can be used to identify strongly reducing conditions and dominating TEAPs in field monitoring (Christensen *et al.* 2000).

Methods to identify TEAPs in groundwater systems have focused on documenting the changes in redox-sensitive species, including consumption of particular TEAs, or alternatively, evolvement of metabolic byproducts, such as methane (CH_4), sulfide (S^{2-}), ferrous iron (Fe^{2+}), and others. The primary redox-sensitive species in groundwater include:

1) dissolved ions, such as SO_4^{2-}, bisulfide (HS^-), Fe^{2+}, manganese (II) (Mn^{2+}), NO_3^-, nitrite (NO_2^-), and ammonium (NH_4^+),
2) dissolved gases, such as CH_4, nitrous oxide (N_2O) and oxygen (O_2), and

3) dissolved organic carbon (DOC) and organic N.

Measuring of redox-sensitive species has been used in identification of reduced and oxidized conditions (Lendvay *et al.* 1998), assignment of redox zones (Lyngkilde and Christensen 1992), and determination of predominant redox reactions (Borden *et al.* 1995). Due to the migration of dissolved redox species and the potential interactions of some aqueous species with solid phase, the evaluation of redox conditions may be only indicative and caution should be taken when using only the aqueous species to estimate the reduction capacity at a contaminated site (Christensen *et al.* 2000).

Dissolved H_2 concentrations have been suggested to be a good indicator of the predominant TEAP in groundwater systems based on the theory that H_2 level is constant and controlled by physiology of the mediating bacteria in a steady-state system, limited by the availability of organic matter (Lovley and Goodwin 1988). This approach has been applied to identify the zonation of TEAPs in several field studies (Chapelle and Lovley 1992; Chapelle and Mcmahon 1991) and H_2 concentration ranges characteristic of different TEAPs have been reported. However, the energetics of the groundwater system may be influenced by temperature, concentrations of dissolved species, and type of solid iron oxides, and thus, may allow iron reduction and sulfate reduction to occur concurrently at the same H_2 concentration (Christensen *et al.* 2000). Therefore, characteristic H_2 levels seem of less general value.

Most probable number (MPN) counts and biomarker measurements can confirm the presence of microorganisms mediating specific TEAPs at a contaminated site, indicating a potential for the occurrence of the redox processes at the site. However, these methods cannot verify the actual redox conditions. In contrast, TEAP bioassays can be used to identify multiple on-going redox processes, and furthermore, to estimate the actual rates of redox processes. Though laborious and time consuming, TEAP bioassays have been considered as the most powerful

approach for characterizing the on-going microbial redox processes at contaminated sites (Christensen *et al*. 2000).

2.2.2 Microbiology of Anaerobic BTEX Biodegradation

Anaerobic biodegradation of BTEX with different TEAPs has been well documented (Widdel and Rabus 2001; Chakraborty and Coates 2004). BTEX can serve as carbon and energy sources for microbial growth phototrophically (Zengler *et al.* 1999), or heterotrophically with NO_3^-, Mn (IV), Fe (III), SO_4^{2-} or CO_2 as the sole electron acceptor (Chakraborty and Coates 2004). Recently, it has also been found that anaerobic biodegradation of BTEX can also be coupled to the respiration of perchlorate or chlorate, or to the reduction of the quinine moieties of humic substances (Coates *et al.* 2001). The initiation reactions, metabolic pathways, and reaction rates of BTEX biodegradation under different redox conditions have been intensively studied and reported (Heider *et al.* 1999; Spormann and Widdel 2000; Widdel and Rabus 2001; Chakraborty and Coates 2004; Aronson and Howard 1997).

The anaerobic toluene degradation has been most intensively studied and is probably most comprehensively understood among all BTEX compounds (Chakraborty and Coates 2004). Since the first evidence of toluene biodegradation was found under nitrate-reducing conditions (Kuhn *et al.* 1985), it has been demonstrated that toluene can be biodegraded by NRBs, IRBs, SRBs, methanogens, and anoxygenic photosynthetic bacteria (Heider *et al.* 1999; Spormann and Widdel 2000; Widdel and Rabus 2001; Chakraborty and Coates 2004).

Geobacter metallireducens GS-15 was the first example of an organism in a pure culture that could anaerobically oxidize toluene (Lovley *et al.* 1989). It was first isolated from freshwater sediments of the Potomac River in Maryland, USA. GS-15 can also use Mn (IV) or NO_3^- as the TEA. MnO_2 was completely reduced to Mn (II), which precipitated as rhodochrosite ($MnCO_3$). NO_3^- was reduced to

ammonia (NH_3). Lovley and Lonergan (1990a) found that GS-15 was able to grow in an anaerobic medium with toluene as the sole electron donor and a poorly crystalline ferric oxide as the TEA. The energy yielded by the oxidation of toluene can support the growth of the microorganisms. Toluene was completely oxidized to CO_2 with ferric oxide being reduced to magnetite. At higher toluene concentrations, there was increased cell growth and more Fe (III) reduction but there was a longer lag period. Since the isolation of GS-15, many pure cultures capable of anaerobic toluene biodegradation have been isolated (refer to Table 2-2).

Studies have revealed that the first step in toluene biodegradation is the addition of fumarate onto the toluene methyl group to form benzylsuccinate, which is further metabolized to benzoyl-CoA (Spormann and Widdel 2000; Widdel and Rabus 2001; Heider *et al.* 1999). This initial activation reaction is mediated by a glycyl radical enzyme benzylsuccinate synthase (BBS) (Leuthner *et al.* 1998). The reduction of benzyol-CoA, an important central intermediate of anaerobic aromatic biodegradation, represents a major energy barrier for anaerobes (Boll and Fuchs 1995), which may be overcome by ATP hydrolysis (Boll *et al.* 1997).

Relatively little is known regarding the anaerobic biodegradation of ethylbenzene. Until now, only five pure cultures utilizing ethylbenzene have been reported, including four NRBs and one SRB (Foght 2008). The isolated NRBs were *Azoarcus* strains EbN1 and PbN1 (Rabus and Widdel 1995) and EB1 (Ball *et al.* 1996) and *D. aromatica* RCB (Chakraborty *et al.* 2005). All strains are facultative anaerobes and can degrade ethylbenzene completely under nitrate-reducing conditions. However, these strains are limited in their capability of oxidizing other aromatic hydrocarbons (Chakraborty and Coates 2004). Ethylbenzene is initially attacked by dehydrogenation of the methylene group of the ethyl side chain to form 1-phenylethanol. The reaction is mediated by ethylbenzene dehydrogenase (Johnson *et al*. 2001). The hydroxyl group of the 1- phenylethanol formed in the initial reaction is derived from water (Ball *et al.* 1996). Strain EbS7,

isolated from Guaymas Basin sediment in the Gulf of California, is the first pure culture strain of a SRB that grows with ethylbenzene as the electron donor and carbon source (Kniemeyer *et al.* 2003). Unlike denitrifying bacteria, the activation reaction for the catabolism of ethylbenzene by EbS7 is a fumarate addition reaction on the side chain to form 1-phenylethylsuccinate.

Anaerobic biodegradation of three xylene isomers has been studied mainly under nitrate and sulfate-reducing conditions. Although it has been demonstrated in sediment or culture enrichment studies that p-xylene is biodegradable under anaerobic conditions (Haner *et al.* 1995; Kuhn *et al.* 1988), no pure culture has been isolated that can mineralize p-xylene to CO_2 (Foght 2008). However, several NRBs that can mineralize m- and o-xylenes have been isolated (Hess *et al.* 1997; Rabus and Widdel 1995). The proposed pathway for anaerobic biodegradation of m- and o-xylenes is an initial reaction of fumarate addition to one of the methyl groups to form 3-methylbenzylsuccinate (mediated by 3-methylbenzylsuccinate synthase), followed by subsequent oxidation to 3-methylbenzoate. The isolated microorganisms are closely related to each other and to the previously identified toluene-degraders. Sulfate-reducing microorganisms capable of anaerobic biodegradation of m- and o-xylenes have also been isolated (Harms *et al.* 1999). Strain mXyS1 and strain oXyS1 use m- and o-xylene, respectively.

The proposed anaerobic biodegradation pathways for TEX are illustrated in Figure 2-1.

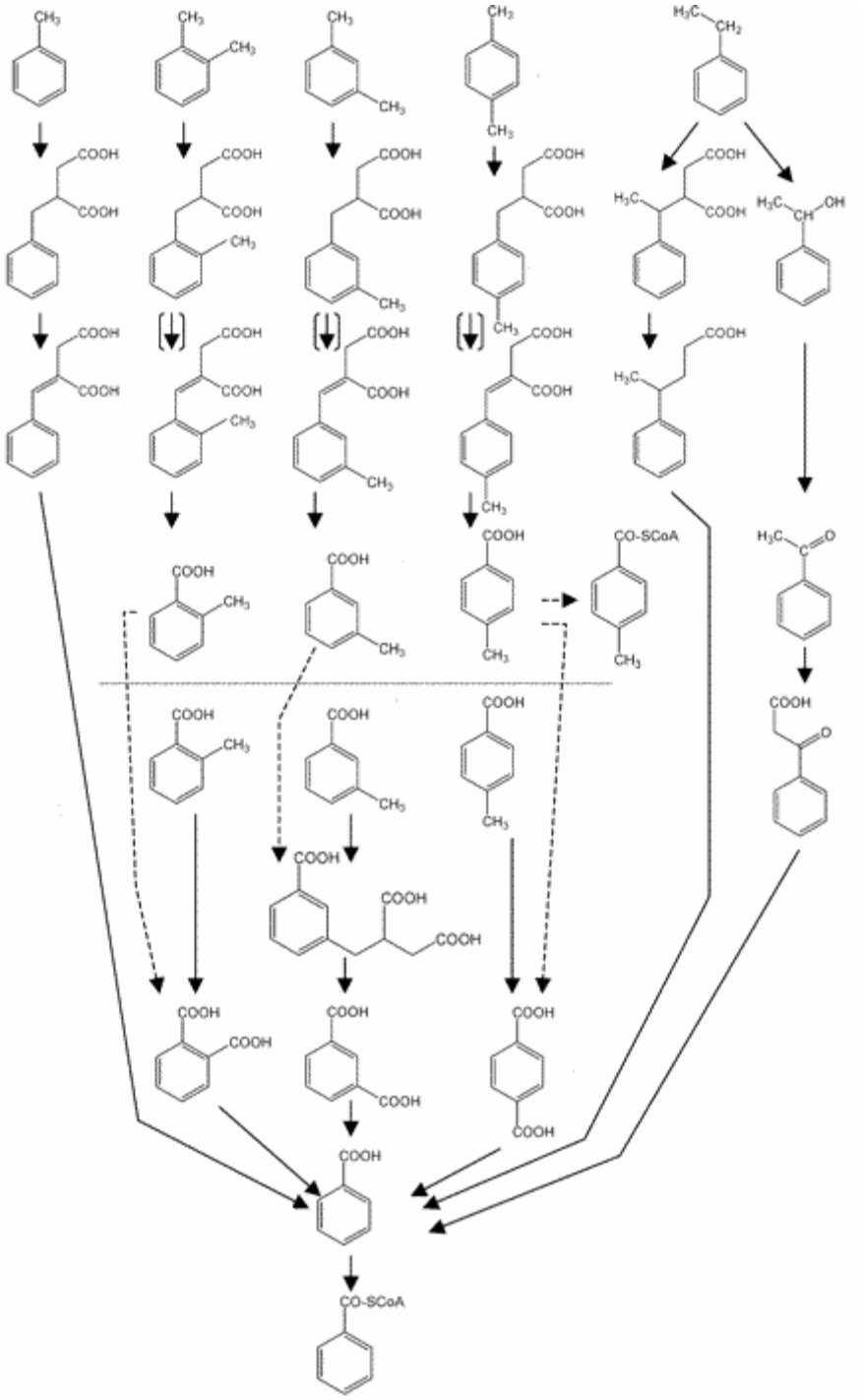

Figure 2-1. Proposed pathways for anaerobic degradation of TEX (adapted from Griebler *et al.* 2004) (Note: Solid arrows indicate proven pathways; dashed arrows indicate suggested transformation steps; and arrows in brackets indicate that the further degradation is not obligatory).

Laboratory and field studies investigating the anaerobic biodegradation of benzene have shown inconsistent results (Lovley 2000). Many studies have indicated that benzene persists under anaerobic conditions; however, anaerobic benzene biodegradation has been observed in sediment studies or with microbial enrichments, under nitrate-reducing, ferric-reducing, sulfate-reducing, and methanogenic conditions (Anderson and Lovley 2000; Coates *et al*. 2002). It was not until recently that the first two organisms capable of anaerobic benzene degradation (*Dechloromonas* strains RCB and JJ) were isolated (Coates *et al.* 2001). Both strains coupled complete benzene oxidation to NO_3^- reduction, while Strain RCB could also couple benzene oxidation to perchlorate reduction. The *Dechloromonas* species and the *Dechlorosoma* species are the predominant perchlorate-reducing bacteria in the environment. These organisms have been found to be ubiquitous, regardless of previous exposure to perchlorate or not (Chakraborty and Coates 2004). These strains are also characterized by their metabolical versatility and their capability of using a broad range of alternative electron donors (Coates *et al.* 2001).

The pathway for anaerobic benzene biodegradation is still under debate (Coates *et al*. 2002; Foght 2008). The common activation reaction of fumarate addition for anaerobic hydrocarbon biodegradation requires a large activation energy to remove hydrogen from the benzene ring. It is highly unlikely that this initial reaction takes place in anaerobic benzene biodegradation (Coates *et al.* 2002). As shown in Figure 2-2, the possible anaerobic benzene degradation pathways include initial reactions of carboxylation, hydroxylation, methylation, or reduction of the benzene ring followed by subsequent transformation to the central intermediate benzoate and ring cleavage.

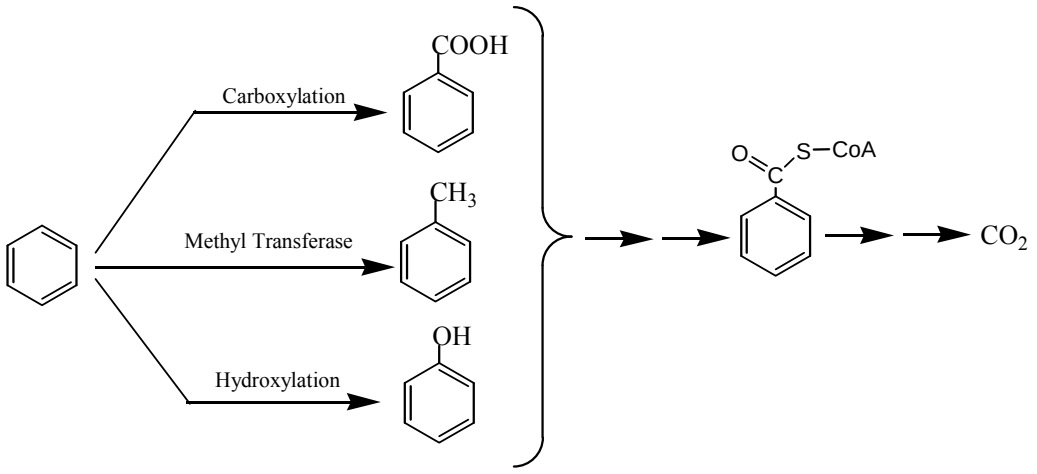

Figure 2-2. Possible pathways of anaerobic benzene degradation (adapted from Coates *et al.* 2002; Chakraborty and Coates 2004).

Some previous studies have indicated that phenol and benzoate are the important intermediates (Caldwell and Suflita 2000; Grbi'c-Gali'c and Vogel 1987; Weiner and Lovley 1998). Studies using radiolabelled compounds or isotopes have indicated that the hydroxyl group may be from water and that the carboxyl group may be derived from the metabolism of benzene. Coates *et al.* (2002) proposed alkylation of benzene to toluene as the first step in anaerobic benzene degradation but provided no direct evidence. Ulrich *et al.* (2005) provided the first direct evidence to support the alkylation of benzene in their study of C-13(6)-benzene biodegradation in nitrate-reducing and methanogenic enrichment cultures.

2.2.3 Investigating *in-situ* Biodegradation Processes

The structure and activity of microbial communities reflects a dynamic interaction between microbes and the environment they inhabit (Zwolinski *et al.* 2000). Due to the indigenous microbial communities and the available TEAs, biodegradation of PHCs tends to be specific to the individual compound and to a given contaminated site (Suarez and Rifai 1999). Furthermore, the biodegradation processes can vary with time and location even for a single field study (Aronson and Howard 1997). Therefore, the anaerobic PHC biodegradation processes

should be cautiously investigated at each specific site using field data and/or laboratory microcosm studies (USEPA 2001).

Rates of biodegradation in contaminated groundwater systems can be measured using field and laboratory methods that track as a function of time (1) the consumption of PHCs, (2) the consumption of TEAs, or (3) the production of microbial metabolites (Chapelle *et al.* 1996). Field studies can provide relevant data for a specific site, essentially showing whether the PHC compound of interest is being biodegraded. The objective of field investigations is to define (both temporally and spatially) the nature and distribution of PHC contamination and to characterize the groundwater plume and its potential impacts on receptors. Data required for a field investigation include the PHC contamination source mass, groundwater flow, PHC phase distribution and partitioning between soil, groundwater, and soil gas, rates of biological and non-biological transformation, etc. To evaluate the biodegradation processes, the nutrients and electron donors and acceptors present in the groundwater and the concentrations of cometabolites and metabolic by-products should be investigated. The microbial populations present at the site may also need to be identified. Normally a conceptual site model will be developed and serve as a foundation for further assessment of the complex NA processes.

In addition to *in-situ* field investigations, static or flow-through microcosm tests are often performed in the laboratory using groundwater and sediment materials collected from the PHC contaminated site. These tests are designed to study the biodegradation processes, in particular to determine the biodegradation rates. It is believed that laboratory microcosm studies can give convincing evidence of the occurrence of biodegradation at a specific contaminated site by direct measurement of metabolites and by a mass balance analysis of PHC contaminants. Wiedemeier *et al.* (1995) state that optimally run laboratory microcosm studies over an 18-month period can resolve biotic and abiotic losses with a rate detection limit of 0.001 to 0.0005 day^{-1}.

However, the results from laboratory-scale microcosm studies can be influenced by many factors, such as (Wiedemeier *et al.* 1995):

- the procedure used to acquire field materials
- the method used to set up microcosms (in particular the ratio of sediment to groundwater used in the microcosms, etc.)
- the incubation conditions, and
- the length of time used for incubation.

It is generally recognized that laboratory microcosm studies often result in higher rates of biodegradation than field studies (Wiedemeier *et al.* 1995). However, the mixing of a natural sample during its collection or during the construction of a microcosm may result in a "disturbance artifact" which can lead to either an increase or a decrease in the microbial activity of the sample (Aronson and Howard 1997). Therefore, rate constants from field studies, if available, should be used to evaluate the time required to achieve the remediation objectives.

Many field and laboratory studies have been conducted on the anaerobic biodegradation of BTEX. Some reported biodegradation rates of BTEX are summarized in Table 2-4.

Table 2-4. Summary of reported first-order anaerobic biodegradation rate constants for BTEX (adapted from Suarez and Rifai 1999).

	Ranges		Average under different redox conditions			
	Field	Lab	Nitrate reducing	Iron reducing	Sulfate reducing	Methanogenic
Benzene	0 - 0.023	0 - 0.089	0.008	0.009	0.008	0.010
Toluene	0 - 4.32	0 - 3.28	0.46	0.012	0.062	0.037
Ethylbenzene	0 - 6.048	0 - 0.48	0.27	0.003	0.002	0.010
m-Xylene	0 - 0.32	0 - 0.49	0.089	0.010	0.081	0.019
o-Xylene	0 - 0.214	0 - 0.075	0.012	0.003	0.027	0.026
p-Xylene	0 - 0.081	0 - 0.44	0.068	0.010	0.011	0.018

The anaerobic biodegradation rates of benzene and m- and p- xylenes were higher in laboratory studies than those estimated from field studies. In contrast, the laboratory anaerobic biodegradation rates of ethylbenzene were lower than the field rates. With regards to toluene and o-xylene, the anaerobic biodegradation rates derived from the field and laboratory studies were quite similar. It can also be seen from the table that the biodegradation rates of BTEX are highly dependent on the individual compound and the different reducing conditions.

2.2.4 Factors Influencing Anaerobic PHC Biodegradation

Biodegradation requires the presence of microorganisms with suitable degradation capabilities at the site as well as favourable site conditions. For anaerobic PHC biodegradation in particular, the absence of DO and availability of PHCs, TEAs, and essential nutrients are critical for hydrocarbon biodegradation. The concentrations of PHCs are an important factor influencing the anaerobic biodegradation processes. If the PHC concentrations are too low, the enzymes involved in the degradation may not be induced (Bauer *et al*. 1994). However, toxicity and inhibition of microorganisms may occur at high PHC concentrations (Sikkema *et al.* 1995). Evans *et al*. (1991a) found that the lag phase and the cell density increased as a function of toluene concentration, whereas toluene concentrations higher than 3 mM inhibited cell growth. For many contaminated sites, nutrients and/or TEAs may be the limiting factors (Braddock *et al.* 2001) and enhanced bioremediation (with amendment of nutrients or TEAs) can be applied.

The following sections will highlight the important factors which influence the biodegradation processes.

2.2.4.1 Bioavailability of PHCs

Biodegradation can only occur if the PHCs are readily accessible to the microorganisms. Bioavailability refers to the fraction of PHCs available for microbial attack and may be a limiting factor for PHC biodegradation at

contaminated sites (Smith *et al.* 1997; De Jonge *et al.* 1997). Two mechanisms control the bioavailability of PHCs: solubilization of PHCs controls their bioavailability at high PHC concentrations, and sorption and diffusion become limiting factors at low PHC concentrations (De Jonge *et al.* 1997).

The subsurface biodegradation of NAPL is critically dependent on their availability to the microorganism populations present in the close environment. Degradation rates for high molecular weight PHCs are believed to be dependent on the aqueous solubility of the NAPL (Leahy and Colwell 1990). The slow dissolution and dispersion rates of the NAPL into the aqueous soil solution are a major problem for successful biodegradation at many contaminated sites (Zoller and Rubin 2001).

Sorption of PHCs to the soil will limit the bioavailability, subsurface transport, and biodegradation of PHCs (Atlas 1995; Pignatello and Xing 1995). Some of the important factors that affect the process of sorption include soil type, organic matter content, soil moisture content, and how long the PHCs have been present in the soil (aging or weathering time) (Pignatello and Xing 1995; Smith *et al.* 1997; Mihelcic *et al.* 1993). There was evidence suggesting that the biodegradation of PHCs could be enhanced, inhibited or negligibly affected by the presence of clays, depending upon the type of clay particle (Mihelcic *et al.* 1993). Manilal and Alexander (1991) found that sorption process correlated well with soil organic matter content and significantly reduced biodegradability.

2.2.4.2 Substrate Interactions

PHC contaminated sites typically involve a complex mixture of organic compounds. It is therefore important to understand the potential interactions among individual PHCs as well as interactions between PHCs and other organic compounds present at the site. Preferential utilization of individual BTEX compounds has been observed at contaminated sites (Chapelle 2001). The substrate interactions among BTEX mixtures could be very complex. The

presence of one BTEX compound may either stimulate or inhibit the degradation of other BTEX compounds, depending on the individual compounds and their concentrations (Dou *et al*. 2008).

Inhibitory interactions have been observed in some previous studies. Barbaro *et al.* (1992) observed the competitive utilization between toluene, ethylbenzene and the xylenes under nitrate-reducing conditions. It was found that toluene inhibited anaerobic biodegradation of o-xylene under sulfate-reducing conditions in sediment and pure culture tests (Meckenstock *et al*. 2004). Da Silva and Alvarez (2004) reported that benzene removal was inhibited by the presence of toluene in methanogenic flow-through aquifer column tests.

Stimulation interactions may be induced by cometabolism of PHC compounds. For example, cometabolism of o-xylene in the presence of toluene appears to be common in anaerobic systems (Evans *et al.* 1991b; Alvarez and Vogel 1995). Cometabolism involves the use of another compound (other than the compound of concern) as the energy and carbon source, while the compound of concern is gratuitously metabolized due to lack of enzyme specificity (Atlas 1995). Some chemicals are only degraded in the soil environment through cometabolic pathways. Therefore an additional carbon source must be present in the contaminated soil for cometabolic degradation to occur (Fiorenza *et al.* 1991). The effects of cometabolism on microorganisms are complex, making the results of *in-situ* biodegradation unpredictable (Atlas 1995).

The presence of other easily biodegradable substances, other than the PHCs, may also affect the biodegradation and persistence of BTEX. Previous studies have shown that the preferential biodegradation of ethanol, a gasoline additive often present at gasoline contaminated sites, accelerated the depletion of available nutrients and TEAs, thus hindered BTEX removal (Corseuil *et al.* 1998; Ruiz-Aguilar *et al.* 2002). Edwards and Grbic-Galic (1994) found that acetate inhibited toluene degradation. It has been observed that the presence of methanol in a

culture fluid slowed toluene and benzene biodegradation under methanogenic conditions (Grbic-Galic and Vogel 1987). However, this negative effect may be offset by the increased microbial growth, which may be conducive to faster degradation rates. Corseuil *et al.* (1998) even found that ethanol enhanced toluene degradation under sulfate-reducing conditions. It was hypothesized that the incidental growth of toluene degraders during ethanol degradation contributed to the enhanced toluene degradation.

2.2.4.3 Nutrients

Microorganisms require macronutrients (such as nitrogen (N) and phosphorus (P)), micronutrients (such as calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}), potassium (K^{+}), and S^{2-}), and co-factors for growth. The requirements for these nutrients are approximately the same as the cell composition of the microorganisms (Suthersan 1997). Based on this approach, the optimal C : N : P ratio is 100 : 10 : 1 (Cookson 1995). However, a wide range of C : N and C : P ratios have been reported for optimal PHC biodegradation (reviewed in Huesemann 2004).

The availability of these nutrients within the same area as the PHCs is critical for PHC biodegradation (Atlas 1981). Depending on the contaminated sites, some of these nutrients might be limiting thus affecting the biodegradation processes. Naturally occurring biodegradation of BTEX is often limited by either the concentration of an appropriate TEA or a nutrient required during the biodegradation (Hunkeler *et al.* 2002). Amendment of TEA and/or nutrients has been found to enhance the natural biodegradation process (Scow and Hicks, 2005). However, there is no consensus on how to best optimize nutrient additions (Head and Seannell 1999).

Previous studies have shown that nutrient addition stimulates PHC biodegradation under aerobic conditions (Atlas 1981; Leahy and Colwell 1990). Graham *et al.* (1999) found that both N and P additions affected biodegradation, but that

stoichiometrically inappropriate nutrient concentrations produced sub-optimal CO_2 yields. However, other studies indicated that nutrient addition had little or negative effects on the biodegradation of hydrocarbons (Chaineau *et al*. 2005; Carmichael and Pfaender 1997). Excessive nutrient concentrations appeared to inhibit the biodegradation activity (Challain *et al*. 2006).

There have been few studies on the effects of nutrient addition on anaerobic biodegradation and the findings were inconsistent. Johnston *et al.* (1996) found no stimulation effects of nutrient amendment on the anaerobic biodegradation of alkylbenzenes in aquifer sediment. In contrast, Cross *et al.* (2006) found that nutrient addition increased the anaerobic biodegradation rates of PHCs present in diesel fuel contaminated groundwater.

2.2.4.4 pH

Most microorganisms are sensitive to the pH. Most heterotrophic bacteria favour a pH near 7. Degradation rates have been observed to decrease if the pH is much higher or much lower than 7. The optimal range has been found to be between 5.0 and 7.8 for the biodegradation of PHCs from fuel found in soil (Dibble and Bartha 1979).

2.2.4.5 Salinity

There are a few published studies on the effects of salinity on bioremediation. Ward and Brock (1978) found that the rates of hydrocarbon metabolism decreased with increasing levels of salinity. Similarly, Rhykerd *et al.* (1995) reported that salt concentration of 200 dS/m in soils decreased the biodegradation of hydrocarbons present in motor oil by up to 44% compared with non-salty control soils. Ulrich *et al.* (2009) studied the effects of salt on aerobic biodegradation of PHCs in groundwater and found that salt concentrations of ≥ 1% (w/v) consistently increased the lag time, but the effects of salt on the biodegradation rates varied depending on the contaminated site being investigated.

2.2.4.6 Temperature

Hydrocarbon biodegradation can occur over a wide range of temperatures. Extreme temperatures may change the physical and chemical properties of the contaminants, and subsequently influence the biodegradation processes. However, both psychrotrophic and thermophilic hydrocarbon utilizing microorganisms have been isolated (Margesin and Schinner 2001). Cold-adapted microorganisms (psychrophiles and psychrotrophs) are able to grow at temperatures around 0°C. Psychrophiles have an optimum growth temperature of ≤15°C and do not grow above 20°C, whereas psychrotrophs have optimum growth temperatures above 15°C and maximum growth temperatures above 20°C (Morita 1975). Cold-adapted indigenous microorganisms play a significant role in the *in-situ* biodegradation of hydrocarbons in cold environments. The reported temperature threshold for significant PHC biodegradation was around 0°C (Siron *et al.* 1995). Most thermophilic microorganisms show a maximum growth temperature between 50 and 70°C (Margesin and Schinner 2001). Hydrocarbon degradation was found to occur at 55°C in the Kuwait desert by indigenous microbes (Al-Awadhi *et al.* 1996). It is generally accepted that the upper temperature limit for anaerobic PHC biodegradation is probably 80°C because biodegradation ceases in oil reservoirs around 75 - 80°C in the zone of thermophilic organisms (Connan 1984).

Temperature affects the type of microorganisms that will flourish at a contaminated site (Atlas 1981) as well as the microbial growth rate. It is generally accepted that the biological reaction rate will double for every 10°C increase in temperature within the temperature range of 5°C to 38°C (Fiorenza *et al.* 1991). However, contrary to this view, it has been found that the biodegradation rates of toluene were not depressed in low-temperature groundwater systems (with an ambient temperature of 5°C) relative to more temperate systems (Bradley and Chapelle 1995). Westermann and Ahring (1987) found that methane production was most sensitive to temperature changes, followed by denitrification and sulfate reduction. The effects of temperature on anaerobic biodegradation of

hydrocarbons, especially at the contaminated sites, are complex and site specific. Therefore, laboratory studies should be conducted at temperatures prevalent at the site.

2.3 Conclusions

PHCs are the most widespread soil and groundwater contaminants in Canada and PHC contamination represents a significant health and environmental risk (CCME 2008). Monitored natural attenuation (MNA) and enhanced attenuation (EA), both relying on natural attenuation (NA) processes, have been investigated as remedial options for the PHC contamination (Wiedemeier *et al.* 1995; ITRC 2008). The biodegradation process is the most important mechanism of NA to reduce the PHC mass at the contaminated site. Since extensive anaerobic zones often develop at the contaminated sites, the anaerobic biodegradation has significant implications for the remediation success of the PHC contamination.

In this chapter, the mechanisms and kinetics of anaerobic PHCs biodegradation were reviewed and summarized, discussing in particular the microbiology, metabolic pathways, and first-order anaerobic biodegradation rates of BTEX. The dynamics of TEAPs and its effects on the biodegradation process were also discussed. Since TEAPs can affect the anaerobic PHC biodegradation rates and the distribution of TEAPs can be very dynamic temporally and spatially, it is important to measure the redox conditions and to characterize the TEAPs at the contaminated sites.

Numerous studies have been undertaken to understand the anaerobic PHC biodegradation processes. It has been demonstrated that anaerobic biodegradation of PHCs can occur under different redox conditions, using NO_3^-, SO_4^{2-}, Fe(III), Mn(IV), or CO_2 as TEA. Diverse strains of microorganisms capable of PHC biodegradation under different redox conditions have been isolated. Potential pathways have been elucidated and some novel initial activation reactions have been identified.

Field and laboratory studies have also been conducted to investigate the *in-situ* anaerobic biodegradation processes at PHC contaminated sites. Anaerobic PHC biodegradation can be affected by the composition and properties of PHC mixtures present at the site as well as the site conditions. Therefore, the anaerobic biodegradation processes could be very specific to each contaminated site. Studies have shown that anaerobic PHC biodegradation is often limited by the availability of an appropriate TEA or a nutrient and enhancement may be achieved by TEA and/or nutrient additions. However, studies on the effects of nutrient addition on anaerobic PHC biodegradation were scarce and the results were inconsistent. Furthermore, the enhancement effects of TEA amendment on anaerobic biodegradation also vary with the specific site conditions and substrate interactions among the PHC mixtures. Laboratory studies should be conducted using the field groundwater and sediment samples to estimate the enhancement effects and determine the potential of using EA as the remedial option for a specific contaminated site.

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Chapter 3. Enhanced Anaerobic Biodegradation of Petroleum Hydrocarbons in Groundwater from a Flare Pit Site

3.1 Introduction

PHCs are the most widespread soil and groundwater contaminants in Canada (CCME 2008). Many PHC contaminated sites are associated with flare pits at upstream oil and gas operating sites. Flare pits are earthen pits that have been used to contain liquid wastes and burn off combustible components from the processing of natural gas and crude oil (Cook *et al*. 2002). The materials released into the flare pits include residues from the flaring of gas and/or occasional inputs of liquid hydrocarbons (condensate and crude oil) and produced water (Amatya *et al.* 2002). The water-soluble PHC and salt contaminants may disperse with groundwater movement and may have a long-term impact on human and environmental health both on and off site (Nublein *et al.* 1994).

NA occurs through a variety of processes including dispersion, sorption, volatilization, and biodegradation, among which anaerobic biodegradation is often the dominant destructive mechanism and can result in significant reduction of contaminant mass (Wiedemeier 1995). MNA and EA are environmental management strategies that rely on NA processes and are regarded as cost-effective approaches for remediation of PHC contamination in the subsurface environment.

PHCs can be biodegraded under anaerobic conditions with nitrate (NO_3^-) (Burland and Edwards 1999; Coates *et al.* 2001), sulfate (SO_4^{2-}) (Coates *et al.* 1996a; Coates *et al.* 1996b; Edwards *et al.* 1992; Kazumi *et al.* 1997; Kropp *et al.* 2000; Lovley *et al.* 1995; Phelps *et al.* 1996), manganese (Mn(IV) and iron (Fe(III)) (Anderson *et al.* 1998; Lovley *et al.* 1994; 1996), and carbon dioxide (CO_2) (Grbic-Galic and Vogel, 1987; Kazumi *et al.* 1997) as terminal electron acceptors (TEAs), but typically at lower degradation rates than aerobic biodegradation (Bhupathiraju *et al.* 2002). The anaerobic biodegradation process may be

stimulated by applying enhancements, for instance, TEA or nutrient amendment. Studies have shown that addition of NO_3^-, SO_4^{2-}, or a combination of both NO_3^- and SO_4^{2-} as TEAs into the contaminated groundwater can enhance *in-situ* PHC biodegradation and is capable of partially or completely removing BTEX (Barbaro *et al*. 1992; Hutchins *et al.* 1991; Anderson and Lovley 2000; Cunningham *et al*. 2000; Cunningham *et al*. 2001). Enhancement effects of nutrient amendment on anaerobic PHC biodegradation have also been demonstrated (Cross *et al.* 2006). However, the *in-situ* enhancement effects of TEA and/or nutrient amendment could be very specific to the contaminated site (ITRC 2008). Thus, field and/or laboratory investigation should be carried out to verify the enhancement effects of TEA and/or nutrient amendment on the anaerobic biodegradation at the specific site.

This chapter presents a laboratory study on the enhancement effects of TEA and nutrient amendment on anaerobic biodegradation of PHC contaminants in the groundwater from a former flare pit site. This study was part of a larger project to evaluate NA as a viable remedial alternative for PHC contamination at upstream oil- and gas-contaminated sites in Alberta, Canada, referred to as the Consortium for Research on Natural Attenuation (CORONA). The objectives of this laboratory study are,

1) to develop a new laboratory anaerobic testing method;
2) to use the developed anaerobic testing method to study the enhancement effects of TEA and/or nutrients amendment on the anaerobic biodegradation of PHCs in the contaminated groundwater from a former flare pit site; and
3) to calculate the enhanced anaerobic biodegradation rates.

The PHCs of interest in the study were benzene, ethylbenzene, and m- and p-xylenes (BEX) and CCME F1 fraction hydrocarbons (F1, C_6 to C_{10} hydrocarbons measured by the CCME method).

3.2 Materials and Methods

3.2.1 Site Description

The study site, denoted as Site 1, is a former flare pit site located in west central Alberta. The hydrocarbon-impacted area is approximately 50 m wide by 60 m long, at depths ranging from approximately 2 to 10 m below ground surface. Groundwater monitoring data showed depleted dissolved oxygen (DO), NO_3^- and SO_4^{2-}, and enriched dissolved iron (Fe(II)), manganese (Mn(II)) and methane (CH_4) compared to background concentrations, indicating the occurrence of *in-situ* anaerobic biodegradation. The estimated *in-situ* attenuation rates were of the order of 10^{-4} d^{-1} (estimated from PHC depletion over time in one monitoring well, Armstrong 2006, personal communication).

3.2.2 Sampling of Groundwater and Sediment at the Site

A custom-made drum was used for the sampling, storage and transport of the groundwater samples. Approximately 200 L groundwater samples were collected from a groundwater monitoring well at Site 1 using a peristaltic pump. The drum was flushed with argon gas prior to groundwater sampling to obtain anaerobic conditions. Contaminated sediments, which would be used as the seed for the mesocosm study, were collected using a hand auger and then put into a 20 L pail and capped with the groundwater. Both groundwater and sediment samples were then transported to the Department of Civil & Environmental Engineering at the University of Alberta and stored at 4°C until use.

3.2.3 Mesocosm Setup in the Laboratory

The size of the containers and the volumes of groundwater and sediment samples used in the test system for this study (>10 L) were much larger compared to the laboratory microcosms commonly used in the anaerobic biodegradation study (<100 mL). The test system is herein referred to as “mesocosm”.

The mesocosms designed for this study are illustrated in Figure 3-1. Each mesocosm used a 13 L glass carboy equipped with a custom-made Teflon stopper.

The stopper was designed to maintain anaerobic conditions in the mesocosm, minimize sorption of PHCs to the stopper, and allow the sub-sampling of water and gas from inside the mesocosm while maintaining anaerobic conditions. The 1/8'' and the 1/16'' stainless steel tubing was used for the sampling of water and headspace gas, respectively. Both were capped with Swagelok® face seal fittings (Edmonton Valve & Fitting Inc., Edmonton, Alberta). The 1/16''gas sampling tubing was also connected to a 1/4'' Swagelok® tube-fitting valve (Edmonton Valve & Fitting Inc., Edmonton, Alberta), which was opened only when the water and headspace gas were sampled. The mesocosm was incubated on its side so that the stopper was completely immersed in water, further preventing gas transfer from the ambient air into the mesocosm.

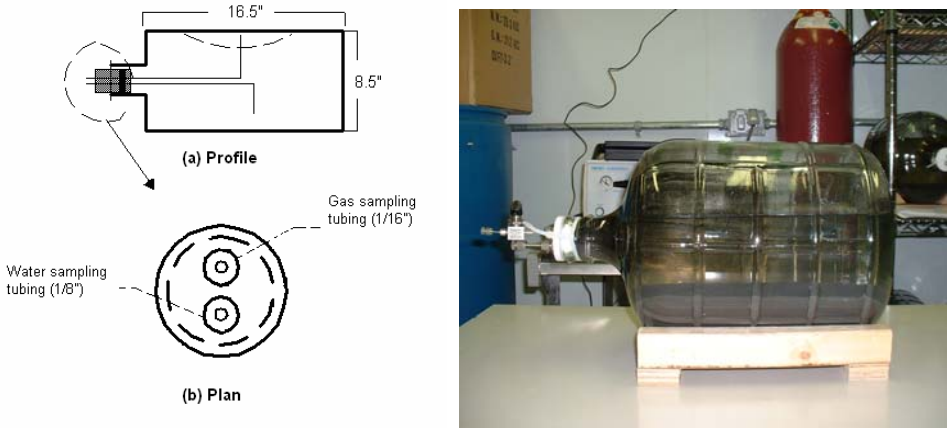

Figure 3-1. Schematics of the mesocosm configuration and photo of a mesocosm.

The mesocosms were set up to achieve a groundwater-to-sediment ratio of 10:1 (by volume) by adding 10 L of groundwater and approximately 1 L of sediment (homogenized in advance) under anaerobic conditions. Anaerobic conditions were established by flushing the mesocosm with nitrogen gas (N_2) prior to, and during the setup. The sterile controls (SCs) can be prepared by autoclaving the samples or by adding biocides to kill the microorganisms. However, adding biocides may not kill all species of microorganisms and may interfere with the geochemical processes in the mesocosm. Therefore, autoclaving was selected to prepare the SC mesocosms. The groundwater and sediment samples were autoclaved at 121ºC for

one hour on three consecutive days. The SCs were spiked with sterilized free product, i.e. liquid PHC contaminants recovered from the site and filtered through a 0.22 μm Millex-FG filter (Millipore Corp; Billerica, MA), to compensate for the PHC loss due to the autoclaving. All the mesocosms were allowed to equlibrate for one week and then selectively amended with TEAs (NO_3^- or SO_4^{2-}) and nutrients, i.e. ammonium (NH_4^+) and phosphate (PO_4^{3-}), except that the SCs were amended with all TEAs and nutrients. The intended amendment concentrations were achieved by injecting 50 mL of each amendment solution through the 1/8" water sampling tubing into the mesocosm. A 4 M solution of KNO_3, a 4 M solution of Na_2SO_4, and a 6 M solution of $NH_4H_2PO_4$ were used for NO_3^-, SO_4^{2-}, and nutrient amendment, respectively. All chemicals were purchased from Fisher Sicentific (Nepean, Ontario).

The experimental matrix and amendment concentrations are summarized in Table 3-1. Losses of ethylbenzene or xylenes in some mesocosms occurred during the mesocosm setup, possibly due to volatilization. Methanol was used as the solvent to add ethylbenzene or xylenes to those mesocosms

Table 3-1. Experimental matrix and amendments in Site 1 mesocosm study (mM/L).

Notation	No.	Amendments			Methanol
		NO_3^-	SO_4^{2-}	NH_4^+, PO_4^{3-}	
SC	**SC1**	2	2	3	-
	SC2	2	2	3	-
Ctrl	**1**	-	-	-	-
	7	-	-	-	10
Ctrl+NP	**2**	-	-	3	-
	8	-	-	3	2.5
NO3	**3**	2	-	-	-
	9	2	-	-	2.5
NO3+NP	**4**	2	-	3	2.5
	10	2	-	3	2.5
SO4	**5**	-	2	-	-
	11	-	2	-	2.5
SO4+NP	**6**	-	2	3	-
	12	-	2	3	2.5

Once prepared, all mesocosms were incubated at 15°C in the dark for 620 days. Mesocosms were mixed weekly by slowly rotating them. The TEAs were re-amended after they were completely depleted in one or more mesocosms.

All glassware, stoppers, laboratory utensils, and amendment solutions used in the experiment were sterilized by autoclaving in advance.

3.2.4 Mesocosm Sampling and Analyses During Incubation

The headspace, water and sediment were sampled for chemical and biological analyses at prescribed time intervals to monitor: (1) the depletion of the petroleum hydrocarbons (specifically, BEX and F1) and TEAs, (2) the production of biogenic gases (mainly CH_4, CO_2, and hydrogen sulfide (H_2S)), (3) the production of metabolites from anaerobic biodegradation of BEX and F1, and (4) the microbial numbers (MPN). The initial conditions prior to incubation (Time 0 data) were determined immediately after nutrient and TEA amendment.

The headspace gases were sampled through the 1/16" stainless steel tubing automatically by a Varian CP-2003P portable Micro-GC approximately twice a month in the first three months of incubation, and then once a month until the end of incubation. Groundwater samples were taken approximately once a month via the 1/8" stainless steel tubing using a peristaltic pump or a syringe with custom-made fittings. With a few exceptions when water samples were stored at 4 °C for less than 3 days before the analysis, all water samples were prepared and analyzed immediately after the sampling, and therefore no preservatives were added. Groundwater-sediment slurry samples were taken from the non-sterile mesocosms (all mesocosms except the SCs) at Time 0, after 6 months of incubation (Day 193), and at the end of incubation (Day 620) for MPN and metabolite analyses. MPN analyses were conducted immediately after sampling, whereas metabolite samples were acidified to pH < 2 with 2 N sulfric acid (H_2SO_4) and sent to the Department of Biological Sciences at the University of Alberta for further analysis.

During the water and sediment sampling, N_2 gas was introduced into the headspace via the 1/16" tubing from a Tedlar® bag (Safety Instrument Ltd., Edmonton, Alberta) filled with N_2 gas to prevent negative pressures developing in the mesocosm due to sample withdrawal.

3.2.4.1 Headspace Gas Analysis

Anaerobic conditions and the production of biogenic gases were identified by the headspace gases analysis, including the quantitative measurements of N_2, oxygen (O_2), CO_2, and CH_4 as well as the qualitative analysis of H_2S. The Micro-GC used for the headspace gas analyses was equipped with TCD detectors and two columns: Column A (Mole Sieve, 10 m length, maximum temperature 180 °C) used for N_2, O_2, and CH_4 measurements and Column B (Hayesep, 0.25 m length, maximum temperature 160 °C) suitable for CO_2 and H_2S analyses. The Micro-GC methods were adopted from Luo (2004). The calibration curves are presented in Appendix A. As a simple way of verifying the calibration for each sampling event, the ambient air was analyzed using the Micro-GC before performing headspace gas analyses.

3.2.4.2 Groundwater Analysis

The groundwater samples were taken from the mesocosms regularly and analyzed for the chemical characteristics, with an emphasis on the depletion of PHCs, TEAs, and nutrients, as summarized in Table 3-2.

Table 3-2. Summary of groundwater chemical analyses.

Characteristics	Analytes	Methods
PHCs	BTEX	Purge-and-trap Gas Chromatography/Mass Spectrometry (GC/MS)
	F1	Purge-and-trap GC
TEAs	NO_3^-, SO_4^{2-},	Ion Chromatography (IC)
Nutrients	NH_4^+, PO_4^{3-}	IC
Others	pH	pH meter
	Alkalinity	Titration
	Major cations	IC
	Other anions	IC

Concentrations of BTEX were analyzed directly from the untreated samples using purge-and-trap and GC/MS based on the USEPA 8260B method. An AQUA Tek 70 liquid autosampler (Tekmar Dohrmann, Cincinnati, OH) was used to transfer the sample to a purge and trap sample concentrator (Velocity XPT^{TM} Sample Concentrator, Tekmar Dohrmann, Cincinnati, OH). The VOCARB 3000 trap installed in the concentrator was operated at a desorption temperature of 250°C and a desorption time of 4 min. Helium was used as the carrier gas with a purge flow of 40 mL/min and a purge time of 11 min. A Varian 3900 GC (Varian Canada Inc., Mississauga, Ontario) equipped with a Chrompack Capillary Column CP- select 624CB column (30m length, 0.32mm internal diameter, and 1.80μm film thickness) was used to separate the individual components. The split ratio was 200:1. The flow rate of the carrier gas (helium) was 1.2 mL/min. The oven temperature was held at 36°C for 4 min, then increased by 12°C per min to 150°C, and then increased by 50°C per min to 240°C and held for 30 sec. A Saturn 2100T MS detector (Varian Canada Inc., Mississauga, Ontario) acquired data in the selective ion storage (SIS) mode. A stock solution of fluorobenzene and 1,2- dichlorobenzene-D4 in methanol (USEPA Standard 524, Sigma-Aldrich Canada Ltd., Oakville, Ontario) was used as internal standards for calibration. Fluorobenzene was used to quantify the BTEX compounds.

The method used to measure F1 hydrocarbons was adopted from the analytical method described in CCME (2001), which was purge-and-trap with GC. The purge and trap instrument used for F1 analysis was a HP Purge and Trap Concentrator (Agilent Technologies Canada Inc., Mississauga, Ontario). However, the purge and trap parameters were similar to those described above for the BTEX analysis. The HP 6890 GC (Agilent Technologies Canada Inc., Mississauga, Ontario) used for F1 analysis was equipped with a DB-1 column (30m length, 0.53mm internal diameter, and 1.50μm film thickness) and a flame ionization detector (FID). The injector temperature was 200°C. The split ratio was 50:1. The flow rate of the carrier helium gas was 1.0 mL/min. The oven temperature was held at 36°C for 4 min, then increased by 5°C per min to 150°C, and then further increased by 15°C per min to 240°C and held for 1 min. The detector temperature was 250°C. The flow rates of H_2 gas and the combustion air were 35 mL/min and 350 mL/min, respectively. Three-point calibration curves were established using the standard solutions containing n-hexane, toluene, and n-decane (Sigma-Aldrich Canada Ltd., Oakville, Ontario). The average response factors of toluene were used to quantify the F1 hydrocarbons. The summary of average response factors of toluene and the calibration records are presented in Appendix A.

TEAs, nutrients and other major ions were measured by IC (USEPA Method 300.0). The DIONEX AS50 system (Dionex Corporation, Sunnyvale, California, United States) was equipped with GP50 Gradient Pump and CD25 conductivity detector. The analytical columns are IonPac® AS14A (4×250mm) for anion and IonPac® CS12A (4×250mm) for cations. The guard columns are IonPac® NG1 (4×35mm) followed by IonPac® AG14A (4×50mm) for anion analysis and IonPac® NG1 (4×35mm) followed by IonPac® CG12A (4×50mm) for cation analysis. The eluents for anions and cations were 8.0 Na_2CO_3 -1.0 mM $NaHCO_3$ and 11 mM H_2SO_4, respectively. The flow rates of both eluents were 1.0mL/min. The calibration standards were run for each batch of samples at five different levels (as summairzed in Appendix A). The calibration standards were prepared

using the Combined Seven Anion Standard II and Combined Six Cation Standard II solutions (Dionex Corporation, Sunnyvale, California, United States).

Alkalinity of each groundwater sample was analyzed by titration to an end point of pH 4.5 with 0.02 N H_2SO_4. An Orion 290 A^+ pH meter (Fisher Scientific Ltd., Nepean, Ontario) was used to measure the pH.

3.2.4.3 Microbial Enumeration and Metabolite Analysis

Three tube MPN methods (Cross *et al.* 2003) were used to enumerate SRB, NRB, IRB, and methanogens in all mesocosms except for the SCs. The recipes of the media are listed in Appendix B. To obtain the initial MPN results, slurry of groundwater and sediment was sampled from four randomly selected mesocosms and enumerated at Time 0. One mesocosm from each treatment was sampled on Day 193 to investigate the changes in MPN numbers. On Day 620, all non-sterile mesocosms were analyzed for the final MPN results. Metabolites were determined by extraction and derivatization followed by GC/MS analysis (Gieg and Suflita 2002). The details of the method are discussed in Chapter 5.

3.2.5 Quality Control

Due to limited sample sizes and due to limitations of the analytical instruments, samples could not be analyzed in duplicate. Some other quality control measures were taken to ensure the accuracy and precision of the analyses. For GC and IC analyses, standard curves were prepared with each batch of samples and checking standards were inserted in each sample sequence at appropriate intervals for quality assurance. Furthermore, a reproducibility test was conducted to determine the standard deviation of all chemical analyses, in which triplicate samples were taken from four randomly selected Site 1 mesocosms and then analyzed for all chemical parameters. The results of reproducibility tests indicated that the standard deviation of all chemical analyses were acceptable (data are summarized in Appendix C)

3.2.6 Decommissioning

At the end of the mesocosm study, all mesocosms were decommissioned for sediment characterization and groundwater analyses, in particular the dissolved redox-specific species. Mesocosms were placed upright under a glove bag that was flushed continuously with N_2 gas. After the mesocosm stopper was removed, DO was measured using a DO meter (YSI Model 50B). Dissolved sulfide (S^{2-}) and ferrous iron (Fe^{2+}) were measured using HACH methods 690 and 255 respectively. Approximately 1 L groundwater samples were taken from each mesocosm using a peristaltic pump, acidified with concentrated HNO_3 to pH < 1, and then stored at 4°C for future study. After pumping out and discarding the remaining groundwater, the sediment was poured into 20 centrifuge tubes, then centrifuged at 3600 rpm for 1 hour using a Heraeus Multrifuge® 3LR Centrifuge (Fisher Scientific Ltd., Nepean, Ontario), and frozen for further analysis. The sediment samples were prepared by microwave acid digestion (details in Appendix A) and then analyzed using inductively coupled plasma-mass spectrometry (ICP-MS) analysis. Concentrated nitric acid (HNO_3, 68-71%, Fisher Scientific Ltd., Nepean, Ontario) was used in the extraction. The microwave acid digestion was performed in the Milestone Ethos SEL Microwave Labstation (ATS Scientific Inc., Burlington, Ontario) following USEPA Method 3051. The digested samples were filtered through a 0.22 μm filter and analyzed using an ELAN 9000 ICP MS along with an ASX-510 autosampler (Agilent Technologies Canada Inc., Mississauga, Ontario). The ICP-MS method was adopted from USEPA Method 6020.

3.3 Results and discussion

Laboratory microcosms and sacrificial sampling have been commonly used to demonstrate the occurrence of anaerobic biodegradation at a contaminated site and to estimate the biodegradation rates. However, sample variability between small individual samples (<100 mL) hampered interpretation of experimental results (Biggar *et al.* 1998; Johns *et al.* 1999; Cross *et al.* 2003). To reduce analytical variability and improve interpretation of experimental results in this

laboratory study, larger volumes (>10 L in mesocosms) were used to facilitate sub-sampling from the same test system multiple times and for a relatively long experimental period.

Anaerobic conditions were maintained in all the mesocosms during the 620-day experimental period. Whereas the headspace O_2 was seemingly high at less than 1% (data shown in Table D-1, Appendix D), the occurence of sulfate reduction and methanogenesis at similar headspace O_2 levels was indicative of the anaerobic conditions. The seemingly high headspace O_2 was possibly caused by the method used to connect the mesocosm headspace and the Micro GC. DO concentrations measured in the groundwater during the decommissioning ranged from 0.1 to 0.3 mg/L in the mesocosms (Table D-7, Appendix D), also verifying that anaerobic conditions were maintained.

3.3.1 BEX and F1 Depletion

The initial concentrations were approximately 400 µg/L of benzene, 40 to 130 µg/L of ethylbenzene, 80 µg/L of m-, p-xylenes, and 2.4 mg/L of $F1_{-BEX}$ in non-sterile mesocosms (Tables D-2 and D-3, Appendix D). The concentrations of BEX and F1 hydrocarbons in the SCs were very low due to losses of volatile hydrocarbons during the autoclaving process. Although sterilized free product was used to spike the SCs, concentrations of the target compounds were still very low (approximately 5 µg/L of each BEX compound and 0.2 to 0.4 mg/L of F1, Tables D-2 and D-3, Appendix D). Therefore, the depletion of PHCs attributed to abiotic attenuation processes could not be identified from the SCs.

Major abiotic processes related to hydrocarbon removal may include adsorption to soil matrix and to the mesocosm inner walls and evaporation to the atmosphere. The sediment samples from the contaminated site had very low organic matter contents (< 0.3% by weight, analyzed by Natural Resources Analytical Lab, University of Alberta). Therefore, the adsorption of hydrocarbons was assumed to be negligible. In addition, it is unlikely that volatilization would significantly

contribute to BEX or F1 hydrocarbon losses. The mesocosms were well-sealed closed systems and the stoppers were always in contact with water in the mesocosm to further prevent any exposure to the atmosphere. Losses of dissolved BEX due to partitioning into the headspace were corrected using the headspace volume change and the respective Henry's Law constant (Sample calculations and the corrected BEX concentrations are presented in Appendix E). The corrections showed only negligible differences in the concentrations. It was thus assumed that the observed losses of BEX and F1 hydrocarbons in the mesocosms were due to microbial activity. Other lines of evidence were investigated to further verify the occurrence of anaerobic biodegradation in the non-sterile mesocosms.

The depletion of BEX and F1 over time in each non-sterile mesocosm was plotted in Figure D-1, Appendix D. At the end of the incubation time, almost complete removal of BEX was obtained in all non-sterile mesocosms except for Ctrl+NP(#8) mesocosm (Table 3-3). High percent removals of F1 (approximately 90%) were observed in the sulfate-amended mesocosms, compared to 22 to 56% in the controls and the nitrate-amended mesocosms, indicating that SO_4^{2-} amendment was more favorable for F1 degradation.

Table 3-3. Percentage removals of BEX and F1 in all Site 1 non-sterile mesocosms after 620-days incubation.

Notation	#	% Removal [a]			
		Benzene	Ethylbenzene	m-, p-Xylenes	F1
Ctrl	1	100	100	100	34
	7	98	99	98	48
Ctrl+NP	2	97	100	100	53
	8	65	16	78	37
NO3	3	89	100	100	56
	9	69	99	100	22
NO3+NP	4	99	100	99	35
	10	80	97	94	24
SO4	5	100	100	100	89
	11	99	100	100	88
SO4+NP	6	100	100	100	90
	12	100	100	100	93

a. Removals of dissolved BEX were based on the concentrations corrected for partitioning into headspace using Henry's Law Constant. F1 concentrations were not corrected.

3.3.2 Lines of evidence for anaerobic biodegradation of BEX

The anaerobic biodegradation of BEX can be coupled to different reducing conditions, using NO_3^-, Fe (III), Mn (IV), SO_4^{2-}, and CO_2 as TEAs, which will generate representative "footprints", i.e. the stoichiometric consumption of BEX and TEAs, and production of the byproducts. Fermentation coupled to methanogenesis is also a potential mechanism of anaerobic BEX biodegradation, which is a two-step process and each step shows different footprints.

Using ethylbenzene as an example, the footprints for different TEAPs are summarized in Table 3-4. For example, the footprint for oxidation of 1 mole of ethylbenzene coupled to nitrate reduction results in the consumption of 8.4 moles of NO_3^-, the generation of 8 moles of inorganic carbon, and the generation of 8.4 equivalents of alkalinity.

Table 3-4. Footprints of anaerobic PHC biodegradation in different TEAPs (using ethylbenzene for illustration).

TEAPs	Stoichiometry of anaerobic biodegradation processes
Nitrate reduction	$C_8H_{10} + 8.4\ NO_3^- + 8.4\ H^+ \rightarrow 8\ H_2CO_3 + 4.2\ N_2 + 1.2\ H_2O$
Sulfate reduction	$C_8H_{10} + 5.25\ SO_4^{2-} + 10.5\ H^+ + 3\ H_2O \rightarrow 8\ H_2CO_3 + 5.25\ H_2S$
Iron reduction	$C_8H_{10} + 42\ FeOOH_{(s)} + 84\ H^+ \rightarrow 8\ H_2CO_3 + 42\ Fe^{2+} + 60\ H_2O$
Fermentation	$C_8H_{10} + 13\ H_2O \rightarrow 2\ CH_3COOH + 4\ H_2CO_3 + 26\ H_2$
Methanogenesis	$CH_3COOH + H_2O \rightarrow CH_4 + H_2CO_3$
	$10\ H_2 + 2.5\ H_2CO_3 \rightarrow 2.5\ CH_4 + 7.5\ H_2O$

Examples of changes in NO_3^- and SO_4^{2-} concentrations in respective TEA-amended mesocosms compared to SCs are shown in Figure 3-2. Rapid depletion of NO_3^- was observed in NO3 and NO3+NP mesocosms (jointly referred to as NO3(+NP) herein); and SO_4^{2-} was rapidly depleted in SO4 and SO4+NP mesocosms (jointly referred to as SO4(+NP) herein) as compared to the SCs. NO_3^- was re-amended to NO3(+NP) mesocosms on the 124th day and on the 497th day of incubation. SO_4^{2-} was re-amended to all SO4(+NP) mesocosms on the 265th day of incubation.

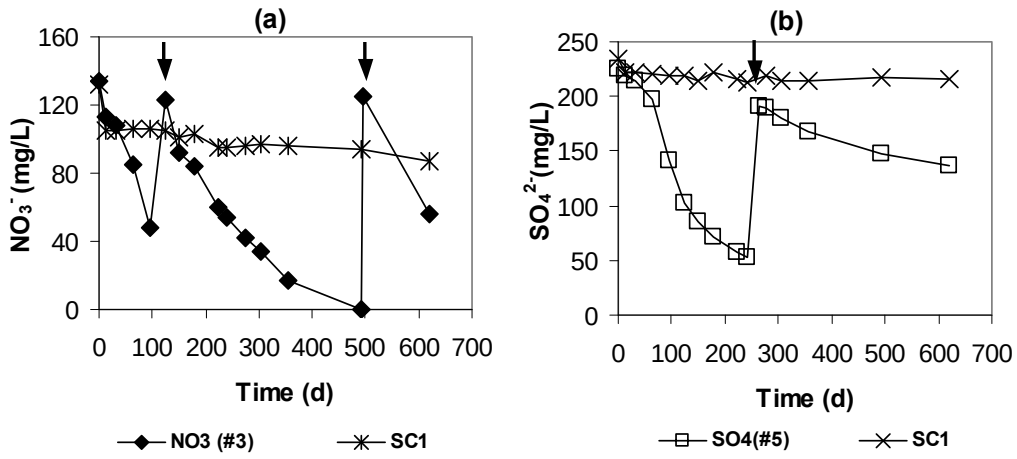

Figure 3-2. Example of depletion of NO_3^- (Panel (a)) and SO_4^{2-} (Panel (b)) with time in TEA-amended mesocosms compared to SCs (where ↓ indicates the day when NO_3^- or SO_4^{2-} was re-amended).

The rapid depletion of TEAs indicated that removal of BEX and F1 might be coupled to nitrate- or sulfate-reduction in these mesocosms. However, limitations in analytical methods and complex geochemical conditions in the testing systems made it difficult to identify the reduced products of these TEAPs and to further determine the stoichiometry.

In SO4(+NP) mesocosms, the smell of H_2S was evident during some sampling events and black precipitate was observed on the inner wall of the mesocosms and on the stainless steel tubing, indicating a possible production of sulfides. However, headspace H_2S data from the Micro GC were inconclusive.

The US-EPA BIOSCREEN Model suggests TEA utilization factors of 4.9 mg NO_3^-, 4.7 mg SO_4^{2-}, and 21.8 mg Fe^{3+} per mg BTEX biodegraded. Assuming the same utilization factors for F1 and applying these factors to the current study, the estimated TEA utilization coupled to degradation of BEX and F1 only accounted for a small portion of TEA depletion (approximately 2 to 4% for NO_3^- and 3 to 7% for SO_4^{2-}). Possible explanations for this include the anaerobic biodegradation of other PHCs or non-PHC carbon sources in the groundwater that consumed TEAs, or abiotic processes that led to TEA reduction. However, there was no

decrease in SO_4^{2-} and only a slight decrease in NO_3^- concentrations in the SCs, suggesting no abiotic loss of TEAs. DOC analyses of the groundwater samples showed that other organic carbon sources were present (Figure 3-3). In addition, methanol added into some of the mesocosms was also a ready carbon source.

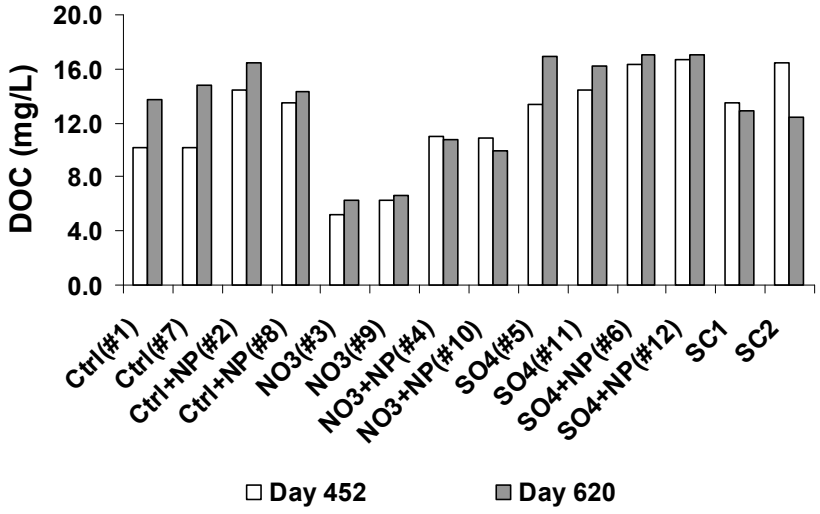

Figure 3-3. DOC concentrations in Site 1 mesocosms measured on Day 452 and Day 620.

Iron reduction also appeared to be a significant TEAP in the non-sterile mesocosms. Dissolved Fe^{2+} analysis during the mesocosm decommissioning showed elevated concentrations in Ctrl(+NP) and SO4(+NP) mesocosms (ranging from 13 to 18 mg/L) compared to SCs (1 mg/L). Sediment characterization at the end of the study also showed abundant iron in the sediment (20 mg Fe/g sediment), suggesting that iron could possibly be an alternative TEA at the site. Although only slight increases in the dissolved Fe^{2+} concentrations (2 to 4 mg/L) were observed in NO3(+NP) mesocosms, the occurrence of iron reduction in these mesocosms could not be completely precluded. It was possible that the reduced iron was oxidized by NO_3^- and thus could not be detected. Previous studies have also demonstrated the anaerobic, nitrate-dependent microbial oxidation of the reduced iron (Straub *et al.* 1996; Weber *et al.* 2001). Lack of monitoring data of reduced iron species over time during the tests limits the verification and interpretation of the iron reduction processes.

CH_4 production was observed in four of the mesocosms that received methanol (Figure 3-4), indicating that methoanogenic conditions occurred in these mesocosms. The highest headspace CH_4 concentration was 10% v/v in Ctrl(#7) which received 4 mL of methanol. The three other mesocosms (Ctrl+NP(#8), SO4(#11), and SO4+NP(#12), each received 1 mL of methanol) showed a maximum of 3 to 5% v/v headspace CH_4.

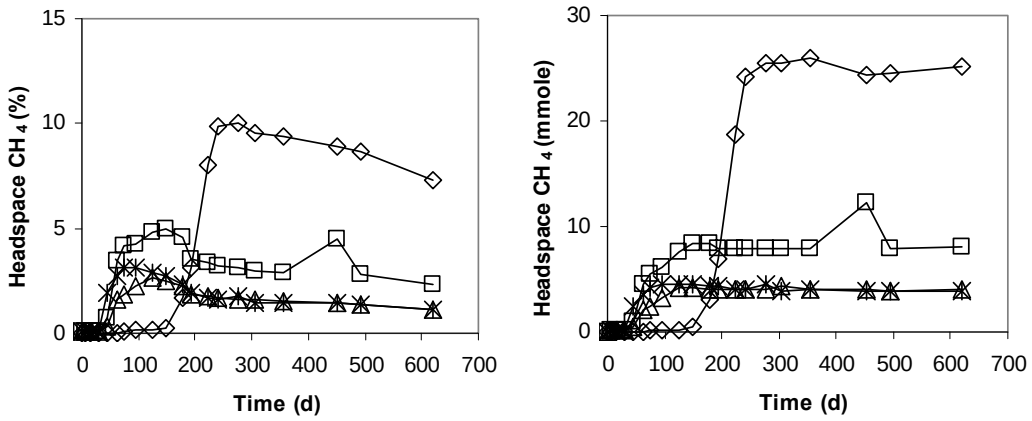

Figure 3-4. The concentrations and cumulative amounts of headspace CH_4 produced in Ctrl(#7) (◊),Ctrl+NP(#8) (□), SO4(#11) (Δ), and SO4+NP(#12) (✳) mesocosms.

Methanol addition apparently induced methanogenesis in these mesocosms. Methanol can be directly used by methylotrophic methanogens to produce CH_4 and CO_2. Or alternatively, in the presence of CO_2, acetogenic bacteria can ferment methanol to acetate, which can then be cleaved to CH_4 and CO_2 by acetoclastic methanogens (Weijma and Stams 2001). In this mesocosm study, IC results indicated a temporary accumulation of acetate (not quantified) before the headspace CH_4 concentrations reached the highest levels in those mesocosms. Carbon isotopic fractionations of CH_4 may help identify the substrate for methanogens because methanol-grown methanogens show higher carbon isotope enrichment factors (~70‰) than hydrogenotrophic methanogens (~40‰) (Oremland *et al.* 1982).

Methanogenesis did not appear to occur in other non-sterile mesocosms, including the three nitrate-amended meosocosms which received 1 mL of methanol. The higher redox potential under nitrate-reducing conditions might have inhibited the methanogens in these mesocosms. Nitrogen oxides were shown to reduce methanogenesis in salt marsh sediments, lake sediments, and waterlogged soils, possibly due to substrate competition, redox changes, or enzyme poisoning (Westermann and Ahring 1987).

Production of biogenic CO_2 and alkalinity are common indicators of the occurrence of biodegradation processes. However, it was very difficult to quantify the biogenic CO_2 and alkalinity evolved in the mesocosms due to the sub-sampling method and the possible geochemical reactions, including partitioning of CO_2 between the water and the headspace, and the possible dissolution/precipitation of carbonate minerals. The cumulative amount of headspace CO_2 and alkalinity (in the form of HCO_3^-) in the non-sterile mesocosms compared with SCs are illustrated in Figure 3-5. The headspace CO_2 amounts in all mesocosms are calculated based on the estimated headspace volumes and the measured CO_2 concentrations and are presented in Appendix E1.

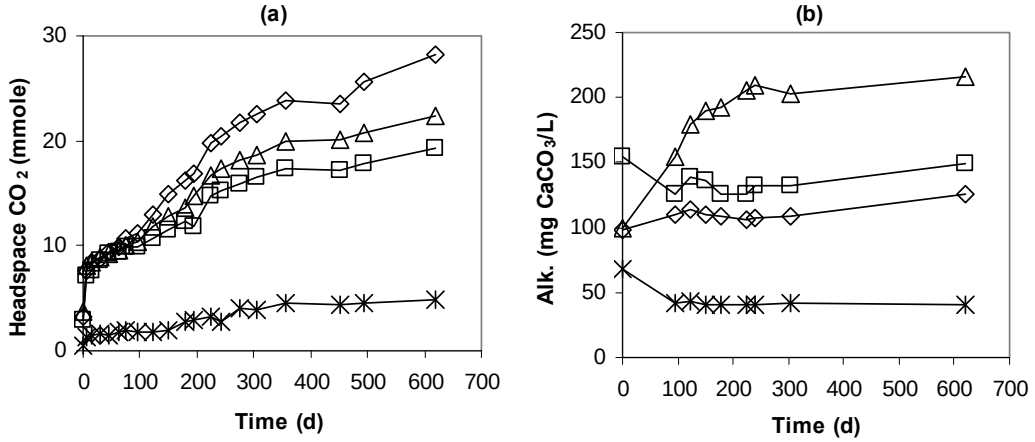

Figure 3-5. Examples of changes in headspace CO_2 amount (Panel (a)) and alkalinity (as mg/L $CaCO_3$, Panel (b)) with time in Ctrl(#2) (□), NO3(#3) (◊), SO4(#5) (Δ), and SC1 (✳) mesocosms.

The initial headspace CO_2 and alkalinity in the SCs were lower than those in other mesocosms, possibly due to the removal of dissolved CO_2 from the groundwater during autoclaving. In all the mesocosms amended with nutrients, although PO_4^{3-} might serve as a pH buffer and interfere with the alkalinity measurement, the trends of alkalinity over time were similar to those in the mesocosms without nutrient addition. Combining the accumulation of CO_2 and alkalinity, the results suggest that anaerobic biodegradation processes were occurring in the non-sterile mesocosms.

The MPN results in the non-sterile mesocosms are summarized in Tables F1-1 and F1-2, Appendix F1. The changes of MPNs with time in the representative mesocosms are shown in Figure F1-1, Appendix F1. The method used to test the significance of the difference between two MPNs is described in Appendix F2. The values of the calculated test statistic are also provided in Appendix F2.

It was assumed that the Time 0 MPN results in all non-sterile mesocosms were consistent. Therefore, four randomly selected mesocosms were analyzed to obtain the initial microbial densities. The MPNs of the redox-specific bacteria in the test mesocosms are shown in Table 3-5.

Table 3-5. MPN results of SRB, NRB, IRB and methanogens at Time 0 (MPN/L).

Mesocosm #	SRB	NRB	IRB	Methanogens
Ctrl+NP(#2)	9.3E+01	2.4E+05	9.3E+04	BDL
NO3(#3)	1.5E+00	2.1E+04	1.5E+05	BDL
Ctrl(#7)	7.0E-01	2.4E+05	4.3E+04	BDL
SO4(#11)	1.5E+00	2.4E+05	4.3E+05	BDL

Using the statistical method described in Cochran (1950), SRBs in Ctrl+NP(#2) and NRBs in NO3(#3) were significantly different from other three test mesocosms at 95% confidence level (results in Table F2-1, Appendix F2). Thus,

there was some variability in the initial MPN results in the testing mesocosm. This variability could be introduced by the mesocosm setup (although all sediment samples were homogenized prior to the setup) or by the MPN technique. There are two principal assumptions for the MPN technique: (1) the organisms are distributed randomly throughout the liquid, and (2) each sample from the liquid is certain to exhibit growth if it contains one organism when incubated in the culture medium (Cochran 1950). The MPN analysis was done on slurry of groundwater and sediment in this study. Thus, if there were clusters of bacteria in the samples, the MPN results might be underestimated.

At Time 0, methanogens were not detected and the MPNs of SRBs were several orders of magnitude lower than those of NRBs and IRBs, suggesting that methanogenesis and the sulfate reduction might not be significant at Site 1.

It is assumed that the changes in microbial population density can be used to assess whether microbial populations are responsible for observed degradation. However, during the 620-day incubation there appeared to be no significant growth of the redox-specific bacteria in the respective mesocosms (Figure F1-1, Appendix F1), although other lines of evidence corroborated that anaerobic biodegradation was occurring in the non-sterile mesocosms. Due to the fact that the relatively low substrate concentrations in the testing mesocosms may not support the growth of bacteria, the MPN results of the redox-specific bacteria may not be correlated with the TEAPs.

MPN technique is biased toward culturable organisms and may also be limited by the growth conditions defined by the choice of medium, substrate, and incubation conditions (Christensen *et al.* 2000). Some organisms are able to use several TEAs and may contribute to the number of bacteria in several redox groups. Therefore, caution should be taken when using the MPN technique because of the inherent limitations of this method. However, MPNs may still provide useful

information regarding the *in-situ* biodegradation processes in that it could confirm the presence of microbial potential for specific microbial redox processes.

In summary, the mesocosms contained multiple phases (sediment, water and headspace gases) and therefore complex physical and geochemical processes may interfere with the identification and interpretation of the biodegradation processes. However, multiple lines of evidence verified that anaerobic biodegradation of hydrocarbons was occurring coupled to respective TEAPs in the mesocosms.

3.3.3 Biodegradation Kinetics and Enhancement Effects

The biodegradation rates were estimated using the concentrations of individual BEX compounds, which were corrected for losses due to partitioning into the headspace. Considering the low substrate concentrations and no increase in MPNs, the first-order kinetics model was used to estimate the biodegradation rates. The linear regression function of Microsoft Excel was used to calcuate the first-order biodegrdation rates.

There was certain length of time (lag period) before the onset of anaerobic biodegradation of benzene and m-, p-xylenes in some mesocosms. Figure 3-5 shows examples of lag periods in the SO4(#6) mesocosm.

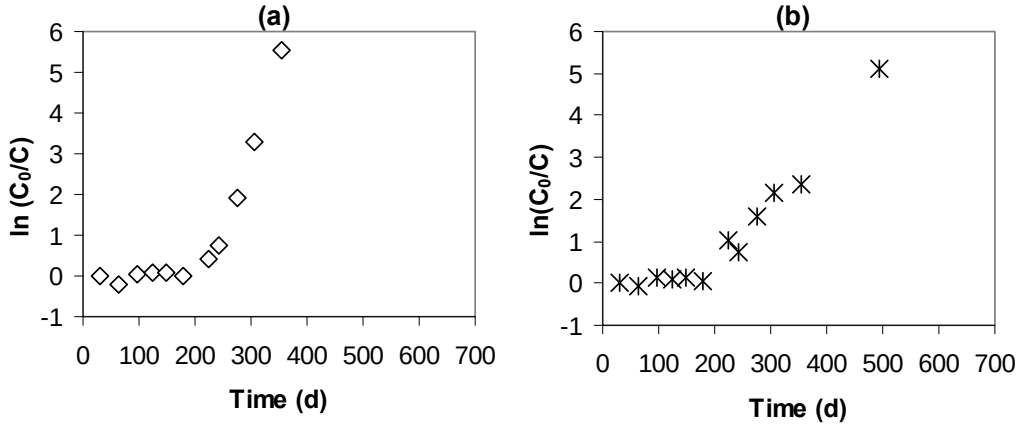

Figure 3-6. First-order anaerobic biodegradation of benzene (Panel (a)) and m-, and p-xylenes (Panel (b)) in SO4(#6) mesocosm.

The observed lag periods for BEX and $F1_{-BEX}$ in all mesocosms are summarized in Table 3-6. Since in-house Time 0 BEX results were missing due to instrumentation problem, when no obvious delay was observed in the biodegradation processes, the lag period was reported as less than 31 days (≤31) when the first BEX analysis was conducted. The lag periods ranged from less than 31 days to as long as 355 days.

Table 3-6. Lag periods before the onset of the anaerobic biodegradation of BEX and $F1_{-BEX}$ (Unit: day).

Treatments	#	Benzene	Ethylbenzene	m-,p-Xylenes	$F1_{-BEX}$
Ctrl	**1**	223	≤31	≤31	≤31
	7	223	≤31	355	≤31
Ctrl+NP	**2**	≤31	≤31	≤31	≤31
	8	≤31	- [a]	≤31	≤31
NO3	**3**	≤31	≤31	≤31	≤31
	9	≤31	≤31	124	≤31
NO3+NP	**4**	179	≤31	≤31	≤31
	10	179	≤31	≤31	≤31
SO4	**5**	179	≤31	≤31	≤31
	11	179	≤31	≤31	≤31
SO4+NP	**6**	179	≤31	179	≤31
	12	179	≤31	179	≤31

a: No obvious biodegradation.

The estimated first-order rates ranged from 0.0032 to 0.033 d^{-1} for benzene, 0 to 0.028 d^{-1} for ethylbenzene, 0.0021 to 0.036 d^{-1} for m-, and p-xylenes, and 0.0006 to 0.0045 d^{-1} for $F1_{-BEX}$ (Figure 3-7).

Large discrepancy was observed between some mesocosms with the same treatment, possibly due to the undesired variability introduced during mesocosm setup, especially the methanol addition to some mesocosms.

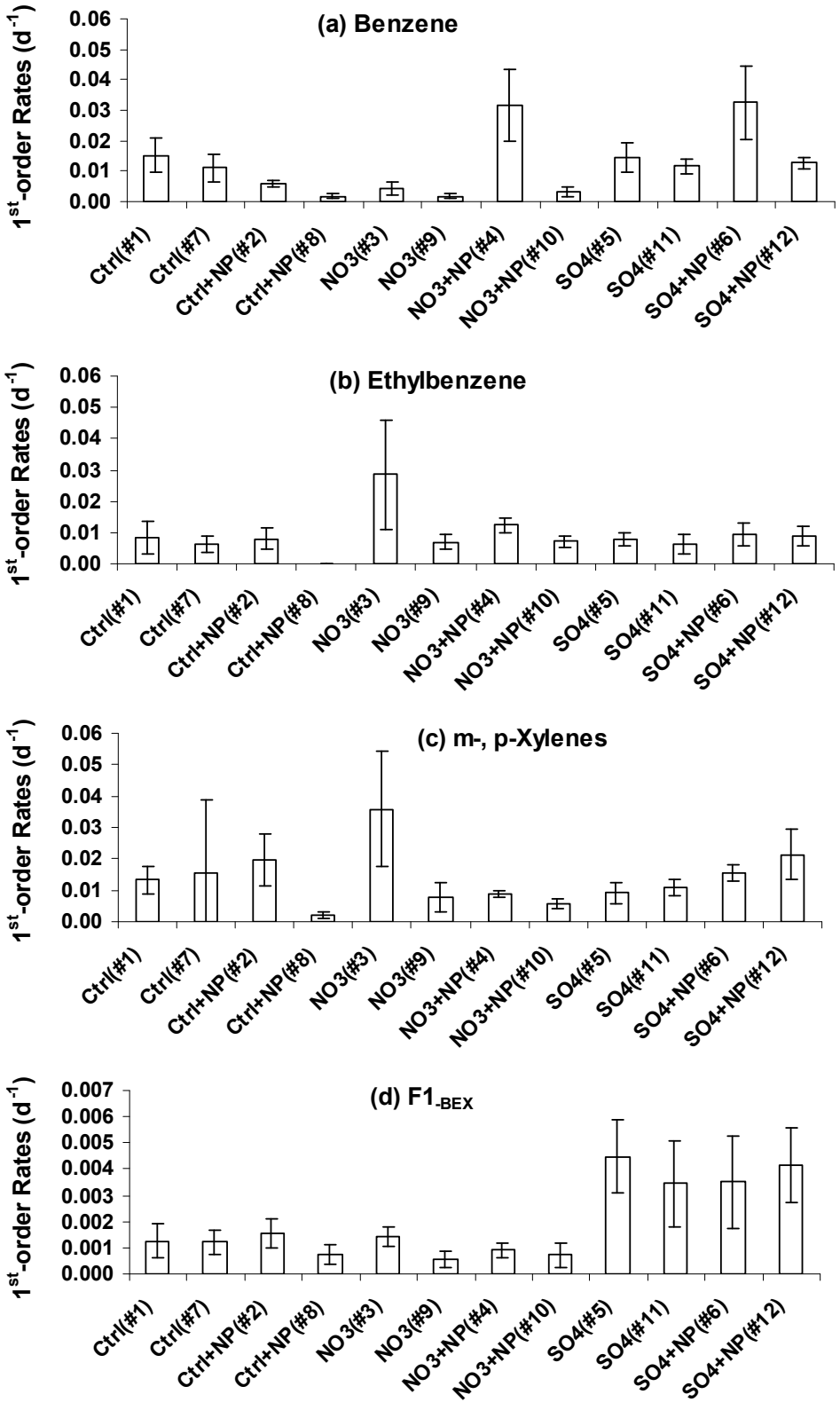

Figure 3-7. The estimated first-order anaerobic biodegradation rates for BEX and $F1_{-BEX}$ (Where error bars represent 95% confidence intervals).

The estimated *in-situ* attenuation rates were of the order of 10^{-4} d^{-1} (Armstrong 2006, personal communication). The mesocosm study was conducted at 15°C, approximately 10°C higher than the field temperature. Appling a Q10 effect of 2 to the field rates, the average first-order laboratory biodegradation rates in the Ctrl(+NP) mesocoms were much higher than the estimated *in-situ* attenuation rates. As discussed previously, iron reduction seemed to be a predominant TEAP in all control mesocosms. The homogenization of sediment before mesocosm setup and weekly mixing during the incubation might have increased the bioavailability of Fe(III) and enhanced the activity of IRBs. The BEX biodegradation rates under other treatment conditions were also one or two orders of magnitude higher than the estimated field rates.

When compared with the published first-order biodegradation rates from other field and laboratory studies (summarized in Table 2-4), it appears that the degradation rates of ethylbenzene and m- and p-xylenes from the mesocosms were on the low side of the reported values, possibly because of the site specificity.

The biodegradation rates for specific PHC compounds are influenced by relative biodegradability, substrate interactions, predominant TEAPs, the availability of TEAs and nutrients, and other site-specific conditions. Many other variables and confounding factors may also affect the biodegradation rates obtained from laboratory studies, including the procedure and methods for sampling field materials, setup of the mesocosms, incubation conditions, and the length of the study period (Aronson and Howard 1997). Although it is generally recognized that laboratory studies often result in higher rates of biodegradation than field studies, the mixing of a natural sample during its collection or during the construction of a microcosm may lead to either an increase (Davis and Olsen 1990) or decrease (Weiner and Lovley 1998) in the microbial activity of the sample. Therefore, caution should be taken when applying the first-order

biodegradation rates from this mesocosm study to predict *in-situ* contaminant removal.

Theoretically, biodegradation rates could also be determined based on the consumption of TEAs and the generation of products over time and the rates should be normalized by the stoichiometry (example equations shown in Table 3-4). As discussed previously, due to the complexity of the geochemical conditions and the presence of other organic carbon in the mesocosms (as is the case of the *in-situ* field conditions), it is impossible to estimate the biodegradation rates of target PHC compounds based on the TEA depletion. However, it is still important to understand the kinetics of the TEA reduction, which may lead to a better estimate of the *in-situ* "effective attenuation capacity" and the possible enhancment effects by TEA amendment.

The estimated first-order TEA consumption rates were also calculated using the linear regression in Microsoft Excel spreasheet, and are presented in Figure 3-8. Initially, very high TEA reduction rates were observed in the mesocosms to which methanol was added (#9, #4, and #10 in Panel (a); #11 and #12 in Panel (b), Figure 3-8). The first-order TEA reduction rates decreased to similar levels after re-amendment of respective TEAs. Thus, methanol might have contributed to the initial TEA depletion.

Methanol can be readily biodegraded under various reducing conditions. Mormile *et al.* (1994) found complete methanol biodegradation coupled to nitrate reduction. Methanol can also be biodegraded in a way where methanogens, SRB, and acetogenic bacteria interact cooperatively or competitively (Weijma and Stams 2001). Some suggest that when methanogens were inhibited, SRB could metabolize methanol (Puhakka *et al.* 1989); while Westermann and Ahring (1987) found that methanol could stimulate both sulfate reduction and methane production.

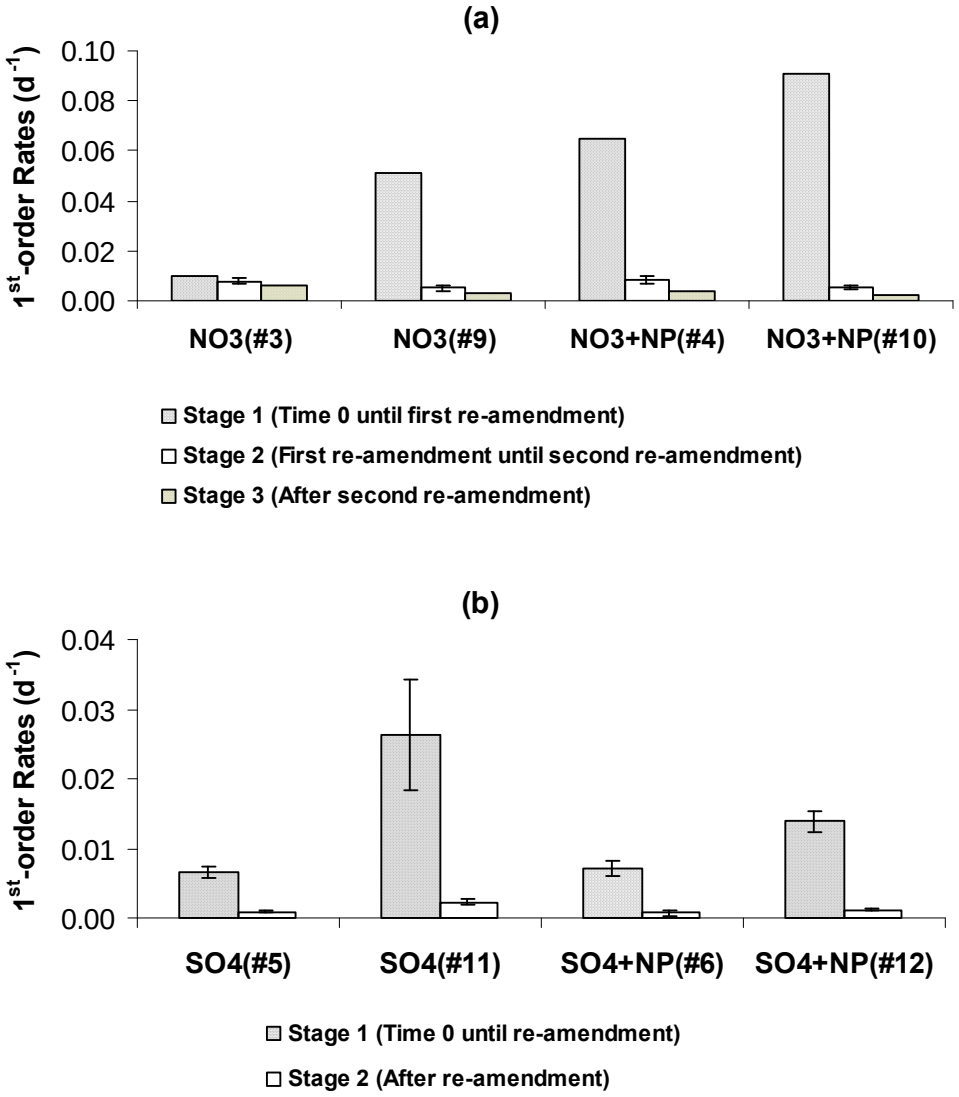

Figure 3-8. Estimated first-order TEA reduction rates of NO_3^- (Panel (a)) and SO_4^{2-} (Panel (b)) in respective mesocosms (Error bars represent 95% confidence intervals).

The added methanol could affect the anaerobic biodegradation of target PHCs with different mechanisms. The preferential utilization of methanol may lead to a depletion of the TEAs and nutrients which would otherwise be available for the degradation of BTEX or other hydrocarbons. As previously discussed in the headspace gas production, methanol addition resulted in methanogenic conditions in unamended and sulfate-amended mesocosms, but not in nitrate-amended mesocosms. The shift of predominant TEAPs may affect the biodegradation of specific contaminants. Methanol addition may stimulate the growth of

microorganisms, and a large microbial population may subsequently enhance the hydrocarbon degradation. In this study, methanol addition appeared to delay or inhibit the BEX or F1 degradation to certain extents in some mesocosms, as shown in Table 3-7 and in Figure 3-7.

It was anticipated that the effect of TEA and/or nutrient amendment on biodegradation could be determined qualitatively and quantitatively by comparing the amended with the unamended mesocosms and by comparing the laboratory and *in-situ* biodegradation rates. Higher biodegradation rates were obtained in both the unamended and the amended mesocosms under the laboratory-controlled conditions, indicating that enhancement of PHC biodegradation could be achieved by increasing the available TEAs (i.e. direct NO_3^- or SO_4^{2-} amendment, or indirect increase in the bioavailability of Fe(III) as in the case of the unamended controls). It can be seen from Figure 3-7 that SO_4^{2-} amendment significantly stimulated degradation of $F1_{-BEX}$, but there was no apparent enhancement effects of NO_3^- or SO_4^{2-} amendment on BEX biodegradation compared to unamended controls, in which iron reduction was probably dominating. Due to the methanol addition and other variability within the mesocosms, the quantification of enhancement effects by TEA amendment was difficult to achieve.

Although there was no apparent enhancement effect of nutrient amendment on the anaerobic biodegradation processes, decreases in PO_4^{3-} and NH_4^+ concentrations were observed in all nutrient-amended mesocosms, including the SCs (data in Appendix D). The loss of PO_4^{3-} might be caused by adsorption, surface complexation and/or precipitation or other reactions (Appelo and Postma 2005). Cation exchange with the sediment may cause a decrease in NH_4^+ concentration. Transport of NH_4^+ in groundwater aquifer is often retarded by cation excation, due to the fact that NH_4^+ has stronger affinity for negatively charged clay and organic matter particles than most other common cations (Erskine 2000). The selective sequence derived by Appelo and Postma (2005) is: $Li^+ < Na^+ < Mg^{2+} < Ca^{2+} < NH_4^+ < K^+$. Corresponding increases in the aqueous sodium concentration

in the nutrient-amended mesocosms (data in Appendix D) appeared to support the occurence of cation exchange.

3.4 Conclusions

The designed mesocosms were determined to be effective for anaerobic biodegradation studies. Anaerobic conditions were obtained during the mesocosm setup and maintained throughout the 620-day laboratory mesocosm study. The relatively large volume enables the monitoring of multiple lines of evidence throughout a long study period. Sub-sampling method allows the monitoring of the biodegradation processes with time in the same mesocosm. The biodegradation of PHCs is determined from one mesocosm, compared to numerous microcosms, which reduce the variability between the replicates in the microcosm studies.

The mesocosm represents a very complex system with a variety of biogeochemical processes occurring simultaneously (similar to the *in situ* conditions to some extent), which adds some variablity to the results and complexity to the data interpretation. Furthermore, variability was introduced into the mesocosm study due to the heterogeneity of the sediments and the methanol addition. A better method to homogenize the sediment materials should be investigated.

Multiple lines of evidence positively indicated that anaerobic biodegradation of BEX and F1 was occurring coupled to nitrate reduction, sulfate reduction and iron reduction in the respective mesocosms. Methanogenesis occurred in unamended and sulfate-amended mesocosms with added methanol. It can be seen that the shifts between TEAPs or co-existence of different TEAPs were possibly occurring in some mesocosms. Undesired variability introduced by the methanol addition affected the biodegradation of target PHC compounds and interfered with the interpretation of the biodegradation data.

The first-order biodegradation rates estimated from depletion of PHCs over time ranged from 0.0032 to 0.033 d^{-1} for benzene, 0 to 0.028 d^{-1} for ethylbenzene, 0.0021 to 0.036 d^{-1} for m-, and p-xylenes, and 0.0006 to 0.0045 d^{-1} for $F1_{-BEX}$, which were higher than the estimated *in situ* attenuation rates of approximately 10^{-4} d^{-1}, but comparable to values reported in the literature.

The first-order TEA reduction rates were also calculated to better understand the kinetics of the TEA depletion. In addition to the anaerobic biodegradation of the target PHCs, some geochemical processes and the anaerobic biodegradation of other carbon sources existing in the groundwater likely also contributed to the TEA reduction. These processes should be further investigated to better understand and to more precisely predict the effective attenuation capacity of amended TEAs.

Sulfate amendment enhanced the biodegradation of $F1_{-BEX}$, but enhanced biodegradation of BEX was not observed in nitrate- or sulfate-amended mesocosms compared to unamended controls. It is postulated that significant iron reduction driven biodegradation occurred in the controls, which could not be quantified. Due to the variability within mesocosms and limitations associated with the analyses, it is difficult to quantify the enhancement effects of TEA amendment. Nutrient amendment showed no apparent enhancement effects. One possible explaination was that the low substrate concentrations could not support significant microbial growth. It was also possible that the *in situ* conditions might not be nutrient limited at Site 1.

The calculated rates and other information obtained from this laboratory study may not be directly used to predict the *in-situ* biodegradation processes and the enhancement effects. However, the results indicate a potential for enhanced anaerobic biodegradation in the PHC contaminated groundwater and sediment at Site1. Further work is needed before a final decision can be made on whether EA will be a viable remedial option for this site. It should be emphasized that the *in*

situ anaerobic biodegradation of PHCs is a more complex system with multiple processes occurring simultaneously. Consequently simple addition of one or more TEAs to attempt to enhance biodegradtion will likely have unpredictable and unintended consequences.

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Chapter 4. Anaerobic PHC Biodegradation Enhanced by TEA and Nutrient Amendment in Natural Gas Condensate Contaminated Groundwater

4.1 Introduction

Natural gas processing is a major industry in Alberta, Canada. Alberta produces approximately 5 trillion cubic feet of natural gas annually, accounting for over 80% of Canada's total natural gas production (Alberta Energy 2009). Natural gas condensate refers to the low-density mixture of liquid phase hydrocarbons separated from natural gas at atmospheric pressure and ambient temperatures. The condensates are composed primarily of aliphatic hydrocarbons, but may also contain substantial amounts of benzene, toluene, ethylbenzene and xylenes (BTEX) (Williams *et al.* 2006). Hawthorne and Miller (1998) reported that samples of condensates collected from sites in Alberta contained hydrocarbons in the range of C_5 to C_{16}, among which BTEX concentrations represented 15.0% of the total condensate weight. Subsurface contamination may occur during the processing, transportation, and distribution of the natural gas products. Groundwater contamination caused by natural gas condensate may represent a significant health and environmental risk.

MNA is considered a cost-effective approach for remediation of PHC contamination in the subsurface environment. The use of MNA for the remediation of contaminated sites at which BTEX are the sole contaminants of concern has been accepted as an adequate approach to address the remediation concerns at certain sites (USEPA 2001). However, under unfavorable site conditions, MNA may not be a viable treatment option and some intervention (i.e. enhancements) should be applied to stimulate the NA processes so that the remedial goals can be achieved within a reasonable timeframem (ITRC 2008).

Previous studies have shown that biodegradation is the key mechanism of NA (Suarez and Rifai 1999) and enhanced anaerobic biodegradation of PHCs could

be achieved by directly amending with TEAs (such as nitrate (NO_3^-) and/or sulfate (SO_4^{2-})) (Cunningham *et al.* 2000; Cunningham *et al.* 2001; Tang *et al.* 2005), or by indirectly increasing the bioavailability of TEAs, for instance, using chelating agents to increase bioavailable iron (Fe(III)) (Lovley *et al.* 1994; 1996).

The Consortium for Research on Natural Attenuation (CORONA) was a joint university, industry, and government research program to evaluate NA as a viable remedial alternative for PHC contamination at upstream oil- and gas-contaminated sites in Alberta, Canada. As part of the CORONA project, two contaminated sites (Site 1 and Site 3) were selected for laboratory studies to investigate the potential of enhanced attenuation of PHCs in contaminated groundwater. The overall objective of these studies was to better understand the anaerobic biodegradation processes after TEA and/or nutrient amendment, the dynamics of different TEAPs, and the enhancement effects on anaerobic PHC biodegradation.

This chapter presents the second laboratory study into the enhancement effects of TEA and/or nutrient amendment on anaerobic biodegradation of PHCs in condensate-contaminated groundwater at Site 3. In this study, the primary PHCs of concern were BTEX and CCME F1 fraction hydrocarbons (F1, C_6 to C_{10} hydrocarbons measured by the CCME method). TEA amended was NO_3^- or SO_4^{2-} and nutrients were amended in the form of NH_4^+ and PO_4^{3-}. The 722-day laboratory mesocosm study was conducted in the Department of Civil & Environmental Engineering at University of Alberta. The objectives of this study are to further apply the developed mesocosm system to the anaerobic biodegradation study, to investigate the enhancement effects of TEA and/or nutrients amendment on the anaerobic biodegradation of the target PHCs, and to calculate the enhanced anaerobic biodegradation rates.

4.2 Methodology

4.2.1 Site Description

The contaminated site in this laboratory mesocosm study, Site 3, is located at an active gas processing facility in southeast Alberta, where the condensate used for fire training was placed in an unlined pit since 1970s and caused groundwater contamination.

Groundwater monitoring data verified that the dissolved-phase organic plume consisted largely of C_3 to C_{10} PHCs, the bulk of which was composed of BTEX. At the contaminated site, the background SO_4^{2-} distribution varied both temporally and spatially, at concentrations ranging from 1,200 to 4,180 mg/L. Background dissolved iron varied from 0.1 to 7.89 mg/L. Field-measured dissolved oxygen (DO) ranged from 0.4 to 1.3 mg/L, and total alkalinity ranged from 392 to 736 mg/L. NO_3^- was generally present in very low concentrations (less than 90 μg/L) in the background wells (Petersmeyer 2006).

Previous site investigations have indicated that, despite the relatively low groundwater temperatures (5 to 10°C), anaerobic PHC degradation is occurring at the site. The estimated first-order attenuation rates were 0.0008 d^{-1} for benzene, 0.0011 d^{-1} for toluene, 0.0002 d^{-1} for ethylbenzene, and 0.0003 d^{-1} for xylenes, based on the depletion of BTEX with time in one monitoring well (Armstrong 2008). Based on the concentrations of the redox-sensitive species in the background and within the plume, aerobic respiration, nitrate reduction, and methanogenesis were not significant at the site, whereas sulfate reduction appeared to be a significant TEAP with iron reduction likely also occurring (Petersmeyer 2006).

4.2.2 Experimental Methods

Anaerobic groundwater and sediment samples were collected from the groundwater monitoring wells at Site 3 following the procedure previously described in Chapter 3. The laboratory mesocosm study was then set up in the

Department of Civil & Environmental Engineering at University of Alberta and carried out for 722 days. The mesocosm configuration, the method of mesocosm setup, the experimental design, and methods of subsequent mesocosm sampling and analyses were adopted from Site 1 mesocosm study and have been described in details in Chapter 3. The experimental matrix and amendment concentrations are summarized in Table 4-1. As with the Site 1 mesocosm study, the NO_3^- and nutrients (NH_4^+ and PO_4^{3-}) were amended at 2 mM and 3 mM, respectively. However, considering the higher SO_4^{2-} background levels at Site 3, 10 mM was chosen as the amendment SO_4^{2-} level in this mesocosm study.

Table 4-1. Experimental matrix and amendment concentrations in Site 3 mesocosm study.

TEAs and Nutrients (mM)	Treatments						
	SC	**Ctrl**	**Ctrl+NP**	**NO3**	**NO3+NP**	**SO4**	**SO4+NP**
NO^{3-}	2	-	-	2	2	-	-
SO_4^{2-}	10	-	-	-	-	10	10
NH_4^+, PO_4^{3-}	3	-	3	-	3	-	3

The methodology for the mesocosm incubation and monitoring is briefly summarized herein. All mesocosms were incubated in the horizontal position at 15°C in the dark for 722 days and mixed weekly by slowly rotating them. Mesocosms were sub-sampled at pre-determined time intervals for chemical and biological analyses. Headspace gases (namely N_2, O_2, CO_2, CH_4, and H_2S) were automatically taken through the 1/16" sampling tubing and measured by Micro-GC (in triplicate). BTEX and F1 in the groundwater were sampled through the 1/8" tubing using a peristaltic pump and analyzed respectively with GC-MS and GC-FID. Due to the high PHC concentrations in the groundwater, BTEX and F1 samples were diluted and therefore, analyzed in duplicate in most cases. TEAs and other major ions present in the groundwater were measured by IC. Three-tube MPN and metabolite analyses were conducted on Day 0, Day 197, and Day 722. Mesocosms were dismantled after the end of incubation. Sediments were

characterized by Microwave Acid Digestion and ICP-MS analysis. DO and some reduced species (Fe^{2+} and S^{2-}) were measured by HACH methods.

4.3 Results and Discussion

Anaerobic conditions were maintained in all mesocosms throughout the experimental period. Generally, headspace O_2 was less than 1% (data shown in Table D-11, Appendix D). As discussed in Chapter 3, the method of connection between the mesocosm headspace and the Micro GC may have caused overestimate of the headspace O_2 levels. Measurements of DO in the groundwater during the decommissioning verified the anaerobic conditions in all mesocosms with DO concentrations of 0.2 to 0.3 mg/L (Table D-18, Appendix D).

4.3.1 Lines of Evidence for Anaerobic Biodegradation of BTEX and F1

4.3.1.1 BTEX and F1 Removal

The total BTEX concentrations at Time 0 in the non-sterile mesocosms were approximately 17 mg/L, which accounted for over 80% of the F1 concentrations. Because BTEX and F1 were measured using different dilutions of water samples and different instruments, the calculated $F1_{-BTEX}$ results were erratic. Therefore, F1, rather than $F1_{-BTEX}$ results, were reported and used for data analysis and discussion.

The initial concentrations and the percentage removal (%) of each PHC component obtained at the end of the experiment are summarized in Table 4-2. The depletion of BEX with time in each non-sterile mesocosm was plotted in Figures D-2 and D-3, Appendix D.

It was anticipated that from the SC mesocosms the losses of PHCs, TEAs and nutrients attributed to abiotic attenuation mechanisms, such as partitioning into the headspace, sorption into/onto the sediment or mesocosm inner walls, and chemical reactions, could be differentiated. However, the PHC concentrations were very low in SCs (approximately 20 μg/L BTEX and 0.1 mg/L F1 at Time 0),

despite that the SCs were spiked with the sterilized condensates recovered from the site. Therefore, the abiotic losses of PHCs could not be directly identified and quantified from SCs.

Table 4-2. Initial concentrations of BTEX and F1 (C_0, mg/L) and percentage removals (%) after 722-day incubation [a].

		B		T [b]		E		m-,p-X [c]		o-X [d]		F1	
	#	C_0	%	C_0	%	C_0	%	C_0	%	C_0	%	C_0	%
	1	6.6	12	2.4	100	0.5	0	5.1	0	1.2	48	19.3	19
Ctrl	7	6.7	16	2.8	100	0.4	0	5.3	0	1.2	89	20.2	25
	2	6.8	22	2.4	100	0.7	19	5.2	0	1.2	50	19.0	21
Ctrl+NP	8	6.9	22	2.8	100	0.8	37	5.3	0	1.3	95	21.6	43
	3	6.9	48	2.3	100	0.7	100	5.3	0	1.3	16	22.8	42
NO3	9	7.2	29	3.0	100	0.7	94	5.2	0	1.2	0	19.9	22
	4	7.0	15	2.7	100	0.8	0	5.5	0	1.3	0	22.2	23
NO3+NP	10	7.0	17	2.9	100	0.7	43	5.1	0	1.2	0	19.6	1
	5	6.6	10	2.3	100	0.8	15	5.3	59	1.2	100	21.9	52
SO4	11	7.2	22	2.9	100	0.7	52	5.1	62	1.2	100	22.5	57
	6	6.6	12	2.7	100	0.6	0	4.8	55	1.2	100	23.4	51
SO4+NP	12	7.1	17	2.7	100	0.7	8	5.1	59	1.2	100	20.9	49

a. % removals for BTEX were based on concentrations corrected for partitioning into headspace using Henry's Law Constant. F1 concentrations were not corrected.

b. Completely depleted within 16 days in most mesocosms, except for NO3 mesocosms (#3 and #9), in which toluene concentrations reached very low concentrations at Day 16 but depleted completely by Day 127 and 197, respectively.

c. Concentrations of m- and p-xylenes leveled off from Day 127.

d. In Ctrl and Ctrl+NP mesocosms, degradation of o-xylene stopped when SO_4^{2-} was depleted; 100% o-xylene depletion occurred within 63 days in SO4 mesocosms and within 30 days in SO4+NP mesocosms.

In the closed-system anaerobic mesocosms, the major attenuation mechanisms for dissolved PHCs may include anaerobic biodegradation, partitioning into the headspace, and sorption to the sediment or mesocosm walls. Sorption was deemed to be negligible in this study because of the low organic content of the sediment. Losses of dissolved BTEX due to partitioning into the headspace were corrected

based on the headspace volume change and the respective Henry's Law constant (A sample calculation and the corrected BEX concentrations are presented in Appendix E2). The corrections showed only slight effects on the % removals and therefore the removals of BTEX (corrected) and F1 (not corrected) were assumed to be mainly due to anaerobic biodegradation.

Other lines of evidence were also investigated to verify the occurrence of anaerobic PHC biodegradation in the mesocosms, including TEA depletion, production of biogenic byproducts (CO_2, CH_4, reduced redox-sensitive species, etc.), MPNs of redox-specific bacteria, and signature metabolites (which will be discussed in Chapter 5). It should be noted that, in most cases, the mesocosms with same TEA conditions are discussed together since no apparent effect of nutrient amendment was observed. The controls, nitrate-amended, and sulfate-amended mesocosms are denoted respectively as Ctrl(+NP), NO3(+NP), and SO4(+NP).

4.3.1.2 TEA Depletion and Identification of TEAPs

The changes in NO_3^-/NO_2^- and SO_4^{2-} concentrations showed similar patterns in the respective TEA-amended mesocosms. Although the Ctrl(+NP) mesocosms were not amended with any TEA, due to the background SO_4^{2-} concentrations (approximately 70 mg/L) in the groundwater samples, the changes in the SO_4^{2-} concentrations were also observed. Examples are shown in Figure 4-1, as compared to the SCs.

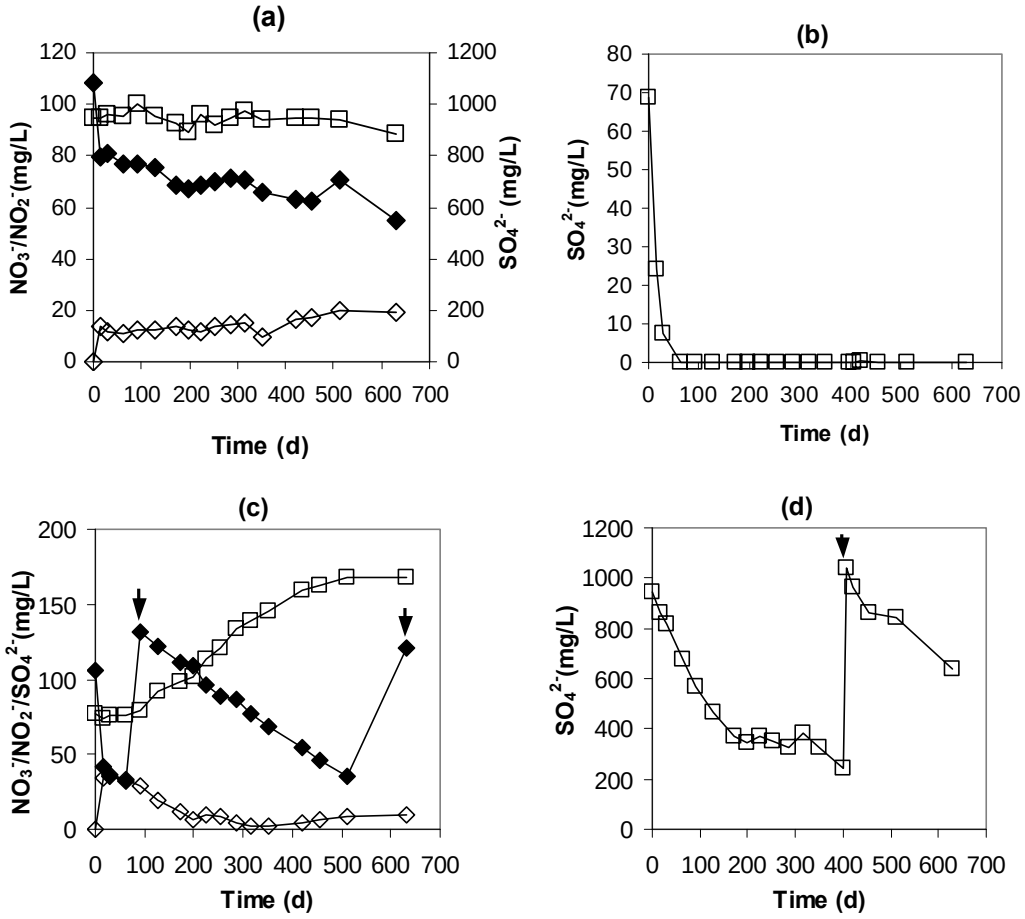

Figure 4-1. Examples of changes in concentrations of NO_3^- (♦), NO_2^- (◊), and SO_4^{2-} (□) in (a) SC2; (b) Ctrl(#2); (c) NO3(#9); and (d) SO4(#11) mesocosms (Note: all ion results on Day 722 were unreasonably high due to some unknown reasons and were not shown in the figure. ↓ indicates the day when NO_3^- or SO_4^{2-} was re-amended).

Rapid depletion of NO_3^- was observed in all NO3(+NP) mesocosms, accompanied by transient accumulation of NO_2^- during the first 30 days. There was slight decrease in the NO_3^- concentration in the SCs, possibly because of changes in the redox potential. SO_4^{2-} depletion was observed in all SO4(+NP) mesocosms as well as in the Ctrl(+NP) mesocosms, in which background SO_4^{2-} was consumed within 63 days. In contrast, increases in SO_4^{2-} concentrations were observed in NO3(+NP) mesocosms, indicating (1) that SO_4^{2-} reduction was repressed by NO_3^- amendment, and further (2) that sulfide oxidation was occurring in these mesocosms.

Previous studies have reported that NO_3^- addition could stop sulfide production and even further remove sulfide from sulfide-laden produced waters in oil fields (Eckford and Fedorak 2002; Gevertz *et al.* 2000; Krishnakumar and Manilal 1999; Okabe *et al.* 2003). According to Schippers and Jorgensen (2001), FeS and FeS_2 were not chemically oxidized by NO_3^- or amorphic ferric oxides under anaerobic conditions. Therefore, the oxidization of sulfide to SO_4^{2-} was an autotrophic denitrification process mediated by nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB). FeS or FeS_2 might be the electron donor and inorganic carbon the carbon source. To investigate the occurrence of autotrophic denitrification processes in the mesocosms, MPNs of NR-SOBs were measured at the end of the incubation (further discussion in Section 4.3.3.4).

The produced SO_4^{2-} was accumulated because SRB activity was repressed under nitrate reducing conditions. It is anticipated that TEAP may shift to SO_4^{2-} reduction once the NO_3^- is depleted. Using Eq. 4-1 and the produced SO_4^{2-}, the estimated NO_3^- consumption accounted for about 40% to 70% of the total NO_3^- depletion. The remaining NO_3^- consumption was probably due to heterotrophic denitrification which might be coupled to the PHC biodegradation. The implication of this observation for the application of NO_3^- amendment in the field is that the existing pool of reduced byproducts from *in-situ* biodegradation processes could consume the amended NO_3^- and result in much lower effective attenuation capacity. On the other hand, SO_4^{2-} produced from the sulfide oxidation may be utilized as an alternative TEA after NO_3^- has been completely consumed.

$$14NO_3^- + 10S^{2-} + 4H^+ \longrightarrow 7N_2 + 10SO_4^{2-} + 2H_2O \qquad \text{Eq. 4-1}$$

Headspace gas measurements indicated no CH_4 production in all mesocosms, suggesting that methanogenesis was not a significant TEAP in the mesocosms. Dissolved Fe^{2+} measured after the decommissioning of the mesocosms showed an increase in Ctrl(+NP) mesocosms (2.0 to 3.2 mg/L, compared to less than 0.1

mg/L in all other mesocosms). Aquifer sediment characterization by sequential extraction (Petersmeyer 2006) showed the presence of bioavailable Fe (III). Therefore, iron reduction could be an important TEAP in Ctrl(+NP) mesocosms. As previously discussed, SO_4^{2-} reduction occurred in Ctrl(+NP) mesocosms until the background SO_4^{2-} was depleted. It was then assumed that the predominant TEAPs in Ctrl(+NP) mesocosms were sulfate reduction followed by, or concurrently with, iron reduction, which might also represent the *in-situ* conditions.

Rapid depletion of amended NO_3^- or SO_4^{2-} suggested that the predominant TEAP in the respective mesocosms shifted to nitrate- or sulfate-reduction after TEA amendment. However, the low dissolved Fe^{2+} concentrations in nitrate- or sulfate-amended mesocosms may not preclude iron reduction as an important TEAP in these mesocosms. It is likely that a series of TEAPs are sequentially or simultaneously occurring in these mesocosms, thus affecting the biodegradation processes of individual PHCs (further discussion in Section 4.3.2). It is therefore important to identify the TEAPs and estimate the overall oxidation capacity provided by all available TEAs when evaluating the biodegradation process for a specific contaminated site.

4.3.1.3 Biogenic Production of CO_2 and Alkalinity

Biogenic production of CO_2 and alkalinity were also indicators of biodegradation processes. However, it is very difficult to quantify the biogenic CO_2 and alkalinity evolved in this closed multi-phase testing system because (1) the sub-sampling method affected the partitioning of CO_2 between water and headspace, and (2) some geochemical reactions might confound alkalinity measurement (i.e. the possible dissolution/precipitation of carbonate minerals). Nevertheless, changes in both measured groundwater alkalinity and estimated amount of headspace CO_2 with time (examples illustrated in Figure 4-2) could still serve as evidence for the occurrence of anaerobic biodegradation. The calculations of cumulative headspace CO_2 amounts in the mesocosms are summarized in Appendix E1.

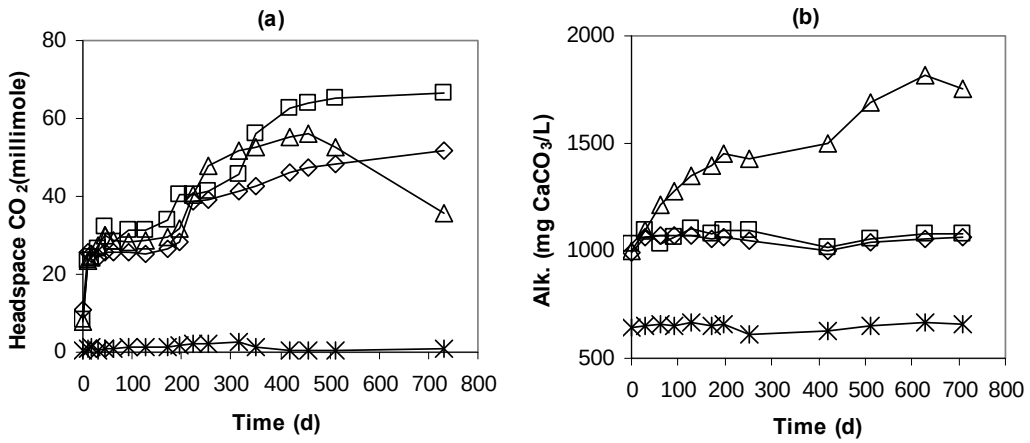

Figure 4-2. Examples of estimated amounts of headspace CO_2 (Panel (a)) and measured alkalinity (Panel (b)) in Ctrl(#1) (□), NO3(#9) (◊),SO4(#11) (Δ), and SC2 (✳) mesocosms.

The lower initial headspace CO_2 and alkalinity in SCs were caused by the autoclaving during the mesocosm preparation. The amounts of headspace CO_2 and the alkalinity in the SCs remained constant throughout the incubation period. Increases in the headspace CO_2 amounts were observed in all non-sterile mesocosms, indicating biogenic CO_2 production. In SO4(+NP) mesocosms, the increases in alkalinity values also suggested the biogenic CO_2 production. There was a decrease in the CO_2 amount in all SO4(+NP) mesocosms since Day 456 due to some unknown reasons.

4.3.1.4 MPN

MPN results of the anaerobic bacteria in the non-sterile mesocosms are summarized in Tables F1-3 and F1-4, Appendix F1. Examples of changes in MPNs of NRBs, SRBs, IRBs and methanogens with time in representative treatment mesocosm are shown in Figure F1-2, Appendix F1. The results of the statistical comparison of MPNs are presented in Appendix F2.

At Time 0, slurries of groundwater and sediments were sampled from three randomly selected mesocosms for the initial micarobial enumeration. The Time 0 MPNs of NRB, SRB, IRB, and methanogens are tabulated in Table 4-3. Using the statistical method described in Appendix F2, there was no significant difference in

the initial microbial numbers among the tested mesocosms, with one exception of the SRB MPNs between mesocosms #3 and #6. The calculated statistic values are summarized in Table F2-2, Appendix F2.

Table 4-3. MPN results of SRB, NRB, IRB and methanogens at Time 0 (MPN/L).

Mesocosm #	SRB	NRB	IRB	Methanogens
#3	2.4E+03	2.4E+04	2.1E+04	4.3E+03
#6	1.1E+02	9.3E+03	1.5E+04	9.3E+03
#7	4.6E+02	4.3E+04	2.8E+04	2.3E+03

The field investigation indicated that sulfate reduction and iron reduction were significant TEAPs at Site 3, whereas nitrate reduction and methanogensis were not significant processes (Petersmeyer 2006). The laboratory MPN results showed higher IRB numbers but very low SRB numbers at Time 0. The MPNs of NRB were significantly higher than those of SRB; and the MPNs of methanogens were also slightly higher (about 5-fold) than the SRB numbers. Therefore, there was no clear correlation between the presence of the redox-specific bacteria and the TEAPs in this study, similar to some previous studies (reviewed in Christensen *et al*. 2000). The MPN results appeared to be low considering the evidence of the occurrence of *in situ* anaerobic biodegradation. As discussed in Chapter 3, since the MPN analysis was done on slurry of groundwater and sediment, the MPN results might be underestimated if there were clusters of bacteria in the samples.

It was assumed that TEA amendment would selectively stimulate the growth of the redox-specific bacteria and consequently alter the composition and structure of the microbial community. However, the MPN results showed that there was no significant increase in microbial numbers of each redox-specific bacterium at the end of the mesocosm study (data in Appendix F). Nonetheless, the MPN results revealed some dynamic interactions between different microbial groups, which were caused by TEA amendment, along the time course.

In Ctrl(+NP) mesocosms, relatively high MPNs of methanogens were detected at the end of the incubation period. Methanogenic conditions might have been established in these mesocosms since no other TEAs were available. High fermentor numbers were found in all mesocosms (data in Appendix F), also suggesting that fermentation and methanogenesis could be potentially very important TEAPs. NO_3^- amendment inhibited methanogens. No methanogens were detected on the 197th day and at the end of the incubation in NO3(+NP) mesocosms. It was also found that NRB outcompeted SRB in NO3(+NP) mesocosms, possibly because nitrate amendment supressed SRB. The possible mechanisms include (1) direct inhibition of SRBs by NO_2^- or nitrous oxides; and (2) interspecies competition for common carbon sources and electron donors, with NRBs outcompeting SRBs. The possible mechanisms could be simply the competitive advantage of NRBs over SRBs due to NO_3^- amendment or toxicity of byproducts of nitrate reduction to SRB (Londry and Suflita 1999). There were more IRBs in NO3(+NP) mesocosms than in Ctrl(+NP) or SO4(+NP) mesocosms, possibly due to the fact that IRB could preferentially use available NO_3^- rather than Fe (III), which might not be readily bioavailable. Although rapid SO_4^{2-} depletion was observed in all SO4(+NP) mesocosms, the final MPNs of SRB were significantly lower than the initial SRB numbers.

MPN of NR-SOBs was measured at the end of the mesocosm study because of the occurrence of the sulfide oxidation coupled to nitrate reduction in the NO3(+NP) mesocosms. However, the results showed that MPNs of NR-SOBs were below detection limit in NO3(+NP) mesocosms (<0.03/L), but of the order of 10^0 to 10^1/L in Ctrl(+NP) and SO4(+NP) mesocosms. The possible explanations of these results could include (1) the medium used for the MPN analysis might not have been suitable, and (2) activity of NR-SOBs had stopped before the sampling event. The fact that no increase in the SO_4^{2-} concentration was observed since Day 420 in all NO3(+NP) mesocosms (Figure 4-1, (c)) supports the suggestion that the activity of the NR-SOBs had stopped before the sampling event.

MPNs may provide useful information regarding the *in-situ* biodegradation processes in that it could confirm the presence of microbial potential for specific microbial redox processes. However, the presence of certain redox-specific bacteria does not necessarily mean that these processes predominate or are actually occurring. Furthermore, MPN technique is biased toward culturable organisms and may also be limited by the growth conditions defined by the choice of medium, substrate, and incubation conditions (Christensen *et al.* 2000). Some organisms are able to use several TEAs, and may contribute to the number of bacteria in several redox groups. Therefore, caution should be taken when using the MPN technique because of the inherent limitations of this method.

In summary, the lines of evidence discussed above suggested positively that the removal of PHCs in the non-sterile mesocosms was due to anaerobic biodegradation processes.

4.3.2 Biodegradation of BTEX and F1 under Different TEAPs

No obvious lag period was identified before the onset of anaerobic biodegradation of target PHC compounds in Site 3 mesocosms. Toluene was rapidly biodegraded under all reducing conditions. Benzene biodegradation was consistently slow in all mesocosms (data presented in Appendix D). However, some selectivity in biodegradation of other PHC compounds was observed associated with different reducing conditions.

It was found in the mesocosm study that ethylbenzene was recalcitrant under sulfate reducing conditions (no significant biodegradation except in one SO4 mesocosm). Almost complete depletion of ethylbenzene (100% and 94%) was observed in NO3 mesocosms, but not in NO3+NP mesocosms (0% and 43%) for unknown reasons (Table 4-2). Thus, no definitive conclusion could be drawn regarding the enhancement of biodegradation of ethylbenzene by NO_3^- amendment for this specific site.

Sulfate-reducing conditions resulted in higher percentage removals of all xylene isomers, whereas there was no degradation of all xylenes under nitrate-reducing conditions in NO3(+NP) mesocosms (Table 4-2). No degradation of m-, and p-xylenes was observed in the Ctrl(+NP) mesocosms, whereas o-xylene was degraded to some extent. Biodegradation of o-xylene appeared to be sulfate-dependent. Complete biodegradation of o-xylene was obtained only in the SO4(+NP) mesocosms (Table 4-2), and in the Ctrl(+NP) mesocosms the biodegradation of o-xylene ceased once SO_4^{2-} was depleted (Figure 4-3). In NO3(+NP) mesocosms, the NO_3^- amendment suppressed the sulfate reduction process, which resulted in no decrease in the background SO_4^{2-} concentrations, nor the o-xylene concentrations (data in Appendix D).

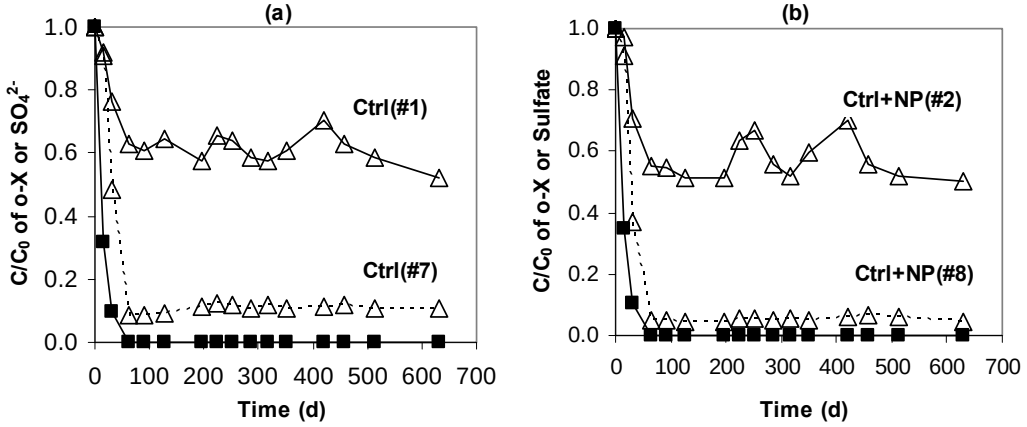

Figure 4-3. Anaerobic biodegradation of o-xylene (Δ, in duplicate mesocosms) coupled to representative SO_4^{2-} (■) reduction in Ctrl (Panel (a)) and Ctrl+NP (Panel (b)) mesocosms.

The reason for the discrepancy of % removal within duplicate mesocosms was unknown. The differences in the initial toluene concentrations between the duplicate mesocosms seemed to be coincident with the discrepancy of o-xylene removals (data in Appendix D). Evans *et al.* (1991) found that o-xylene was cometabolized in the presence of toluene under nitrate reducing conditions. Therefore, the biodegradation of o-xylene in the mesocosm study appeared to be both sulfate-dependent and cometabolized with toluene.

Over 50% F1 removal was observed in all SO4(+NP) mesocosms, compared to approximately 20% F1 removals in Ctrl(+NP) and NO3(+NP) mesocosms (except 43% in one Ctrl+NP and 1% in one NO3+NP), suggesting that sulfate reducing conditions were more favorable for F1 biodegradation. It was also found from Site 1 mesocosm study that SO_4^{2-} amendment stimulated the anaerobic biodegradation of $F1_{-BTEX}$ PHCs (as discussed in Chapter 3).

4.3.3 Kinetics of Anaerobic BTEX Biodegradation

The biodegradation rates were estimated using the concentrations of individual BTEX compounds (corrected for losses due to partitioning into the headspace). The first-order biodegradation rates were calculated using the linear regression in Microsoft Excel spreadsheet. First-order models assume a steady-state biomass density or little or no increase in microbial cell numbers (Schmidt *et al.* 1985). As discussed in the previous section, the MPN results showed no increase in the numbers of redox-specific bacteria and the assumption was satisfied. All the estimated first-order biodegradation rates are compared in Figure 4-4.

As shown in Figure 4-4, the estimated first-order biodegradation rates ranged from 0 to 0.0003 d^{-1} for benzene (except for 0.0009 d^{-1} in one NO3 mesocosm) and from 0 to 0.0008 d^{-1} for ethylbenzene (except for 0.0038 d^{-1} and 0.011 d^{-1} in NO3 mesocosms), which were comparable to the observed field attenuation rates, i.e. 0.0008 d^{-1} for benzene and 0.0002 d^{-1} for ethylbenzene. There was no definitive evidence from the laboratory mesocosm study that NO_3^- or SO_4^{2-} amendment could stimulate the biodegradation of benzene and ethylbenzene. It was evident that SO_4^{2-} amendment significantly enhanced the anaerobic biodegradation of xylenes. The estimated first-order field attenuation rate was 0.0003 d^{-1} for xylenes, whereas the enhanced laboratory rates ranged from 0.0013 to 0.0016 d^{-1} for m-, and p-xylenes and larger than 0.076 to 0.15 d^{-1} for o-xylene in SO4(+NP) mesocosms.

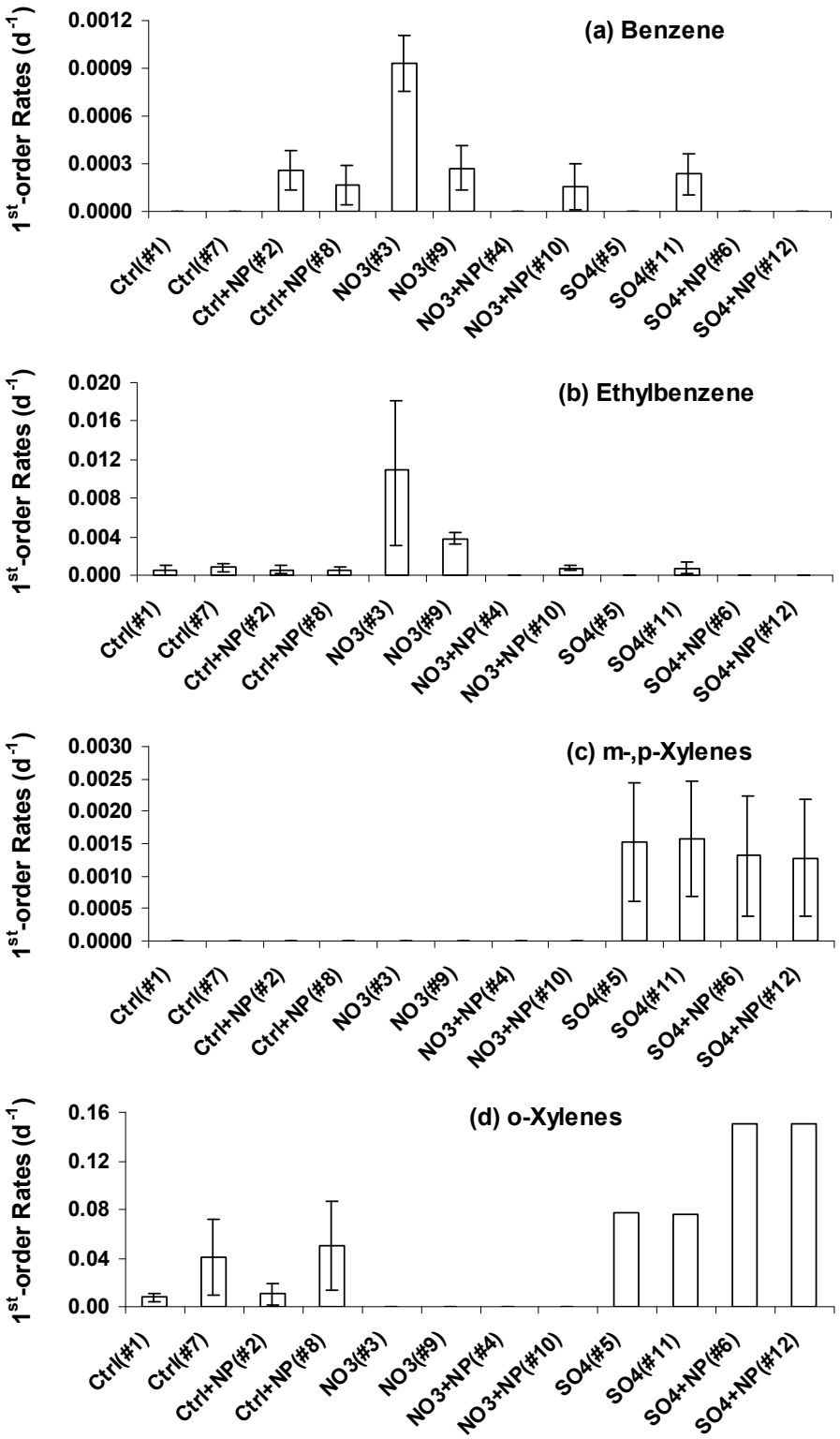

Figure 4-4. Estimated first-order anaerobic biodegradation rates for BEX (Error bars represent 95% confidence intervals. No column indicates that there was no measurable biodegradation. In panel (d), the o-xylene degradation rates in Ctrl(+NP) were estimated during the time when SO_4^{2-} was present and rates in SO4(+NP) were estimated minimum rates).

It appeared that nutrient amendment showed no enhancement effects under all reducing conditions. Also, the PO_4^{3-} concentrations in all mesocosms showed no biological PO_4^{3-} consumption. There was only a slight decrease in PO_4^{3-} concentration in SCs and SO4+NP mesocosms. Although PO_4^{3-} decreased continually in Ctrl+NP and NO3+NP mesocosms (data in Appendix D), this loss of PO_4^{3-} might simply be caused by geochemical reactions.

Theoretically the first-order biodegradation rates could be estimated from the depletion of TEAs and should be stoichiometry-normalized. However, based on the utilization factors of 4.9 mg NO_3^-/mg BTEX and 4.7 mg SO_4^{2-}/mg BTEX, only a small portion of the TEA depletion could be coupled to the biodegradation of BTEX in the mesocosm study. Other processes which might have contributed to the TEA reduction include the metabolism of other readily biodegradable organic compounds present in the groundwater (DOC results are presented in Appendix D) and some physical/chemical processes. Simple fermentation products could also serve as common substrates for anaerobic bacteria. As discussed previously, chemolithotrophic nitrate reduction also contributed to NO_3^- depletion. Hence, the estimated first-order rates from TEA depletion could not represent the biodegradation kinetics of target PHC compounds. Nevertheless, it still has important implications for the environmental management strategies which rely on natural attenuation processes, especially when TEA amendment is selected as the enhanced attenuation strategy at a contaminated site.

The first-order attenuation rates estimated from TEA depletion are presented in Figure 4-5. The fact that the attenuation rates decreased after the re-amendment of TEAs (Day 91 for NO_3^- re-amendment and Day 407 for SO_4^{2-} re-amendment) indicated that anaerobic biodegradation of readily biodegradable substrates and chemical redox reactions not kinetically-controlled may have falsely resulted in the higher initial reduction rates. All these processes should be differentiated from the TEA reduction coupled to biodegradation of target compounds in order to

better determine the biodegradation potential and to estimate the actual "effective attenuation capacity" more precisely.

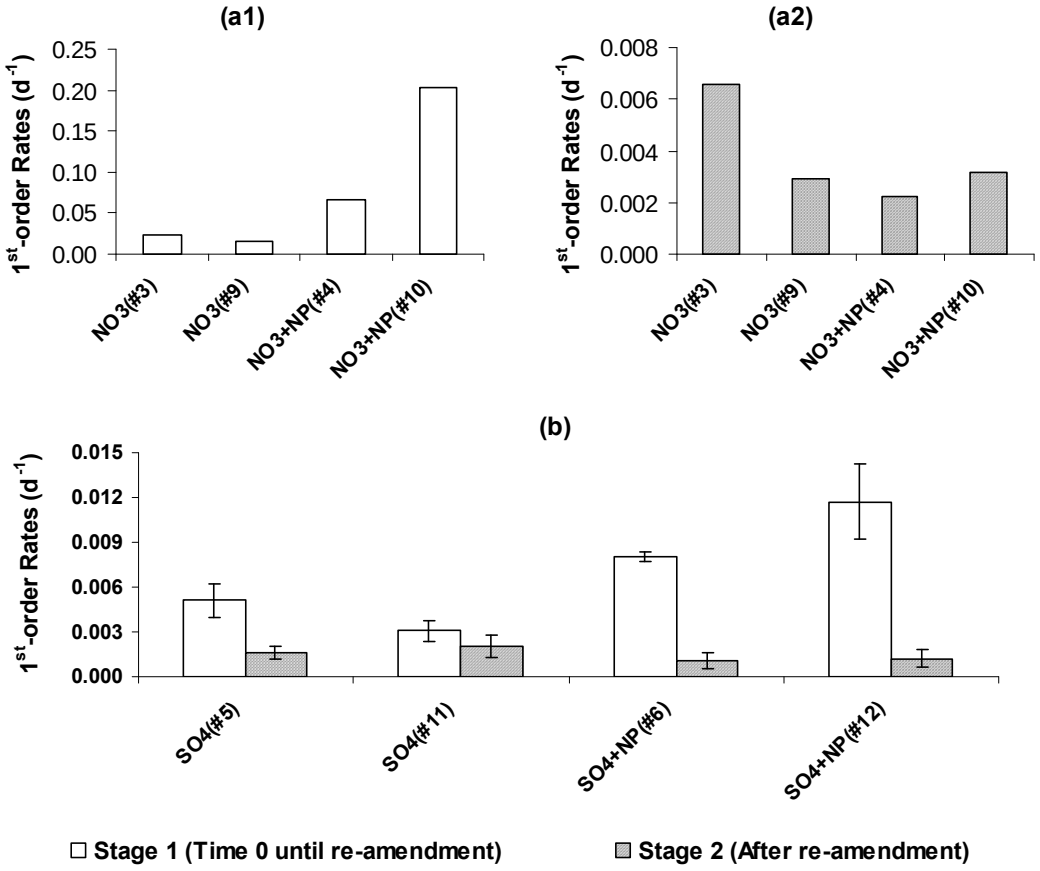

Figure 4-5. First-order attenuation rates estimated from depletion of NO_3^- ((a1) and (a2)) and SO_4^{2-} (Panel (b)) in respective mesocosms (Error bars represent 95% confidence intervals).

It should be emphasized that the experimental temperature was approximately 10°C higher than the average temperatures at the site. Thus, when applying the laboratory biodegradation rates to predict *in-situ* attenuation potentials, the temperature effects should be taken into account. A Q10 value of 2 could be employed for this purpose. However, further investigation might be necessary to verify the temperature effects on the biodegradation processes.

4.4 Conclusions and Recommendations

This two-year laboratory study demonstrates that the carefully designed mesocosms and the sub-sampling method are suitable for anaerobic biodegradation study. The larger size of the mesocosm allows the monitoring of multiple lines of evidence for the anaerobic biodegradation processes. Sub-sampling from the same mesocosm throughout the experiment may reduce some of the variability associated with the numerous replicates used in the microcosm studies. Useful information could be obtained to understand the anaerobic biodegradation processes and the influencing factors.

Nitrate reduction or sulfate reduction were identified as predominant TEAPs in the respective amended mesocosms. Sulfate reduction followed by, or concurrently with iron reduction might be the sequential TEAPs occurring in unamended controls, which also represented the *in-situ* conditions. Lack of ferrous iron data as a function of time limits the certainty in this regard. Selective biodegradation of certain PHCs under different TEAPs was observed. Biodegradation of o-xylene appeared to be sulfate dependent and likely cometabolized with toluene. Nitrate amendment inhibited sulfate-dependent o-xylene biodegradation.

The first-order biodegradation rates estimated from PHC depletion over time ranged from 0 to 0.0009 d^{-1} for benzene, 0 to 0.011 d^{-1} for ethylbenzene, 0 to 0.0016 d^{-1} for m- and p-xylenes, and 0 to 0.15 d^{-1} for o-xylene. There was no conclusive evidence that nitrate or sulfate amendment could enhance the anaerobic biodegradation of benzene and ethylbenzene, whereas sulfate amendment enhanced the biodegradation of xylenes. Sulfate reducing conditions seemed also favorable for removal of F1 hydrocarbons. Nutrient amendment showed no enhancement effects.

The amended nitrate may have been first used to oxidize the existing reduced byproducts from the previous or ongoing anaerobic biodegradation, which

decreased the effective attenuation capacity of NO_3^- and thus diminished the enhancement effects. Autotrophic denitrification appeared to be occurring in the mesocosms. Further studies should be conducted to better understand the nitrate reduction- sulfide oxidation process. Furthermore, SO_4^{2-} generated from this process may serve as an alternative TEA after NO_3^- is completely depleted. Sediment analysis indicated the presence of abundant bioavailable Fe(III) and the elevated Fe(II) concentrations measured in Ctrl(+NP) mesocosms after the decommissioning of these mesocosms suggested that iron reduction might be an important TEAP at the site. Hence, iron reduction merits further investigation; for instance, the feasibility of using chelating agents to stimulate iron reduction at the site. Individual PHC compounds showed different biodegradability under different reducing conditions, implying that amendment of different TEAs might be incorporated into the management strategy as a treatment train to achieve the remedial objective at a specific site.

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Chapter 5 Detection of Signature Metabolites in the Laboratory Mesocosm Studies of Enhanced Anaerobic Biodegradation of Petroleum Hydrocarbons (PHCs) in Groundwater

5.1 Introduction

Signature metabolites, also known as biomarkers, are chemicals that are produced during the biodegradation of a target contaminant. Good signature metabolites should be (1) intermediate products of the degradation pathways and highly specific to the process being monitored, (2) not a normal component of industrial wastes, (3) neither a product of cometabolism nor a dead-end product, and (4) biologically and chemically stable (Griebler *et al.* 2004; Phelps *et al.* 2002). Detection of specific biomarkers can be used in conjunction with other lines of evidence to confirm that bioremediation is occurring at the site. For example, benzylsuccinates have been shown to be useful indicators of *in-situ* anaerobic alkylbenzene biodegradation (Gieg and Suflita 2002).

The identification of benzylsuccinate as the intermediate metabolite of toluene biodegradation under sulfate- and nitrate-reducing conditions (Beller *et al.* 1992; Evans *et al.* 1992) formed the basis for the elucidation of the anaerobic metabolism of toluene and other hydrocarbons. Numerous studies have demonstrated that fumarate addition is the initial activation mechanism employed by anaerobic microorganisms for the biodegradation of alkane, alkylbenzene, and alkylated PAHs under different reducing conditions (Annweiler *et al.* 2000; Elshahed *et al.* 2001; Krieger *et al.* 1999; Kropp *et al.* 2000; Rabus *et al.* 2001). A different metabolic pathway has been reported for ethylbenzene metabolism under nitrate reducing conditions (Ball *et al.* 1996; Rabus and Widdel 1995). The proposed metabolic pathways for anaerobic biodegradation of TEX are illustrated in Figure 2-1.

Metabolic pathways for unsubstituted aromatic hydrocarbons appear to be different (Coates *et al*. 2002; Foght 2008). The initiation reaction of benzene is still unclear. The proposed anaerobic benzene metabolic pathways include initial reactions of carboxylation, hydroxylation (Caldwell and Suflita 2000; Weijma *et al.* 2000), or methylation (Coates *et al.* 2002; Ulrich *et al.* 2005), followed by subsequent transformation to the central intermediate benzoate and ring cleavage as presented in Figure 2-2. Carboxylation of naphthalene has been reported under sulfate reducing conditions yielding naphthoic acid (Zhang and Young 1997). The proposed anaerobic metabolic pathway for naphthalene and alkylated naphthalene is illustrated in Figure 5-1.

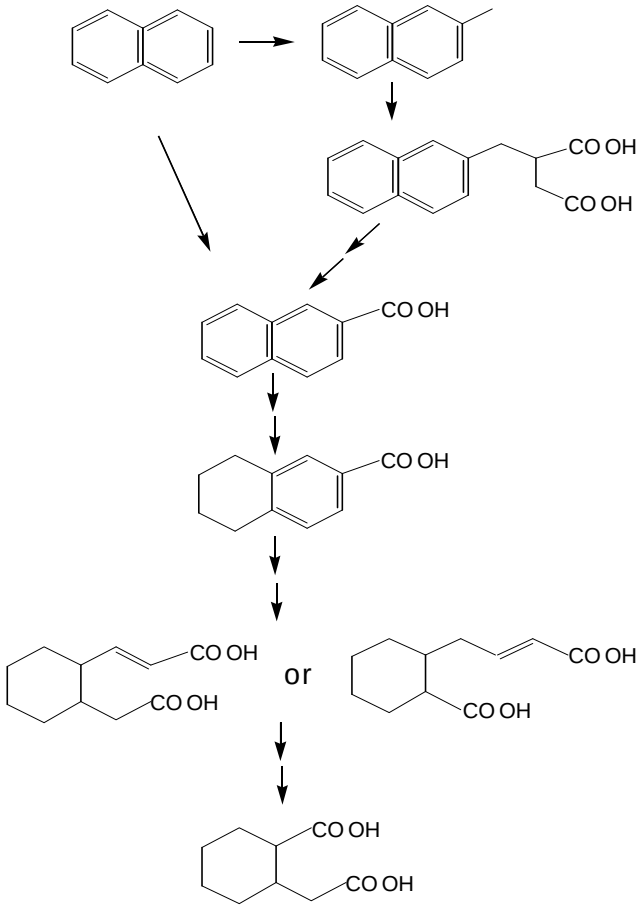

Figure 5-1. Proposed metabolic pathway for anaerobic biodegradation of naphthalene and alkylated naphthalene (Meckenstock *et al.* 2004; Safinowski and Meckenstock 2006; Zhang *et al.* 2000).

Two laboratory mesocosm studies were conducted in the Department of Civil & Environmental Engineering at University of Alberta to investigate the enhanced anaerobic biodegradation of PHCs in contaminated groundwater by TEA and/or nutrient amendment. These studies have been presented in detail in Chapters 3 and 4. In summary, nearly 100% removal of BEX was achieved in Site 1 mesocosm study and high percentage of F1 removals were observed in sulfate-amended mesocosms. In contrast, in Site 3 mesocosms, which had much higher substrate concentrations, much lower % removals of BTEX and F1 was achieved. Benzene and ethylbenzene were persistent in most active mesocosms. It was also found that o-xylene degradation was sulfate-dependent. There was evidence suggesting that xylenes were cometabolized with toluene. Polycyclic aromatic hydrocarbons were not monitored; however previous field studies indicated the presence of anaerobic naphthalene metabolites at Site 1 (Gieg and Suflita 2002).

In the laboratory mesocosm studies all purported metabolites were analyzed as a function of time. The objective of the metabolite analyses in the laboratory mesocosm study is to identify the signature metabolites and to provide supplementary evidence of the occurrence of anaerobic biodegradation of specific PHC contaminants and to better understand the metabolic pathways of anaerobic biodegradation of these PHCs under certain TEAPs.

5.2 Methodology

5.2.1 Sampling and Analysis

At Time 0, 6 months after incubation, and at the end of incubation (Day 620 for Site 1 study and Day 722 for Site 3 study), 1 L groundwater and sediment samples were pumped into clean glass bottle from the mesocosms using a peristaltic pump and acidified immediately to pH < 2 with 2 N H_2SO_4. The large sample volume (1 L) was used to ensure the detection of trace metabolites. The samples were then sent to the Department of Biological Sciences at University of Alberta for metabolite analysis.

All samples were extracted 3 times with 300 mL ethyl acetate. Prior to extraction, 4-fluoro-1-naphthoic acid (4F1NA) was added to each sample as an extraction standard. The extracts were dried over anhydrous sodium sulfate and concentrated to 100 µL using a RotoVap®, then derivatized with *N, O*-bis(trimethylsilyl)trifluoroacetimide (BSTFA) at 70°C to form trimethylsilyl (TMS) esters. Derivatized samples were injected into a HP 5890 GC coupled to HP 5970 MS detector for identification and quantification of selected metabolites.

The GC was equipped with a HP- 5MS column (30m length, 0.25mm internal diameter, and 0.25µm film thickness) to separate all the derivatized components. The oven temperature was held at 65°C for 2 min, then increased at 4°C per min to 280°C and held for 5 min. The MS detector used the total ion chromatogram (TIC) mode to acquire data of all fragment ions within the mass units of 50 to 550 m/z. The reconstructed ion chromatogram (RIC) function was used to probe the total ion chromatograms for specific characteristic fragment ions to identify the metabolites.

5.2.2 Identification and quantification

Authentic standards for most supposed anaerobic hydrocarbon metabolites were analyzed using the same method for metabolite analysis to determine the respective retention time and to obtain the mass spectra. The metabolites were then positively identified by matching both GC retention times and mass spectra with those of the standards. When no standards were available, metabolites were identified by comparing the mass spectra with the published MS profiles.

The determination of the metabolite concentrations was conducted in a semi-quantitative manner. 100 nM 4F1NA, which has a unique characteristic ion of 247 m/ z, was added to each sample as an extraction standard. The mass spectrum of 4F1NA is shown in Figure 5-2. Metabolite concentrations were estimated by multiplying 100 nM with respective Relative Response Factors $(M-15)^+/247$ ratio, i.e. comparing the peak area of the specific metabolite peak (at the correct

retention time and having the characteristic $(M-15)^+$ ion, integrated using RIC) with that of 4F1NA (247 m/z ion).

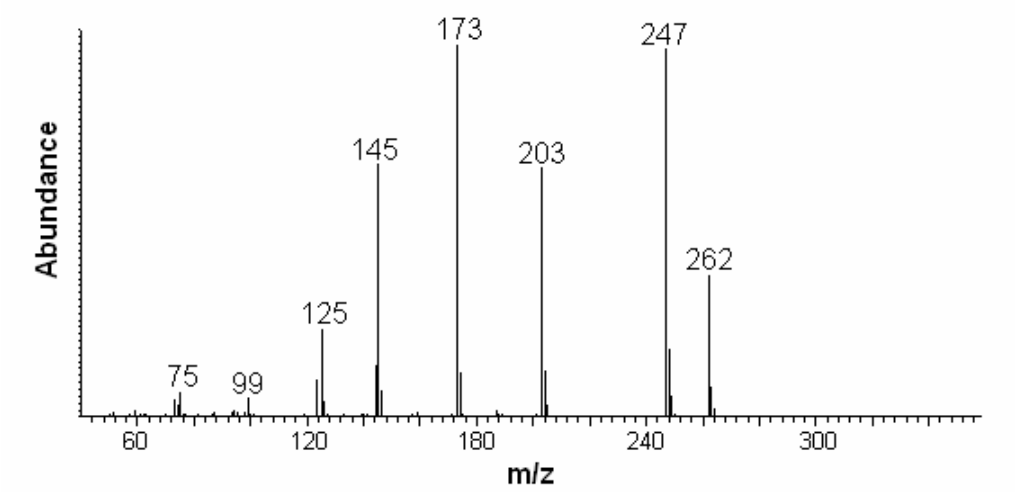

Figure 5-2. Mass spectrum of 4-fluoro-1-naphthoic acid (with a characteristic 247 m/z ion).

5.2.3 Extraction efficiency

To ensure the metabolites were extracted sufficiently from water and derivatized properly, extraction efficiency of all standards were determined. Authentic standards (100 nM) were derivatized (with no extraction) and injected into the GC/MS to determine the $(M-15)^+/247$ ratios without extraction. Standards were then added to tap water at the same concentration and analyzed by extraction, derivatization, and GC/MS. The $(M-15)^+/247$ ratios with extraction were also determined. The extraction efficiency of each standard was determined by comparing the $(M-15)^+/247$ ratio with extraction and that without extraction.

The GC chromatograms of the standards with or without extraction are compared in Figure 5-3. The extraction efficiencies of all standards are summarized in Table 5-1. It was found that approximately 100 % recovery of all standards (except for methylsuccinate, which showed only a 15% recovery) was achieved.

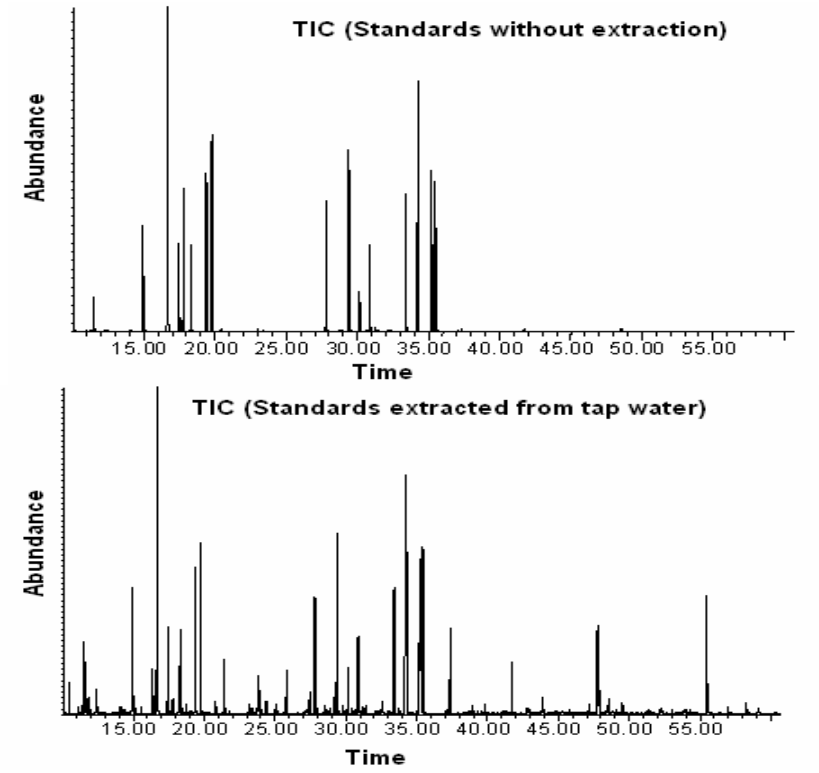

Figure 5-3. Comparison of total ion chromatograms of standards with and without extraction.

Table 5-1. Extraction efficiency of all standards from water (% recovery of authentic standards determined by $(M-15)^+$/247 ratios).

Standards	RT (min)	$(M-15)^+$ Ions	$(M-15)^+$/ 247 Ratios (without extraction)	$(M-15)^+$/ 247 Ratios (with extraction)	Extraction efficiency (%)
4F1NA	29.4	262	0.39	0.40	102.3
n-Octylsuccinate	34.3	359	0.76	0.71	92.7
Benzylsuccinate	33.4	337	0.21	0.20	94.6
Ethylbenzylsucciate	34.2	351	0.26	0.25	95.7
m-methylbenzylsuccinate	35.2	351	0.30	0.33	110.2
p-methylbenzylsuccinate	35.5	351	0.12	0.11	93.5
o-Toluate	17.5	193	0.73	0.70	96.2
p-Toluate	18.3	193	1.17	0.96	81.5
1-naphthoate	30.2	229	0.37	0.37	99.6
2-naphthoate	30.9	229	0.75	0.67	88.3
benzoate	15.0	179	1.10	1.01	91.9
methylsuccinate	17.8	261	0.34	0.05	14.7
1,2,3,4-tetrahydronaphthoate	27.8	233	0.27	0.27	99.9

RT = retention time

5.3 Results and Discussion

5.3.1 Identification of the anaerobic PHC metabolites

All anaerobic PHC metabolites identified from Site 1 and Site 3 mesocosm studies are summarized in Table 5-2. The mass spectra of the metabolites produced during anaerobic biodegradation of TEX and C_6 to C_{10} PHCs (saturated and unsaturated) are shown in Figure 5-4.

Table 5-2. List of anaerobic PHC metabolites identified from the laboratory mesocosm studies.

Parent compounds	Metabolites	No. [f]	$(M-15)^+$ Ion (m/z)	Confirmatory Ion (m/z)	RT (min)
TEX					
Toluene	Benzylsuccinate	(a)	337	205, 221, 234	32.9
Ethylbenzene [a]	Ethylbenzylsuccinate	(b)	351	159, 204, 235	33.6
Xylenes	o-, m-Methylbenzylsuccinate	(c)	351	159	34.8
	p-Methylbenzylsuccinate	(c)	351	159	35.0
	o-Toluate	(d)	193	119, 149	16.7
	m-Toluate	(d)	193	119, 149	17.6
	p-Toluate	(d)	193	119, 149	18.0
	Cyclohexane carboxylate	(e)	185		13.2
	Benzoate [b]	(f)	179	105, 135	14.5
CCME F1 PHCs					
Hexane (n-C_6)	Hexylsuccinate	(g)	262	331	28.2
C_7 [c, d] (Unsaturated)		(h)	262	343	30.2
C_9 [c, e] (Unsaturated)		(i)	262	371	34.5
Other PHCs					
Propane (n-C_3)	Propylsuccinate [c]	(j)	262	289, 217, 147	21.8
Butane (n-C_4)	Butylsuccinate [c]	(k)	262	303	24.2
Pentane (n-C_5)	Pentylsuccinate [c]	(l)	262	317	25.6
Naphthalene	1-Naphthoate	(m)	229	127, 155, 185	29.6
	2-Naphthoate	(n)	229	127, 155, 185	30.4
	5,6,7,8-Tetrahydronaphthoate [c]	(o)	233	159, 189	30.1

a: Different pathways were reported for ethylbenzene under nitrate- or sulphate-reducing conditions. Ethylbenzylsuccinate is believed to be the metabolite under sulphate-reducing conditions, whereas 1-phenylethanol was the metabolite under nitrate-reducing conditions, which was not detected in our laboratory mesocosm study.

b: Benzoate is a common metabolite in anaerobic PHC biodegradation, not specific to one particular parent compound.

c: No standards available; identified by comparison with published MS spectra or analog analysis.

d: Most abundant in Site 1 mesocosms

e: Most abundant in Site 3 mesocosms

f: Notation as in Figure 5-4.

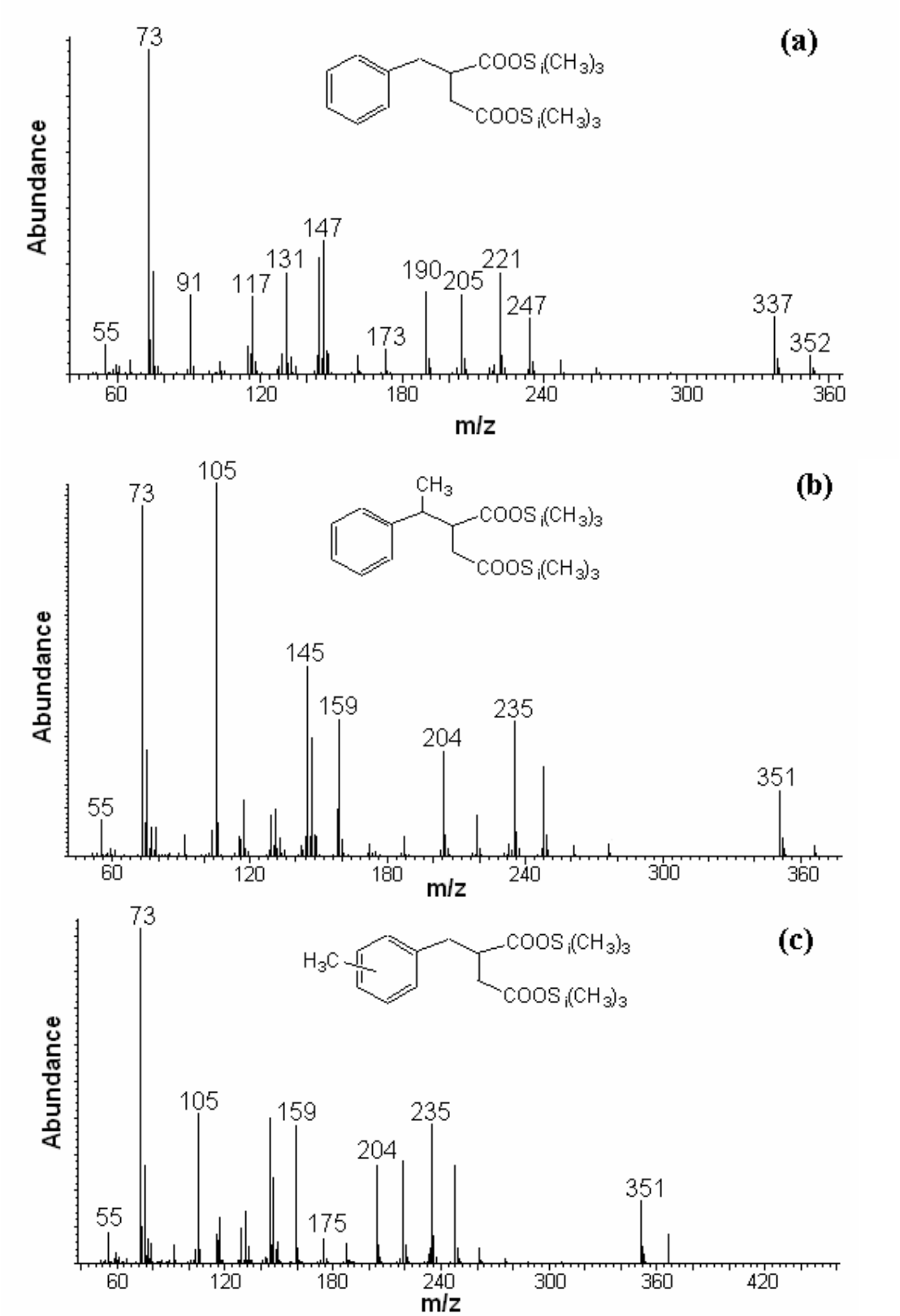

Figure 5-4. Representative mass spectra of all anaerobic PHC metabolites detected from Site 1 and Site 3 mesocosms. The notations, parent compounds, characteristic ions, and retention times are summarized in Table 5-2.

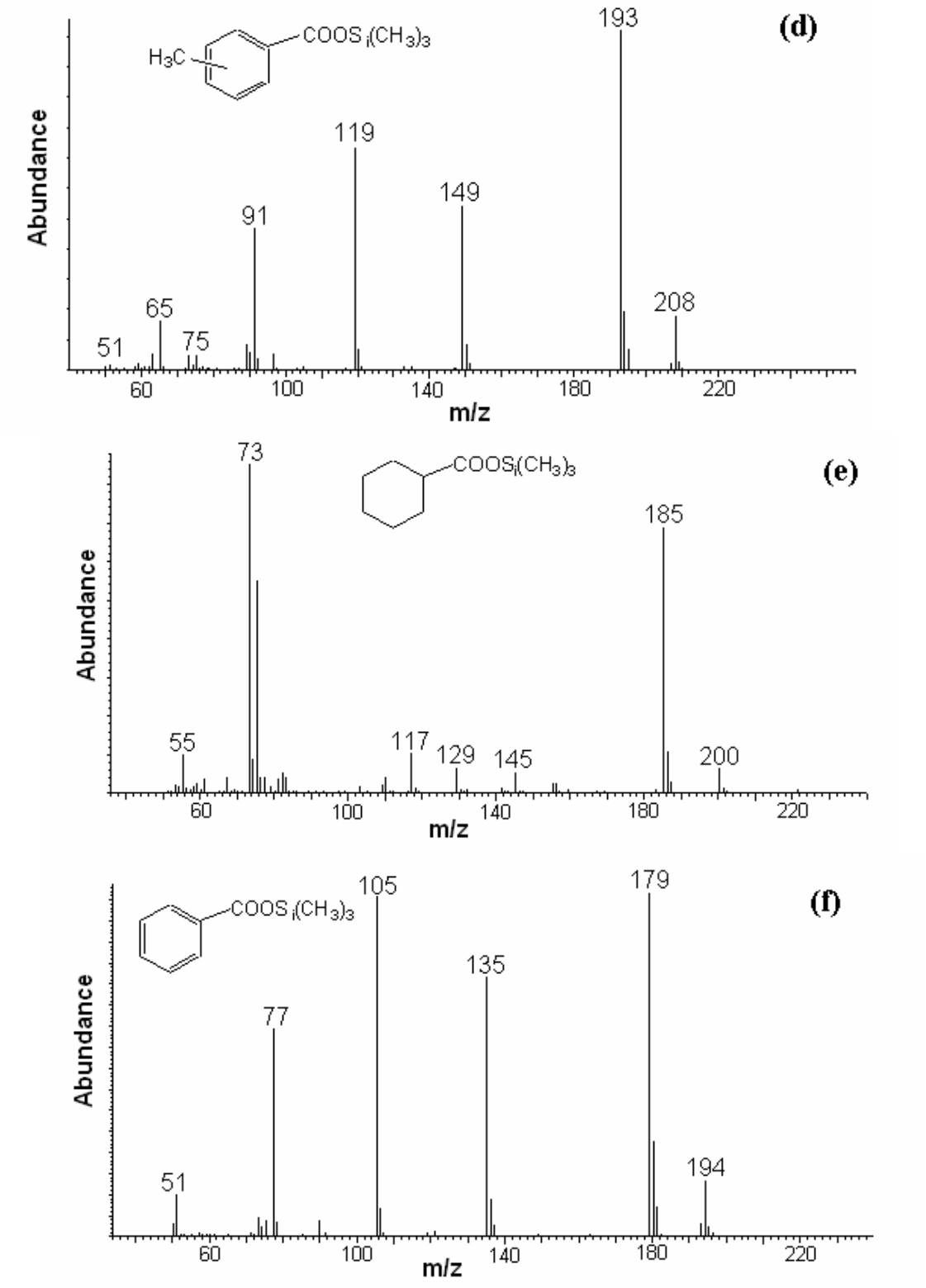

Figure 5-4. (Continued).

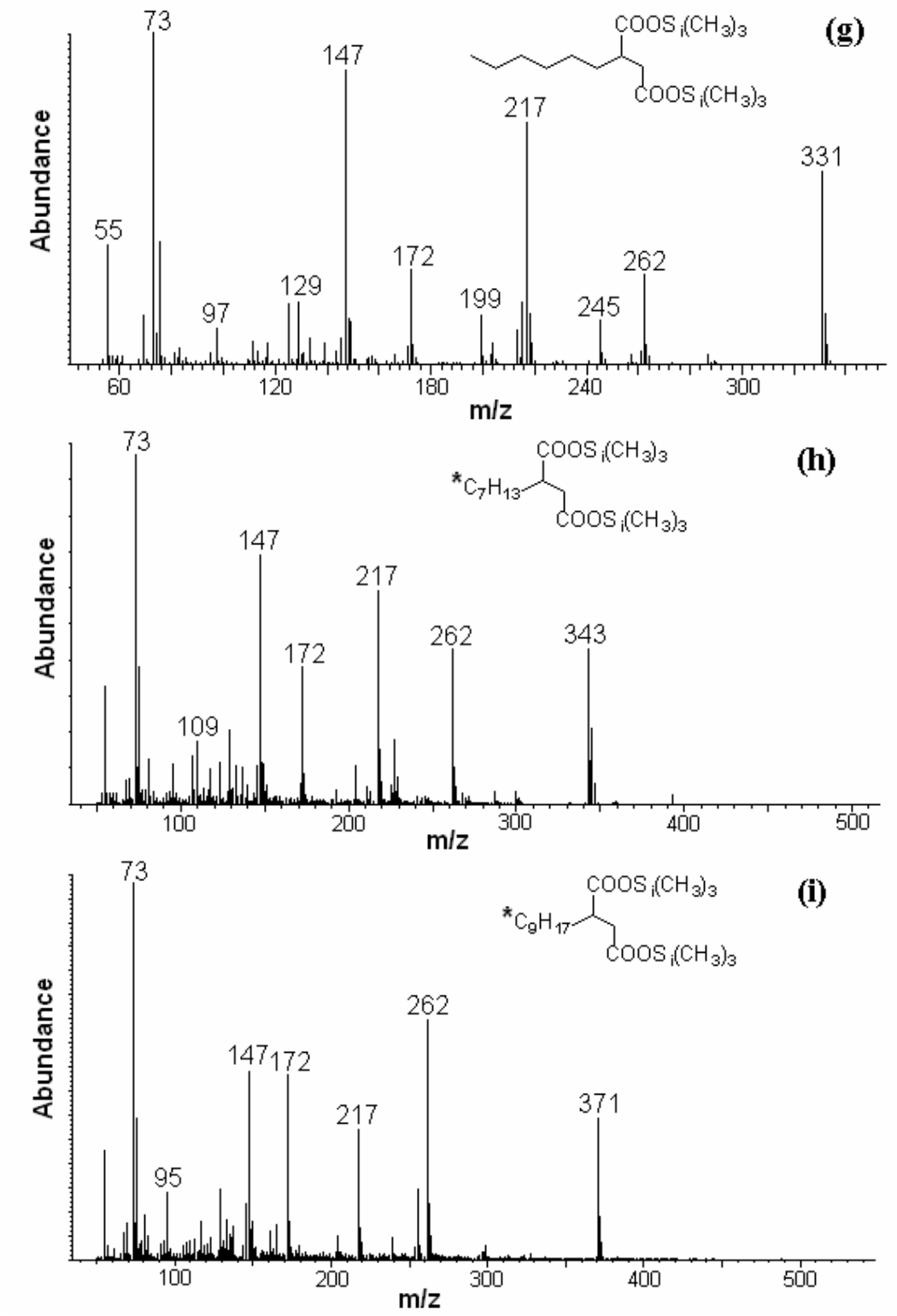

Figure 5-4. (Continued).

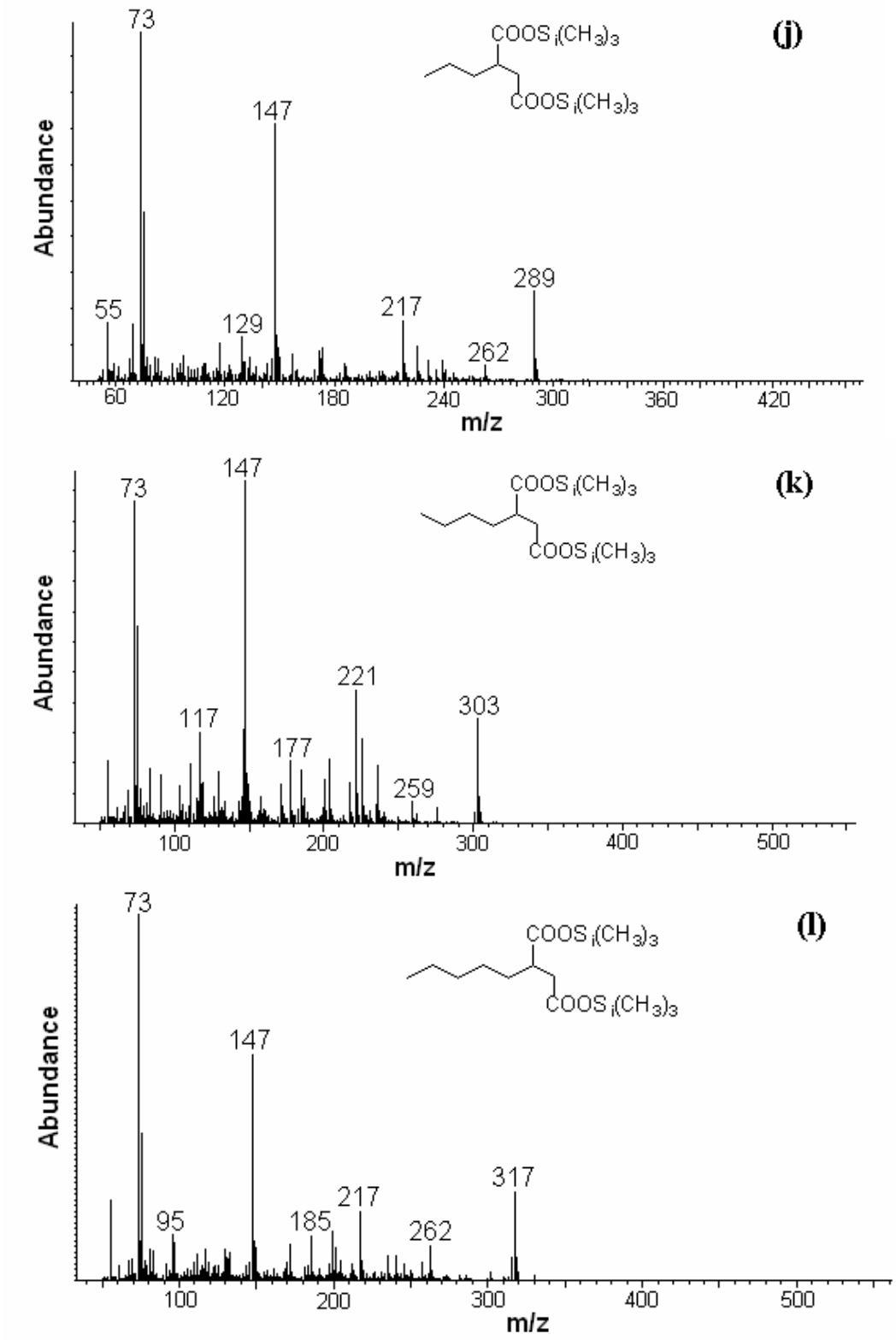

Figure 5-4. (Continued).

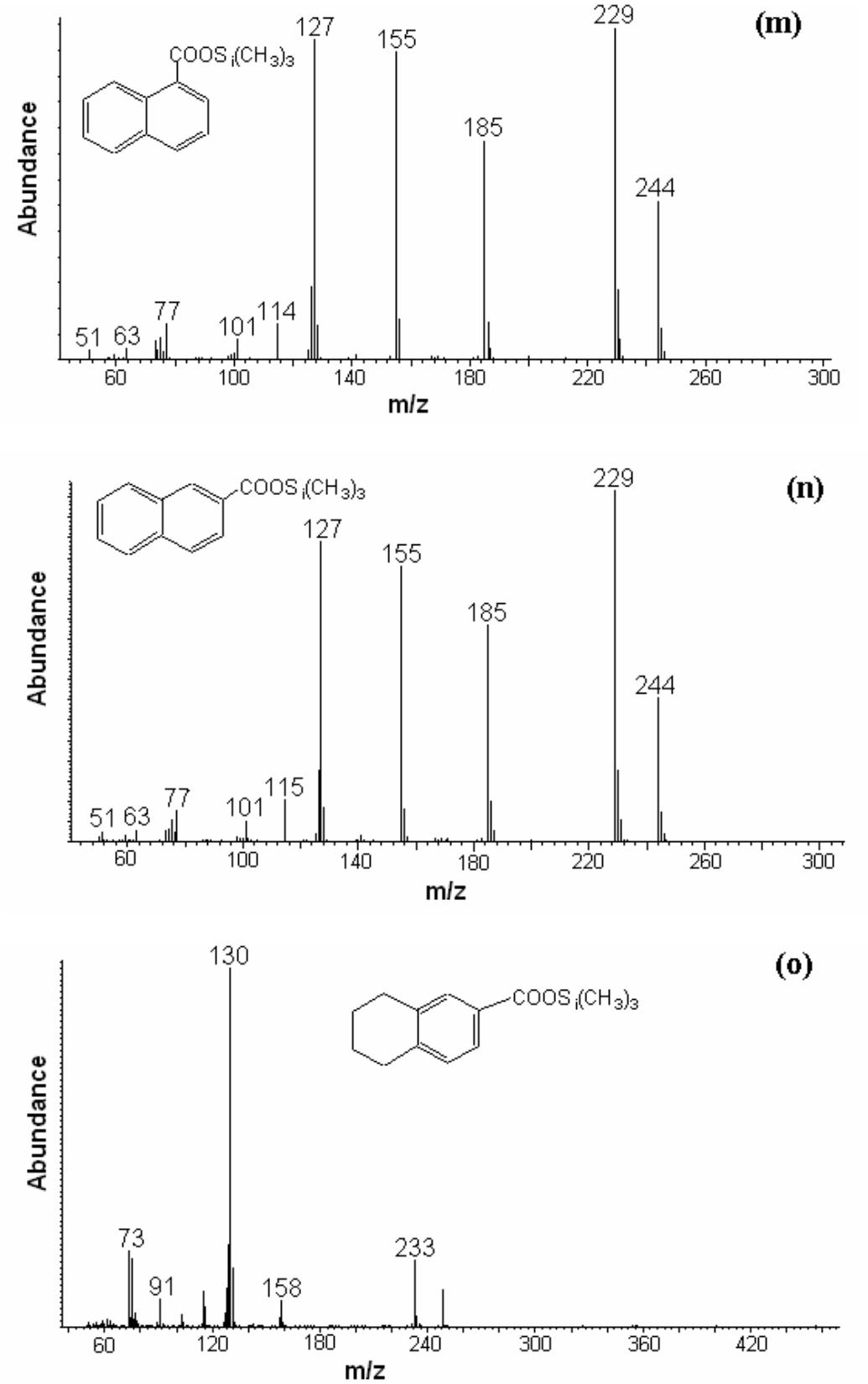

Figure 5-4. (Continued).

5.3.2 Identification and Quantification of metabolites at Time 0

A variety of metabolites were detected at estimated concentrations of nanomoles/L in both studies at Time 0, indicating the occurrence of *in-situ* anaerobic biodegradation of PHC contaminants at both sites. The presence and estimated concentrations of Time 0 metabolites (if available) are tabulated in Table 5-3.

Table 5-3. Presence and approximate concentrations of anaerobic PHC metabolites at Time 0 in the mesocosm studies.

Metabolites	Site 1		Site 3	
	Presence	C (nM)	Presence	C (nM)
Propylsuccinate	-	-	+	NQ
Butylsuccinate	+	NQ	-	-
Pentylsuccinate	+	NQ	+	NQ
Hexylsuccinate	+	NQ	+	NQ
C7 (unsaturated)	+	42	-	-
C8 (unsaturated)	+	NQ	+	NQ
C9 (unsaturated)	-	-	+	115
Benzylsuccinate	-	-	+	3
Ethylbenzylsuccinate	+	7	+	79
o-,m-Methylbenzylsuccinate	-	-	+	28
p-Methylbenzylsuccinate	-	-	+	56
o-Toluate	-	-	+	27
m-Toluate	+	10	+	130
p-Toluate	+	28	+	46
Cyclohexane carboxylate	+	267	+	260
1-Naphthoate	-	-	+	5
2-Naphthoate	+	11	+	9
5,6,7,8-tetrahydronaphthoate	+	62	+	70
Benzoate	+	30	+	137

+: Presence
-: None presence
C: concentration (average of 12 values)
NQ: Not quantified due to lack of standard or other reasons

Metabolites identified in Site 1 mesocosms included alkylsuccinates from C_4 to C_6 alkanes and C_7 to C_8 alkanes with unsaturation, metabolites from anaerobic biodegradation of ethylbenzene and m-, and p-xylenes. In Site 3 study, the presence of alkylsuccinates from anaerobic biodegradation of C_3 to C_9 alkanes and metabolites from anaerobic biodegradation of toluene, ethylbenzene and xylenes was identified. Metabolites of anaerobic naphthalene biodegradation were detected in both studies.

Gieg and Suflita (2002) investigated *in-situ* anaerobic metabolites of saturated and aromatic hydrocarbons at Site 1. Their study identified the presence of alkylsuccinates from C_5 to C_{11} alkanes and C_7 alkane with unsaturation. The metabolite of ethylbenzene was also detected, but no metabolites from xylenes or naphthalene biodegradation were found (Gieg and Suflita 2002). The metabolites identified from the Site 1 laboratory mesocosm study were consistent with the published results.

Both sites have a long history of contamination by PHC mixtures and therefore the indigenous microorganisms have adapted to the biodegradation of these PHC contaminants. The metabolites showed some differences because the compositions and the concentrations of the PHC contaminants at these two sites were different. Using ion 262 (characteristic ion for alkylsuccinate (TMS ester)) for example, the differences between two sites are shown in Figure 5-5.

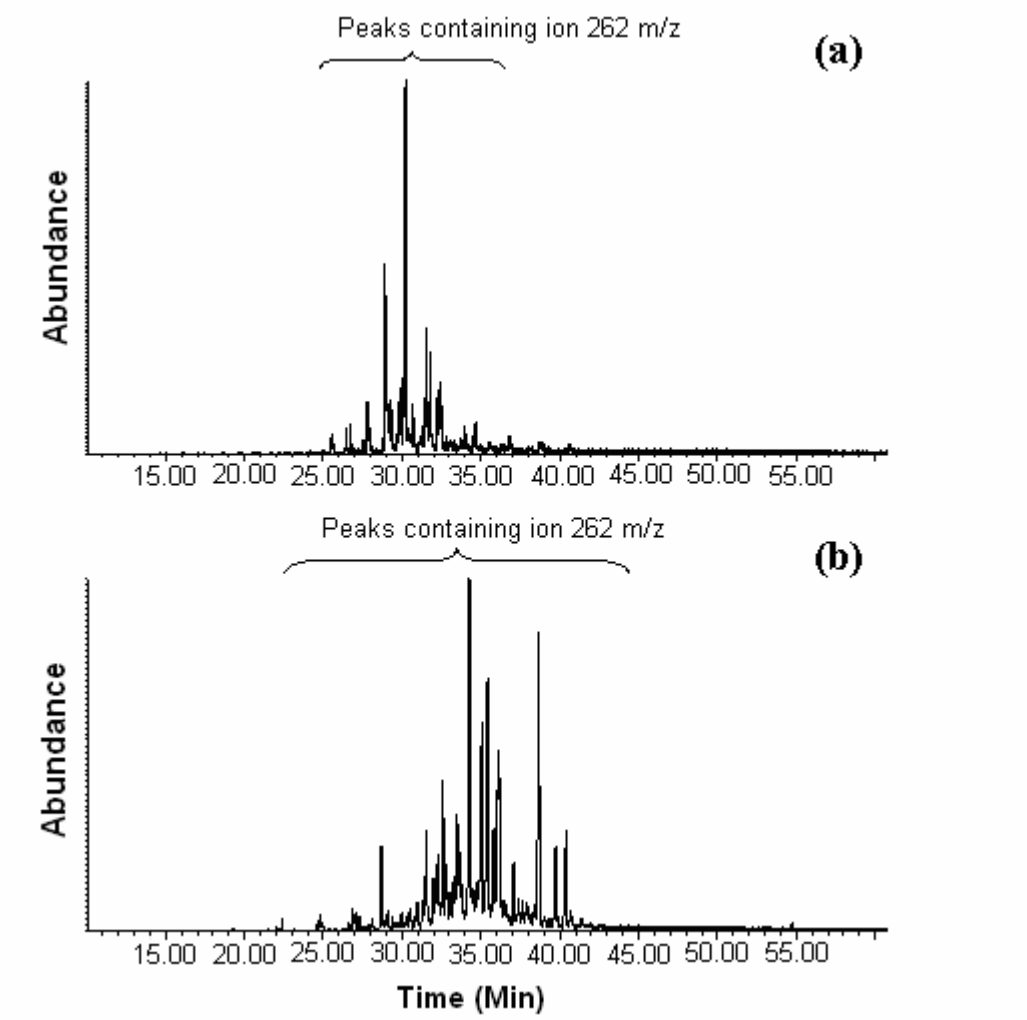

Figure 5-5. RIC of all peaks containing ion 262m/z in representative Site 1 (Panel (a)) and Site 3 (Panel (b)) mesocosms.

The free phase PHCs collected from Site 1 (a flare pit site) consisted of approximately 56% (mole fraction) of C_6 to C_{10} hydrocarbons and approximately 38% of C_{11} to C_{30} hydrocarbons (data shown in Appendix D). However, the concentrations of the dissolved hydrocarbon compounds in the groundwater were relatively low. There were fewer metabolites detected from all Site 1 mesocosms at Time 0, also at relatively lower abundance.

The condensates recovered from Site 3 (a gas condensate contaminated site) consisted of light end PHCs, approximately 46% of C_6 to C_{10} and approximately 54% of C_5 (data presented in Appendix D). The concentrations of dissolved PHC

contaminants in the groundwater were quite high. Accordingly, abundant C_3 to C_9 metabolites and TEX metabolites were detected from Site 3 mesocosms.

5.3.3 Changes in metabolite concentrations with time and implication of the biodegradation processes

Previous studies have suggested that anaerobic biodegradation of PHCs are selective under different TEAPs and are often specific to site conditions at the contaminated site (Hutchins 1991; Wiedemeier *et al.* 1995; Suarez and Rifai 1999; Aronson and Howard 1997). As discussed in the previous chapters, the mesocosm studies also indicated that different TEAPs impacted the biodegradation process of specific PHC compounds in the PHC mixtures. The metabolite results from these mesocosm studies all revealed that different patterns of metabolites were observed under different TEAP conditions. Metabolites of ethylbenzene and xylene isomers identified from the Site 3 mesocosm study are shown in Figure 5-6 as examples. The metabolite results from all non-sterile mesocosms are plotted and compiled in Appendix G.

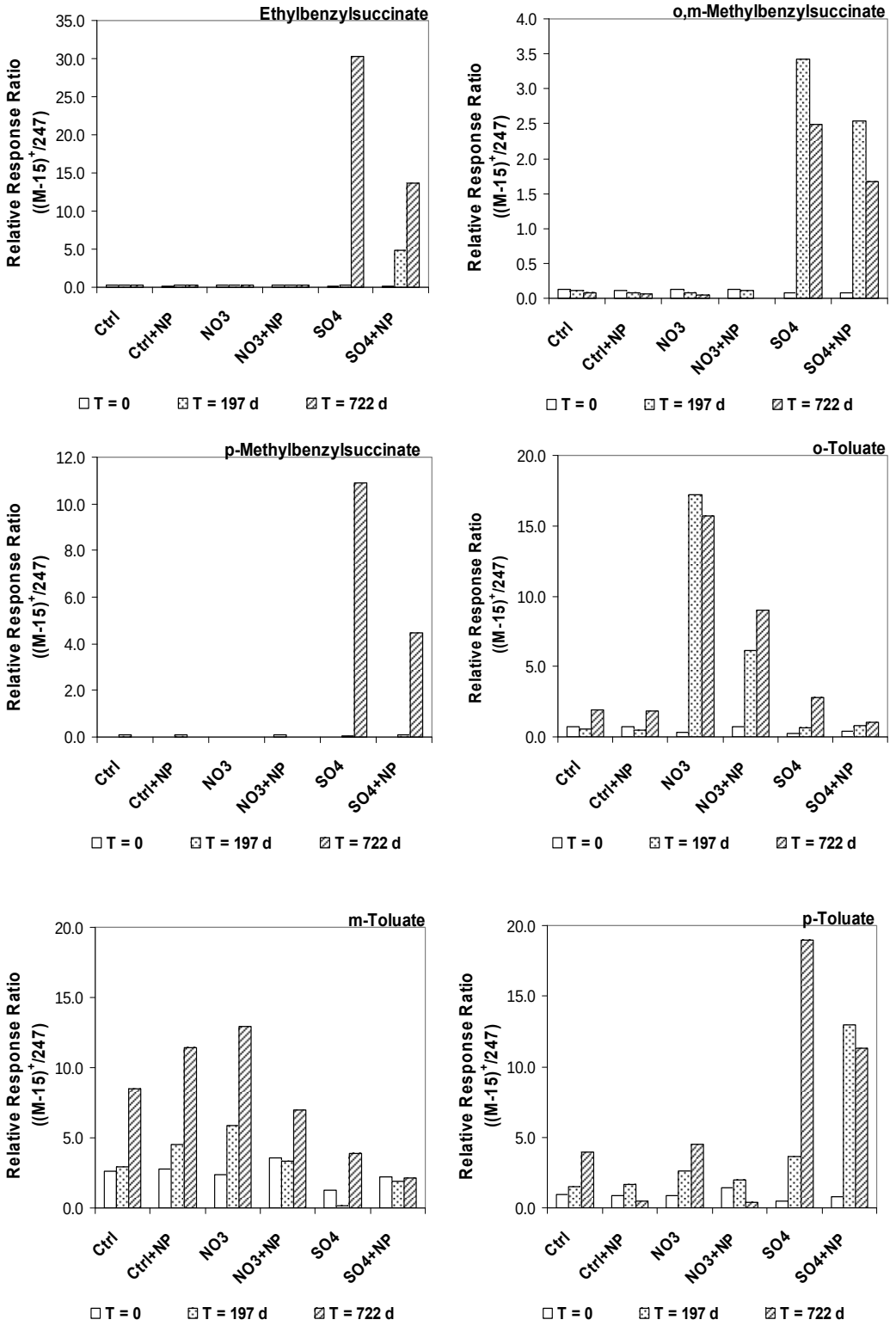

Figure 5-6. Changes in the abundance of metabolites of xylenes (as relative response ratio) with time in Site 3 mesocosms (Note: the figures are not at the same scale; the m-, and o-methylbenzylsuccinate could not be resolved by the GC/MS).

It has been identified that the methylbenzylsuccinate isomers and the toluate isomers are anaerobic biodegradation metabolites of xylenes (Table 5-2). As shown in Figure 5-6, elevated levels of p-methylbenzylsuccinate and p-toluate were observed in Site 3 SO4(+NP) mesocosms. In contrast, higher o-toluate levels were detected in Site 3 NO3(+NP) mesocosms. Ethylbenzylsuccinate was more abundant in SO4(+NP) mesocosms, consistent with literature reports that the pathway yielding this metabolite is dominant under sulfate reducing conditions (Widdel and Rabus 2001). These different patterns indicated that anaerobic biodegradation of PHCs was closely related to specific TEAPs. However, the detection of some metabolites did not appear to be correlated with the depletion of their parent compounds in the mesocosms, as described below.

Two representative examples from the Site 3 mesocosm study are presented in Figure 5-7, which shows little to no change in the concentrations of ethylbenzene and o-xylene and the increases in the abundance of their signature metabolites in Site 3 mesocosms.

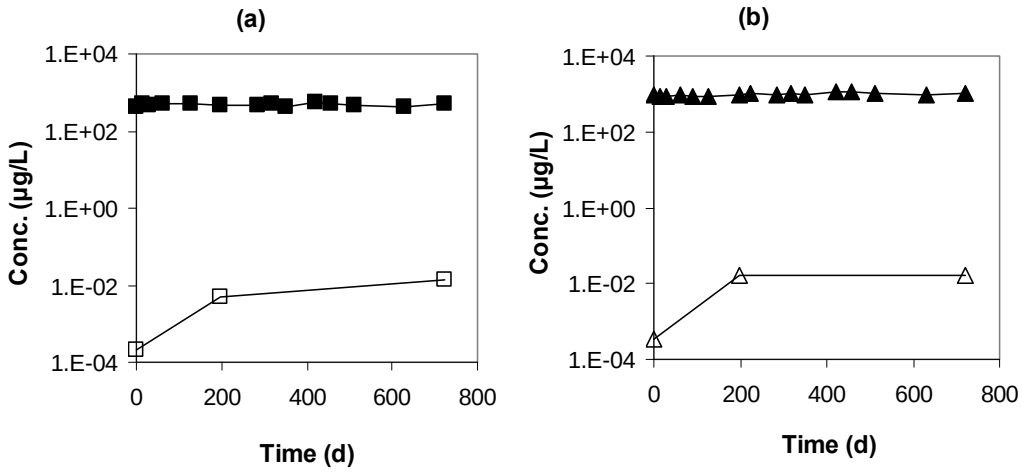

Figure 5-7. Comparison of the changes in the concentrations of parent compounds and the relative abundance of specific metabolites. Where (a): Ethylbenzene (■) vs. ethylbenzylsuccinate (□) in SO4+NP(#6); (b): o-Xylene (▲) vs. o-toluate (Δ) in NO3(#9).

Up to 10-fold increases in the abundance of ethylbenzylsuccinate were detected at the end of the experiment in Site 3 SO4(+NP) mesocosms, however, no apparent decrease in the ethylbenzene concentration was observed (Figure 5-7, Panel (a)). Similarly, the increase in o-toluate was not accompanied by a corresponding decrease in the o-xylene concentrations in Site 3 NO3 (Figure 5-7, Panel (b)) and NO3+NP mesocosms (data not shown). This observation might be explained by the relative magnitudes of the metabolites with respect to the parent compounds. The possible reason for the accumulation of the metabolites is that these metabolites are dead-end metabolites under the specific conditions, or that the metabolites were continuously excreted from the cells and were not taken up efficiently for further metabolism after being released (Beller 2000; Safinowski and Meckenstock 2006).

Although changes of the identified metabolite abundance with time were observed in the mesocosm studies (also shown in Figure 5-6), it is impossible to correlate the changes in the metabolites to the extent of biodegradation processes. Due to limitations of sample volume and considering that metabolite analyses are time consuming and effort demanding, the frequency of metabolite analyses was very low. Metabolites were measured only at Time 0, 6 month after incubation, and the end of incubation, which might not be concurrent with the occurrence of anaerobic biodegradation processes. For instance, the total depletion of toluene in all Site 3 mesocosms and o-xylene in Site 3 SO4(+NP) mesocosms were obtained within 16 to 63 days. Therefore, the metabolite results might not be sufficient to represent the entire anaerobic biodegradation process. Thus, caution should be employed to interpret the metabolite results and correlate the metabolite results to biodegradation processes. To better achieve this type of correlation, metabolite analysis must be done more frequently.

The interpretation of the metabolite results are very complicated due to the fact that complex PHC mixtures are present at the contaminated site and also that some metabolites (benzoate and toluates in particular) may arise from more than

one parent compound. Detection of the metabolites may only suggest the potential of the anaerobic biodegradation processes, but it does not necessarily indicate that the metabolism of the parent compound is ongoing, or occurring to an appreciable extent. On the other hand, no increase in the abundances of the metabolites or even no detection of the metabolites specific to the parent compounds may suggest that there is no accumulation of the intermediate metabolites whereas the anaerobic biodegradation process is still occurring. Therefore, the metabolite analysis cannot be used as a stand-alone, definitive line of evidence for the occurrence of anaerobic biodegradation processes.

5.4 Conclusions and Recommendations

During the long-term laboratory mesocosm studies of enhanced anaerobic biodegradation of PHCs by TEA and/or nutrient amendment, metabolite analyses were conducted using 1 L water samples, which enabled the detection of trace metabolites. A variety of anaerobic metabolites of TEX, alkanes (C_3 to C_9), and naphthalene biodegradation were detected in both studies. The estimated concentrations of the metabolites were at levels of nanomole/L.

Metabolites detected from these two sites showed different patterns, indicating that metabolites were site-specific. The detection of metabolites in the mesocosms and the observed changes of some metabolites over time provided supplementary information on the occurrence of anaerobic metabolism in the mesocosms. The comparison of metabolites between different treatments indicated that the biodegradation of specific PHC compounds was related to certain TEAPs. However, there was no definitive correlation between the abundance of metabolites and the biodegradation of the parent compounds.

These metabolites might be used as indicators of *in-situ* anaerobic PHC metabolism. Together with other lines of evidence, the occurrence of *in-situ* biodegradation could be verified. However, further studies may be necessary to

demonstrate that these metabolites are not dead-end metabolites and are suitable as signature metabolites of *in-situ* anaerobic PHC biodegradation.

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Chapter 6 General Discussion

PHCs are the most widespread soil and groundwater contaminants in Canada. PHC contamination often represents a significant health and environmental risk and PHC contaminated sites should be remediated (CCME 2008). MNA is considered a cost-effective approach for remediation of PHC contamination in the subsurface environment (USEPA 2001). However, under unfavorable site conditions MNA may not be a viable treatment option and some enhancements should be applied so that the remedial goals can be achieved within a reasonable timeframe (ITRC 2008). Both MNA and EA rely on NA processes to degrade or immobilize contaminants. Among all NA processes, anaerobic biodegradation is often the dominant NA mechanism that can result in significant reduction of contaminant mass (Wiedemeier 1995).

This project was a part of the CORONA study to evaluate NA as a viable remedial alternative for PHC contamination at upstream oil- and gas-contaminated sites in Alberta, Canada. Laboratory mesocosm studies were conducted using groundwater and sediment materials collected from two PHC contaminated sites in Alberta to investigate the enhancement of anaerobic PHC biodegradation by TEA and nutrient amendments at each specific site.

The two contaminated sites chosen for the laboratory mesocosm studies have a long history of PHC contamination. Previous site investigations have shown evidence of the anaerobic biodegradation of PHCs at both sites, despite the relatively low groundwater temperatures (approximately 5 °C). The estimated first-order *in situ* attenuation rates of BTEX were of the order of 10^{-4} d^{-1} (Armstrong 2006, personal communication; Armstrong 2008).

The results from each laboratory mesocosm study have been discussed in the previous chapters. In this chapter, an integration and comparison of the Site 1 and the Site 3 mesocosm studies will be presented and the studies will also be related

to the field of anaerobic PHC biodegradation studies. The discussion will focus on the lines of evidence, substrate biodegradability and interactions, the site-specificity of anaerobic biodegradation processes, and the enhancement effects of TEA and nutrient amendments on the anaerobic biodegradation of PHCs. The applicability of mesocosm and sub-sampling methods in the anaerobic biodegradation study will also be discussed.

6.1 Using mesocosms and sub-sampling for anaerobic PHC biodegradation studies

To determine whether NA processes are sufficiently efficient to achieve the remedial objectives at a contaminated site, the site-specific degradation processes should be characterised, quantified and evaluated.

Laboratory microcosm studies are used to confirm specific biodegradation processes that cannot be conclusively demonstrated with field data alone and/or to estimate site-specific biodegradation rates that cannot be conclusively demonstrated with field data alone (Wiedemeier 1995; ITRC 2008). If properly designed, implemented, and interpreted, laboratory microcosm studies can provide convincing documentation of the occurrence of biodegradation processes at a contaminated site. To achieve a rate detection limit of 0.001 to 0.0005 d^{-1}, microcosms with an optimal solid/water ratio should be incubated and sampled for up to 18 months (Wiedemeier *et al.* 1995).

Sacrificial sampling is typically used in laboratory microcosm studies to monitor the anaerobic biodegradation processes. Thus, numerous microcosm replicates are required to be prepared in microcosm studies. However, it has been found that the sample variability between the replicates often hampers the interpretation of experimental results (Wilson *et al.* 1997; Cross *et al.* 2006).

A mesocosm system was therefore developed in this thesis to study anaerobic PHC biodegradation. The use of mesocosms (i.e. microcosms with large volumes,

>10L in this study) allows for the same analyses as with microcosms, but, because of their larger volume, sub-sampling from the same mesocosm is possible. The sub-sampling method used in this study allowed the monitoring of the anaerobic biodegradation processes from the same mesocosm over the experimental period, presumably overcoming some of the variabilities associated with microcosm studies and sacrificial sampling.

The designed mesocosm system appeared to be suitable for the anaerobic biodegradation study. The anaerobic conditions were maintained throughout the long experimental periods (620 days and 722 days, respectively for the two studies). It was also demonstrated that the mesocosm system enabled direct monitoring of multiple lines of evidence. Direct measurements of PHC depletion, TEA consumption, headspace gases, geochemical conditions, microorganisms, and metabolites were all conducted. In paticular, the use of mesocosms allowed the measurement of trace metabolites. The detection of trace metabolites at concentrations of nM/L requires 1 L of samples and would not have been possible if microcosms had been used.

The mesocosms used in these studies were a complex system, in that they contained materials of different phases (sediment, water, and headspace gases) and a variety of biogeochemical processes were occurring concurrently. Some inherent variability and uncertainty was unavoidably associated with this complex system. For instance, the potential partitioning of the PHCs and biogenic gases into the different phases added uncertainty to the data interpretation. The heterogeneity of the sediment also introduced some variability between the mesocosms.

The complexity in the biogeochemical processes resulted mainly from the presence of a complex mixture of PHCs and other organic compounds and the existing dynamic redox conditions in the contaminated sites. The TEA and/or

nutrients amendment in both studies, as well as the methanol addition in Site 1 mesocosm study, added more complexity to the geochemical conditions.

Some experimental variability could be attributed to the lack of experience with the novel setup procedures and some challenges with the analytical instrument. For instance, autoclaving is the preferred sterilization method for long-term laboratory studies (Wilson *et al*. 1997). However, the autoclaving resulted in losses of PHCs in the groundwater samples. Although efforts was exerted to replenish the concentrations of the target PHCs using sterilized free product in both Site 1 and Site 3 mesocosm studies, the achieved BTEX and F1 concentrations in the SCs were very low so that the SCs could not be used to differentiate the abiotic losses of PHCs. Further improvements of the experimental method are required to reduce the experimental variability.

6.2 Lines of evidence for anaerobic PHC biodegradation in the mesocosm studies

The anaerobic biodegradation of PHC compounds coupled to reduction of different TEAs will generate representative "footprints", i.e. the stoichiometric consumption of PHCs and TEAs, production of the byproduct compounds, and microbial growth (NRC 2000; Maurer and Rittmann 2004). Bhupathiraju *et al*. (2002) used the following lines of evidence for assessment of *in-situ* bioremediation at PHC contaminated sites: (1) decreasing PHC concentrations, (2) TEA reduction and production of their reduced products, (3) detection of metabolites, and (4) distinct elevation of biomass concentration and activities. In this mesocosm study, all the lines of evidence were adopted to investigate the anaerobic PHC biodegradation processes.

6.2.1 PHC removal

Both biodegradation and abiotic processes may contribute to the PHC removal in laboratory biodegradation studies. The stoichiometric consumption of PHCs accompanied by depletion of TEAs and production of byproducts can be used to

verify the occurrence of biodegradation processes (NRC 2000). Sterile controls can also be used to differentiate the biodegradation removal from the abiotic losses of PHCs (Wilson *et al*. 1997). After the abiotic PHC losses has been identified and quantified, the depletion of PHCs attributed to biodegradation processes can be quantified and the biodegradation rates can be determined.

The depletion of BTEX and $F1_{-BTEX}$ compounds were observed in both Site 1 and Site 3 mesocosm studies. At the end of the incubation time (Day 620) in the Site 1 mesocosm study, high percentage removals of BEX were obtained in all non-sterile mesocosms. Approximately 90% removal of $F1_{-BEX}$ was observed in the sulfate amended mesocosms, compared to only 22 to 56% in other non-sterile mesocosms. In the Site 3 mesocosm study, the percentage removal at the end of incubation (Day 722) varied from 0 to 100 % for different PHCs under different reducing conditions. Unfortunately, the depletion of PHCs attributed to abiotic attenuation processes could not be identified due to the low concentrations of the target PHCs in the SCs. Mass balance calculations were also impossible because of the complexity of the mesocosm system in this study. However, the depletion of PHCs was attributed to the biodegradation processes for the following reasons:

(1) The losses of PHCs due to sorption and volatilization were deemed minimal, since (i) the materials used in the construction of the mesocosms (i.e. glass containers, Teflon stoppers and stainless steel) would result in minimal adsorption of the PHCs to the testing system itself; (ii) the sedimentwas low in organic content, thus low in sorption potential; and (iii) theoretical calculations showed that the volatilization of BTEX into the headspace was negligible.

(2) For ethylbenzene and m- and p-xylenes, which have relatively higher Henry's Law constants and K_{oc} values as compared to other BTEX compounds (as shown in Table 2-1) and thus are more susceptible to abiotic losses, the concentration of these compounds in some Site 3 mesocosms remained constant throughout the

experiment (0% removal at the end of the incubation), suggesting that abiotic processes such as sorption and volatilization did not contribute to the PHC losses.

(3) Other lines of evidence indicated the occurrence of anaerobic biodegradation of PHCs in both mesocosm studies.

6.2.2 TEA utilization

Rapid depletion of amended TEAs was observed in the non-sterile mesocosms in both Site 1 and Site 3 mesocosm studies, in comparison with the slight or no changes in concentrations of NO_3^- and SO_4^{2-} in the SCs, indicating that the TEA depletion was due to the biodegradation processes.

The consumption of TEAs coupled to BTEX and F1 biodegradation (estimated from the utilization factors suggested in the US-EPA BIOSCREEN Model) contributed to a small portion of the total TEA depletion. The nonstoichiometric reduction of NO_3^- and SO_4^{2-} coupled to BTEX biodegradation has been reported in both field (Schreiber and Bahr 2002; Cunningham *et al*. 2001) and laboratory microcosm studies (Ball and Reinhard 1996; Dou *et al.* 2008a). The observed stoichiometry between the utilization of NO_3^- and BTEX degradation was influced by whether NO_3^- is reduced to NO_2^- or N_2 or both (Dou *et al.* 2008a). The nonstoichiometric utilization of NO_3^- and SO_4^{2-} was mainly attributed to the oxidation of other organic compounds in the aquifer (Cunningham *et al*. 2001; Schreiber and Bahr 2002).

In both Site 1 and Site 3 studies, the presence of other organic compounds in the groundwater samples and their biodegradation might have contributed to the observed TEA depletion. Therefore, the TEA utilization, in both mesocosm studies, could only be used as an indicator of the occurrence of anaerobic biodegradation processes but could not be used to quantify the anaerobic biodegradation of the target PHCs.

6.2.3 Production of biogenic gases and alkalinity

The generation of biogenic gases and alkalinity can be used as indicators of the biodegradation processes. In the Site 1 mesocosm study, the production of CH_4 was a definitive evidence of the occurrence of the methanogenesis in the methanol-added, SO4(+NP) and Ctrl(+NP) mesocosms. Unfortunately the production of biogenic N_2 gas could not be used to identify the denitrification process in the mesocosm studies, due to the introduction of N_2 gas during sampling to equilibrate the headspace pressure. If argon or other inert gases were used for this purpose, the biogenic N_2 production might serve as an indicator of denitrification process.

In general, the total alkalinity increases with the PHC biodegradation because of the production of CO_2 in the biodegradation processes. Changes in alkalinity are most pronounced during nitrate reduction, iron reduction, and sulfate reduction and less pronounced during methanogenesis (Morel and Hering 1993).

In this study, the biogenic CO_2 production and the alkalinity were difficult to quantify because of the closed multi-phase system and the sub-sampling method. Many factors should be taken into account when interpreting the data, including the volume changes in the headspace, the introduction of the N_2 gas, and the relationship between the CO_2 and alkalinity. In both Site 1 and Site 3 mesocosm studies, the combining evidence of the estimated cumulative CO_2 amounts in the headspace and measured alkalinity supported CO_2 production and therefore provided evidence that biodegradation processes were occurring in the non-sterile mesocosms.

6.2.4 Occurrence of an active microbial population

The MPN technique has been used to characterize the biodegradation processes at some contaminated sites (Christensen *et al.* 2000). Previous studies have shown that higher numbers of SRBs occurred in the sulfate-reducing zone and higher numbers of methanogens were observed where methane production was observed

(Harris *et al.* 1999; Ludvigsen *et al*. 1999). However, little success has been achieved to correlate the different bacteria groups with the dominant redox processes (Essaid *et al*. 1995; Ludvigsen *et al*. 1999). Kao and Borden (1997) found no correlation between the number of NRBs enumerated by the MPN technique and the degradation rates of PHCs under nitrate reducing conditions.

The MPN analysis in these mesocosm studies did not prove to be useful evidence for the occurrence of the anaerobic PHC biodegradation. The MPNs of the redox-specific bacteria did not appear to increase with the anaerobic biodegradation processes in either Site 1 or Site 3 mesocosms. The MPN of methanogens were below the detection limits even when significant methanogenesis appeared to be occurring in some Site 1 mesocosms, in which up to 10 % headspace CH_4 concentration was detected. There was no correlation between the MPN results and the anaerobic biodegradation processes, possibly attributed to the fact that the PHC biodegradation could not support the bacteria growth, or some inherent limitations of the MPN technique. Low microbial growth rates have been observed in the PHC biodegradation under sulfate-reducing conditions due to small energy yields (Aeckersberg *et al*. 1998; Rabus *et al*. 1993; Beller *et al*. 1996). The MPN technique is biased toward culturable organisms and may also be limited by the growth conditions, i.e. the choice of medium, substrate, and the incubation conditions (Christensen *et al.* 2000).

There are two principal assumptions for the MPN technique: (1) random distribution of the organisms in the sample; and (2) one single organism will exhibit growth when incubated in the culture medium (Cochran 1950). In the mesocosm study described here, the MPN analysis was conducted on a slurry sample of groundwater and sediment. If there were clusters of bacteria in the sediments, the MPN results might underestimate the number of organisms present.

6.2.5 Additional evidence of anaerobic PHC degradation: Signature metabolites

Signature metabolites have been proposed as indicators of occurrence of the anaerobic biodegradation (Beller *et al*. 1995; Phelps *et al.* 2002; Griebler *et al.* 2004). For example, the alkylbenzylsuccinates have been adopted as indicators of anaerobic BTEX biodegradation (Foght 2008). The metabolites are typically detected at concentrations several orders of magnitude lower than the parent compounds (Smets and Pritchard 2003; Griebler *et al*. 2004). The detection of trace metabolites involved solvent extraction of relatively large volumes of groundwater with subsequent derivatization and GC-MS analysis (Elshahed *et al.* 2001; Gieg *et al*. 1999). These methods are well established and definitive, but can be costly and time-consuming (Foght 2008). Because of their transient nature (Beller 2000), the signature metabolites should be interpreted in conjunction with other chemical and biological evidence (Gieg *et al.* 1999; Elshahed *et al*. 2001).

In this study, metabolites were analyzed using a modification of the methods described by Gieg and Suflita (2002). Extraction of 1 L of samples enabled the detection of trace metabolites. In both Site 1 and Site 3 mesocosm studies, anaerobic metabolites of BTEX, C_3 to C_9 alkanes, and naphthalene biodegradation were detected at concentrations of nanomole/L. The metabolites identified from the Site 1 mesocosm study, excluding the naphthalene metabolites, were consistent with the published field results (Gieg and Suflita 2002). A variety of anaerobic metabolites were detected from the mesocosm studies at Time 0, suggesting the occurrence of *in-situ* anaerobic biodegradation at both sites.

The changes of metabolite abundance over time were observed in both mesocosm studies. The accumulation of metabolites in the growth medium has been found in some previous studies (Beller 2000; Safinowski and Meckenstock 2006). It was speculated that the metabolites were continuously excreted from the cells and were not taken up efficiently for further metabolism after being released. The mechanism of the excretion and uptake of the metabolites may affect the biodegradation of the parent compounds (Foght 2008).

There was no definitive correlation between the anaerobic biodegradation of the parent compounds and the changes in the metabolite concentrations. In Site 3 NO3(+NP) mesocosms, the increase in the o-xylene metabolite abundance was not accompanied by the depletion of o-xylene. This might be explained by the fact that the concentrations of metabolites were several orders of magnitude lower than the parent compound concentrations or that these metabolites are dead-end metabolites under the specific conditions. The production of dead-end metabolites through cometabolism has been reported previously in anaerobic biodegradation of naphthalene (Safinowski and Meckenstock 2006) and xylenes (Beller *et al*. 1996; Beller 2000). Furthermore, due to limitations of the sample volume and other laboratory resources, the metabolite analysis was conducted only at Time 0, at 6 months after incubation and at the end of incubation. Therefore, the limited analysis of metabolite abundance might not be sufficient to represent the biodegradation processes.

Technical difficulties with inconsistent detection and identification of signature metabolites still hamper characterization of *in situ* anaerobic biodegradation (Foght 2008). The interpretation of metabolite results can be very complex, especially for sites contaminated with PHC mixtures for a long history. From the mesocosm studies, detection of the metabolites seems appears to indicate the potential of anaerobic biodegradation, but does not necessarily indicate that the metabolism of the parent compound is ongoing, or occurring to an appreciable extent. Therefore, in this study, the metabolite analysis cannot be used as a stand-alone line of evidence. However, coupled with the analysis of substrate and TEA loss, the detection of signature metabolites can be used to verify the occurrence of anaerobic biodegradation of specific PHCs.

6.2.6 Summary

In summary, multiple lines of evidence verified the anaerobic biodegradation of BTEX and $F1_{-BTEX}$ PHCs in these laboratory mesocosm studies. No single line of

evidence can be used as definitive evidence for the anaerobic biodegradation of the target PHCs because of the complexity of the mesocosm system. MPN and metabolite analyses cannot be used as a stand-alone line of evidence for the anaerobic biodegradation processes. The field conditions in the contaminated sites are often more complex compared to the mesocosms. Multiple lines of evidence must be carefully investigated to obtain a reliable interpretation on the *in situ* anaerobic PHC biodegradation.

6.3 Anaerobic biodegradation of BTEX and F1

The anaerobic biodegradation of the target PHC compounds appeared to be influenced by its relative biodegradability, the substrate interactions, the predominant TEAPs, and other site-specific conditions in this study.

6.3.1 Substrate biodegradability

PHC contaminants typically contain a complex mixture of different compounds at the contaminated sites. The PHC composition at a contaminated site is a function of the source, site conditions, and time since release (CCME 2008). Initial oxidation of individual PHCs under anaerobic conditions largely depends on the chemical structure of the compound (Wiedemeier *et al.* 1995). Although the contaminants can be quite similar in structure, their respective biodegradability under different reducing conditions could be different. For instance, the differential biodegradibility of xylene isomers (Morasch *et al*. 2004) and ethyl- and dimethyl-substituted naphthalenes (Townsend *et al.* 2003) to anaerobic biodegradation has been reported.

Anaerobic biodegradation of each BTEX compound has been well documented under a variety of reducing conditions (Widdel and Rabus 2001; Chakraborty and Coates 2004). It has been found that the biodegradability of individual BTEX compounds was different. Among the BTEX compounds, toluene is the most readily degraded under all reducing conditions among BTEX compounds, whereas many studies have indicated that benzene persists under anaerobic

conditions (Langenhoff *et al.* 1996; Phelps and Young 1999; Anderson and Lovley 2000). The occurrence of benzene biodegradation appears to be more site-specific (Johnson *et al*. 2003; Nales *et al*. 1998) and the biodegradation of benzene is usually slow, incomplete and subject to long lag times (Edwards and Grbic-Galic 1992). Earlier work suggested that the biodegradation of BTEX under denitrifying conditions occured in the following order: toluene > p-xylene > m-xylene > ethylbenzene > o-xylene (Norris *et al.* 1994). Dou *et al.* (2008a) reported that the biodegradability decreased with toluene > ethylbenzene > m-xylene > o-xylene > benzene > p-xylene under both nitrate reducing and sulfate reducing conditions.

In the Site 1 mesocosm study, there were long lag periods before the onset of the benzene degradation under all reducing conditions. However, there was no apparent difference in the first-order biodegradation rates of benzene, ethylbenzene, and m-, and p-xylenes under different reducing conditions. The results indicate that biodegradation of $F1_{-BEX}$ was more favourable under sulfate-reducing conditions. It should be noted that the variability associated with the methanol addition interfered with the interpretation of the biodegradation data (further discussion in Section 6.3.2).

No apparent lag periods were observed for all BTEX compounds in the Site 3 mesocosm study. Toluene was readily biodegraded under all reducing conditions in the Site 3 mesocosms, whereas selective biodegradation of BEX compunds under different TEAPs was observed. Although anaerobic biodegradation of xylenes coupled to nitrate reduction has been observed (Rabus and Widdel 1995; Hess *et al.* 1997), all xylenes were not biodegraded under nitrate reducing conditions in the Site 3 mesocosm study. This was also contrary to the Site 1 mesocosm study, in which m- and p-xylenes were biodegraded under nitrate reducing conditions (o-xylene was not present in the Site 1 goundwater samples), further indicating that anaerobic biodegradation could be TEA and site-specific.

Under sulfate reducing conditions, the biodegradation rates of BEX appeared to increase with benzene < m-, and p-xylenes < o-xylene in the Site 3 mesocosm study. It was found in this study that the anaerobic biodegradation of o-xylene was sulfate-dependent. It also appeared that nitrate amendment inhibited the sulfate-dependent o-xylene degradation in the Site 3 nitrate-amended mesocosms (in which SO_4^{2-} was available at background concentrations in the contaminated groundwater). Previous studies have shown anaerobic o-xylene biodegradation coupled to sulfate reduction (Edwards *et al*. 1992; Jahn *et al*. 2005).

6.3.2 Substrate interactions

The interactions between different compounds in a PHC mixture also influence the biodegradation of individual compounds. For instance, it has been found that benzene is subject to inhibition by co-contaminants, such as toluene (Johnson *et al.* 2003) or other BTEX compounds (Nales *et al*. 1998). Substrate interactions have often been observed for the anaerobic biodegradation of BTEX mixtures, but there appeared to be no general rule for predicting the substrate interactions (Dou *et al.* 2008b). Previous studies have shown inhibition of anaerobic BTEX degradation by toluene (Phelps and Young 1999; Da Silva and Alvarez 2004) and some competitive utilization among BTEX compounds (Barbaro et al. 1992) under different reducing conditions. Dou *et al*. (2008b) found that the presence of one BTEX compound may either stimulate or inhibit the degradation of other BTEX compounds, depending on the individual compounds and their concentrations. In this mesocosm study, it was difficult to identify the substrate interactions due to the complexity of the mesocosm system and substrate interactions.

In the Site 3 mesocosm study, there was evidence suggesting that the degradation of o-xylene was cometabolized with toluene. Cometabolism of PHC compounds may induce stimulatory substrate interactions. It has been found that cometabolism of o-xylene in the presence of toluene appears to be common in anaerobic systems (Evans *et al.* 1991a; Alvarez and Vogel 1995). However, the

effects of cometabolism on the biodegradation processes are complex and unpredictable (Atlas 1995). The cometabolic pathway may only produce dead-end products and not support growth (Beller 2000). In addition, the question of toxicity of the dead-end cometabolites to the microorganisms has not yet been addressed (Foght 2008).

The effects of other hydrocarbons or other non-hydrocarbon organic compounds on the biodegradation of BTEX could be either inhibitory (Ruiz-Aguilar *et al.* 2002) or stimulatory (Prince and Suflita 2007). Ruiz-Aguilar *et al.* (2002) suggested that the preferential biodegradation of ethanol accelerated the depletion of available nutrients and TEAs, thus hindered BTEX removal. However, Corseuil *et al.* (1998) found that ethanol enhanced toluene degradation under sulfate-reducing conditions, possibly due to the incidental growth of toluene degraders during ethanol degradation. In the Site 1 mesocosm study, it was found that the methanol addition inhibited or delayed the anaerobic biodegradation of BEX and $F1_{-BEX}$. Grbic-Galic and Vogel (1987) also found that the presence of methanol in a culture fluid slowed toluene and benzene biodegradation under methanogenic conditions.

In the Site 1 mesocosm study, the methanol addition resulted in methanogenic conditions in Ctrl(+NP) and SO4(+NP), but not in nitrate-amended mesocosms. Since PHC biodegradation appeared to be selective under different TEAPs (Wiedemeier *et al.* 1995; Suarez and Rifai 1999), this shift in the TEAPs might also have influenced the biodegradation of individual compounds.

6.3.3 Effects of site-specific conditions on the mesocosm studies

The dominant biodegradation processes are ultimately determined by the environmental conditions and the microbial competition at a contaminated site (Wiedemeier *et al.* 1995). The two studied sites both had a long history of PHC contamination, but showed different site conditions and thus different characteristics of *in situ* attenuation processes. Comparison of the Site 1 and the

Site 3 mesocosm studies indicated that the differences in the composition and concentration of the PHCs and the different dynamics of the TEAPs affected the biodegradation processes in each of the Site 1 and Site 3 mesocosm studies.

Site 1 was a former flare pit and the PHC contaminants were composed of C_6 to C_{30} hydrocarbons. The contamination source in Site 3 was natural gas condensate, and thus the contaminants were composed of the lighter end PHCs ($\leq C_{10}$). In addition to the difference in the compositions of the PHCs, the concentrations of the PHCs were also different at these two sites. Accordingly, the initial concentrations of the target PHCs in the mesocosms were different. In Site 1 non-sterile mesocosms, the initial BTEX and $F1_{-BTEX}$ concentrations were approximately 600 µg/L (composed of 400 µg/L benzene, 40 to 90 µg/L ethylbenzene and 80 µg/L m-, p-xylenes) and 2.4 mg/L respectively. In contrast, the initial concentrations in all non-sterile Site 3 mesocosms were approximately 17 mg/L BTEX (composed of 7 mg/L benzene, 3 mg/L toluene, 1 mg/L ethylbenzene, 5 mg/L m-and p-xylenes, and 1 mg/L o-xylene) and 21 mg/L F1 hydrocarbons.

In this study, a higher percentage removal of target PHCs at higher degradation rates was observed in the Site 1 mesocosms. At the end of the incubation period, total removal of BEX was achieved in the Site 1 study except for Ctrl+NP(#8) mesocosm (620 days), in comparison to low percentage removal of BEX in the Site 3 mesocosom study (722 days). The estimated first-order rates for anaerobic BEX biodegradation were also higher in Site 1 mesocosms, ranged from 0.0032 to 0.033 d^{-1} for benzene, 0 to 0.028 d^{-1} for ethylbenzene, and 0.0021 to 0.036 d^{-1} for m-, and p-xylenes, as compared to 0 to 0.0009 d^{-1} for benzene, 0 to 0.011 d^{-1} for ethylbenzene, and 0 to 0.0016 d^{-1} for m- and p-xylenes. In Site 3 mesocosms, rapid depletion of toluene was observed in all non-sterile mesocosms, and rapid biodegradation of o-xylene was observed in SO4(+NP) mesocosms (no presence of toluene and o-xylene in the Site 1 groundwater). The first-order biodegradation rates for o-xylene varied from 0 to 0.15 d^{-1} in Site 3 mesocosms. The estimated

first-order biodegradation rates from these two studies were within the range reported in the literature (Aronson and Howard 1997; Suarez and Rifai 1999).

The composition and concentrations of PHCs are important factors influencing the anaerobic biodegradation processes. As discussed in the previous sections, the composition of the PHC mixtures affects the biodegradation processes due to the different biodegradability of individual compounds and the complex substrate interactions. Sikkema *et al.* (1995) identified that toxicity and inhibition of microorganisms may occur at high PHC concentrations. Evans *et al*. (1991b) found that the lag period increased as a function of toluene concentration, and high toluene concentrations inhibited cell growth.

The dominant TEAPs were also different at these two contaminated sites. Geochemical data at Site 1 showed depleted NO_3^- and SO_4^{2-} and elevated concentrations in dissolved Fe(II), Mn(II) and methane (CH_4); whereas at Site 3 high background SO_4^{2-} concentrations were detected, and sulfate reduction appeared to be the dominant TEAP with iron reduction likely also occurring at Site 3 (Petersmeyer 2006). The dynamics of the TEAPs in the Site 1 and the Site 3 mesocosm studies were different, partially due to the site-specific conditions, and partially due to the difference in the methodologies, e.g. higher SO_4^{2-} amendment concentrations in Site 3 mesocosm study and methanol addition in the Site 1 study. The dominant TEAPs in the Ctrl(+NP) mesocosms were postulated to be iron reduction in Site 1 study, and sulfate reduction (before the depletion of the background SO_4^{2-}) and iron reduction in Site 3 mesocosm. In TEA amended mesocosms, the dominant TEAP shifted to nitrate reducing or sulfate reducing accordingly, after NO_3^- or SO_4^{2-} amendment in both studies, but other TEAPs were also identified. For example, the methanol addition induced methanogenic conditions in Site 1 SO4(+NP) and Ctrl(+NP) mesocosms. The TEAP dynamics may have affected the anaerobic biodegradation processes in these two mesocosm studies.

The metabolites appeared to be reflective of the site-specific anaerobic biodegradation of PHC compounds in the Site 1 and the Site 3 mesocosm studies. Metabolites identified in Site 1 mesocosms included alkylsuccinates from C_4 to C_6 alkanes and C_7 to C_8 alkanes with unsaturation, metabolites of anaerobic biodegradation of ethylbenzene and m-, and p-xylenes. In Site 3 mesocosms, the presence of alkylsuccinates from anaerobic biodegradation of C_3 to C_9 alkanes and metabolites from anaerobic biodegradation of toluene, ethylbenzene and all three xylenes was identified. Metabolites of anaerobic naphthalene biodegradation were detected in both studies. Overall, more species of metabolites were identified in Site 3 mesocosms, and at higher abundances.

6.4 TEA Amendments

The mechanisms for enhancing anaerobic biodegradation by TEA amendment are to increase the concentrations of the available TEAs that could be utilized for the PHC biodegradation, and to shift the *in situ* TEAPs to a higher energy yield or more efficient TEAP that could stimulate the PHC biodegradation (Wiedemeier *et al*. 1995; ITRC 2008).

Because of the high water solubility, both NO_3^- and SO_4^{2-} can be added at very high concentrations, thus can provide high electron accepting capacities. However, high NO_3^- concentrations in drinking water may represent an environmental health concern. The Canadian guideline for NO_3^- in drinking water is 45 mg/L (Health Canada 2008). Therefore, the NO_3^- amendment concentrations may be constrained at specific sites. SO_4^{2-} is non-toxic and generally abundant in geologic formations. However, the sulfide produced from sulfate reduction could be toxic, if there is not enough reduced metal ions to precipitate the excessive sulfide. Relatively high concentrations of hydrogen sulfide have been shown to be inhibitory to BTEX biodegradation under sulfate-reducing conditions (Reinhard *et al.* 1997).

Previous studies have shown that addition of NO_3^-, SO_4^{2-}, or a combination of both NO_3^- and SO_4^{2-} as TEAs into the contaminated groundwater can enhance *in-situ* PHC biodegradation and is capable of partially or completely removing BTEX (Anderson and Lovley 2000; Cunningham *et al*. 2000; Cunningham *et al*. 2001). However, the *in-situ* enhancement effects of TEA amendment could be very specific to the contaminated site (ITRC 2008).

In the Site 1 and Site 3 mesocosm studies, nitrate reduction or sulfate reduction was identified to be the dominant TEAP in the respective TEA-amended mesocosms. Other TEAPs were occurring concurrently in some TEA-amended mesocosms. For example, iron reduction appeared to be an important TEAP in both studies. Methanogenic conditions were established in those Site 1 SO4(+NP) mesocosms added with methanol. Due to the high background concentrations of SO_4^{2-} at Site 3, the shift and dynamics of TEAPs appeared to be quite complex in the nitrate amended Site 3 mesocosms. In these mesocosms, the nitrate amendment inhibited the SRB activity and consequently the degradation of xylene isomers which was coupled to sulfate reduction. On the other hand, SO_4^{2-} was produced after nitrate amendment through autotrophic denitrification process in these nitrate amended mesocosms, which may serve as an alternative TEA when nitrate is completely depleted.

The estimated first-order TEA reduction rates from both studies suggested that other easily biodegradable organic compounds or other geochemical processes also contributed to the TEA reduction, and thus affected the effectiveness of the TEA amendment. Therefore, the effective electron accepting capacity achieved after any TEA amendment should be evaluated at the specific contaminated sites.

In the Site 1 mesocosm study, it was found that the anaerobic biodegradation of $F1_{\text{-BEX}}$ was enhanced by sulfate amendment. The first-order rates obtained from the laboratory mesocosm study were higher than the estimated *in-situ* attenuation rates, indicating the potential for enhanced anaerobic biodegradation at the

contaminated site. However, when comparing the TEA-amended mesocosms with the unamended controls, in which iron reduction might be the predominant TEAP, there was no enhancement effect on BEX biodegradation by amendment of either NO_3^- or SO_4^{2-}. The laboratory manipulation may have increased the bioavailability of ferric iron in the unamended controls and resulted in a stimulatory effect on the anaerobic biodegradation. Lovley *et al.* (1994) showed enhanced biodegradation of aromatic hydrocarbon by increasing the bioavailability of Fe(III).

The Site 3 mesocosm study showed no conclusive evidence that NO_3^- or SO_4^{2-} amendment could enhance the anaerobic biodegradation of benzene and ethylbenzene. The estimated first-order biodegradation rates for benzene and ethylbenzene were comparable to the observed field attenuation rates. However, it was conclusive in Site 3 mesocosm study that sulfate amendment enhanced the biodegradation of xylenes. The fact that all xylenes were not biodegraded under nitrate reducing conditions demonstrated that higher energy-yield TEAP was not necessarily more efficient for anaerobic biodegradation of certain PHC compounds.

Due to the variability within the mesocosms, i.e. sediment heterogeneity and presence of other organic compounds (including the added methanol in the Site 1 mesocosms), the enhancement effects of TEA amendment cannot be quantified in both the Site 1 and the Site 3 mesocosm studies. The comparison of these two studies showed that the effects of TEA amendment are site specific and that no TEAP will be universally beneficial for the anaerobic biodegradation of all PHCs at the contaminated sites. Simple addition of TEAs to attempt to enhance biodegradtion will likely have unpredictable and unintended consequences at a contaminated site.

6.5 Nutrient Amendments

Nutrient amendment has been applied to stimulate the biodegradation process at PHC-contaminated sites. Although in most cases nutrient addition stimulates aerobic biodegradation of hydrocarbons, sometimes the addition of nitrogen inhibits the aerobic biodegradation of aromatic and aliphatic hydrocarbons (Alexander 1994). Braddock *et al*. (1997) showed that the degradation rates were inversely proportional to the absolute amount of nutrients added for a N : P ratio of around 2 in Arctic soils. There have been few studies on the effects of nutrient addition on anaerobic biodegradation and the findings were inconsistent. Powell *et al.* (2006) reported the stimulatory effect of nutrients on denitrifying hydrocarbon degraders in nutrient-poor Antarctic soils. Cross *et al.* (2006) also observed enhanced anaerobic degradation of PHCs by nutrient amendment in a laboratory microcosm study. On the contrary, Johnston *et al.* (1996) found no stimulation effects of nutrient amendment on the anaerobic biodegradation of alkylbenzenes in aquifer sediment.

In the Site 1 and the Site 3 mesocosm studies, $NH_4H_2PO_4$ was added as the source of N and P (i.e. macronutrients required for microbial growth during the anaerobic biodegradation processes) to investigate the effects of nutrient amendment on the anaerobic PHC biodegradation. Although NO_3^- was added as the TEA amendment, it might also serve as a source of nitrogen nutrient. There were previous studies on the effect of the type of nitrogen sources on the aerobic biodegradation. Wrenn *et al*. (1994) found that biodegradation of crude oil begins more quickly when nitrogen was supplied as NH_4^+ than when it was supplied as NO_3^-, but the acid production accompanied by NH_4^+ utilization could inhibit the rate of oil biodegradation or cause it to cease entirely under some conditions. On the contrary, Kwapisz *et al.* (2008) found that there was no significant difference in biomass production in media with NH_4^+ or NO_3^- ions as the nutrient. In these studies, the rapid depletion of NO_3^- indicated that NO_3^- was utilized mainly as a TEA.

Although changes in the PO_4^{3-} and NH_4^+ concentrations were observed in the mesocosms, there was no apparent enhancement effect of nutrient amendment on the anaerobic biodegradation of target PHCs in both studies. In the Site 1 mesocosm study, decreases in PO_4^{3-} and NH_4^+ concentrations were observed in non-sterile and SC mesocosms, indicating possible abiotic losses. Many geochemical reactions, such as adsorption, surface complexation and/or precipitation, could cause a decrease in the PO_4^{3-} concentration (Appelo and Postma 2005). Cation exchange with the sediment may cause a decrease in NH_4^+ concentrations (Erskine 2000; Appelo and Postma 2005). In the Site 3 mesocosm study, the NH_4^+ concentrations were constant in all nutrient amended mesocosms. There was a slight decrease in PO_4^{3-} concentration in SC and SO4+NP mesocosms, in comparison to a gradual decrease in PO_4^{3-} concentration in Ctrl+NP and NO3+NP mesocosms, possibly due to some abiotic processes.

There are several possible reasons why there was no enhancement effect of nutrient amendment on the anaerobic biodegradation of the target PHCs in the mesocosm studies. The results may simply indicate that nutrients were not limiting at these two contaminated sites. Recent laboratory and field research have found that nutrient levels, and their relative concentrations, influence the composition of hydrocarbon-degrading microbial populations, which in turn influences the biodegradation rate of aliphatic and aromatic hydrocarbons (Head and Swannell 1999). It is possible that the nutrient concentrations and their relative ratio applied in the mesocosm studies might not be optimal for the anaerobic biodegradation. Furthermore, the occurrence of some geochemical reactions might have reduced the bioavailbility of the amended nutrients, particularly phosphate, to the microorganisms.

6.6 References

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Chapter 7 Conclusions and Recommendations

7.1 General Conclusions

Laboratory microcosms and sacrificial sampling have been commonly used to demonstrate the occurrence of the anaerobic biodegradation at contaminated sites and to estimate the biodegradation rates. However, sample variability between small individual samples in the microcosm study may hamper interpretation of experimental results (Biggar *et al.* 1998; Cross *et al.* 2003). The designed large-volume mesocosms (>10 L) used in these laboratory studies made it possible to sub-sample from the same mesocosm multiple times for a relatively long experimental period. This sub-sampling method allowed the monitoring of substrate and TEA depletion and generation of biodegradation byproducts from the same mesocosm over the long experimental time period. Therefore, the variability associated with small microcosms could be mitigated.

It was demonstrated from these studies that the designed laboratory mesocosms were applicable for the study of anaerobic biodegradation processes. Anaerobic conditions were obtained and well maintained during the two years of experimental period. Nitrogen gas (N_2) was employed to obtain the anaerobic conditions during mesocosm setup. During each sub-sampling event, N_2 was also introduced into the mesocosm to prevent the build-up of negative pressure due to withdrawal of water samples and to equilibrate the headspace. The disadvantage of this protocol is that the biogenic N_2 gas production, a possible end product of nitrate reduction process, could not be differentiated. Another inert gas, such as argon for example, might be used instead of N_2 to maintain the anaerobic conditions and the headspace pressure.

The mesocosm was a very complex system. Some inherent variability and uncertainty was unavoidably associated with the complex mesocosm system and rendered some uncertainty to the data interpretation.

Multiple lines of evidence verified that anaerobic biodegradation of target PHC compounds were occurring in both Site 1 and Site 3 laboratory mesocosm studies. However, due to some site-specific conditions, the biodegradation processes showed different characteristics.

Both sites have a long history of contamination by PHC mixtures. However, the composition and concentrations of PHCs were different at these two sites. Site 1 groundwater was contaminated by heavier PHCs (C_6 to C_{30}) at relatively low concentrations, whereas the groundwater at Site 3 was contaminated by light end PHCs ($\leq C_{10}$) at much higher concentrations.

The predominant TEAPs were identified by depletion of TEAs, production of specific byproducts, and the presence of according microorganisms. Shift of TEAPs due to the available background TEAs and the TEA amendment was dynamic, which will have great implications in the enhanced anaerobic biodegradation practice. The background sulfate concentrations at Site 3 provided an opportunity to look into the interactions between the nitrate amendment and the sulfate amendment. It was found that nitrate amendment inhibited the sulfate reduction and the sulfate-dependent o-xylene biodegradation. Nitrate amendment also induced the sulfide oxidation and sulfate production through autotrophic denitrification process.

Removal of BTEX and F1 were observed from in Site 1 and Site 3 mesocosm studies under different reducing conditions. Higher percent removals of BEX were obtained at the end of incubation in Site 1 meosocosms as compared to Site 3 mesocosms. Higher first-order biodegradation rates of benzene, ethylbenzene, and m-, p-xylenes were also obtained for Site 1 mesocosms, seemingly associated with the lower substrate concentrations.

The substrate interactions appeared to be affected by both the composition and the concentration of the PHC mixture and by the predominant TEAPs. Selective

biodegradation of PHCs under different reducing conditions was observed. However, there was no conclusive evidence that one reducing condition will universally favor the biodegradation of the specific PHCs. This may have significant practical implication in the field conditions where TEA amendment may be considered because of the apparent specificity of individual TEAPs.

There was no conclusive evidence that NO_3^- or SO_4^{2-} amendment could enhance the anaerobic biodegradation of benzene and ethylbenzene in both mesocosm studies by comparing the TEA amendment mesocosms with the unamended controls. However, it was conclusive that sulfate amendment enhanced the biodegradation of xylenes in Site 3 mesocosm study. It was found that o-xylene degradation was dependent on the sulfate reduction in the Site 3 study, which has not been reported. In the Site 1 mesocosm study, it was found that the anaerobic biodegradation of $F1_{-BEX}$ was enhanced by sulfate amendment. Similarly in the Site 3 study, sulfate amendment was also favourable for F1 biodegradation. In both studies, nutrient amendment showed no enhancement effects.

Anaerobic metabolites of BTEX, C_3 to C_9 alkanes, and naphthalene biodegradation were detected in both Site 1 and Site 3 mesocosms at concentrations of nanomoles/L. The metabolites appeared to be site-specific and reflective of different TEAPs. However, there was no definitive correlation between the changes in the metabolite levels and the anaerobic biodegradation processes of the parent compounds. Therefore, the metabolite analysis cannot be used as a stand-alone line of evidence. However, together with the evidence of substrate and TEA depletion, the detection of signature metabolites can be used to verify the occurrence of anaerobic biodegradation of specific PHCs.

7.2 Implications in PHC Bioremediation Practice

It is generally recognized that laboratory microcosm studies often result in higher rates of biodegradation than field studies (Wiedemeier *et al.* 1995). The first-order biodegradation rates estimated from the laboratory tests cannot be used directly to

predict *in-situ* attenuation processes and estimate the attenuation capacity. However, laboratory mesocosm studies will help better understand the site-specific natural attenuation processes and the influencing factors, which is of great significance for the success of bioremediation of PHC contamination. When comparing the first-order rates obtained from the laboratory mesocosm studies to the first-order *in-situ* attenuation rates (estimated from the depletion of BEX over time in the groundwater monitoring well), the Site 1 laboratory rates for BEX were higher than the field rates, whereas the Site 3 biodegradation rates for benzene and ethylbenzene were comparable to the observed field attenuation rates.

Selective biodegradation of individual PHC compounds under different TEAPs has been observed from these laboratory mesocosm studies. The biodegradation rates of the same PHC compound vary with the reducing conditions and with the sites. Therefore, the most recalcitrant compound of concern should dictate the selection of remediation technology as well as the prediction of timeframe for attenuation to meet the remediation goals. Selection of the favorable TEAP(s) should also take into account the site-specific geochemical conditions, i.e. the availability of potential TEAs. Multiple TEAPs, concurrent or sequential, may happen at a specific site due to its specific geochemical conditions. The temporal and spatial dynamics of TEAPs at a site should be considered for the decision-making on the enhancement of attenuation processes. The shift of the dominant TEAP after the TEA amendment suggests that TEAPs may be achieved by intentionally adding specific TEAs. The sequential TEAPs might be utilized to optimize the anaerobic biodegradation process. The results from the mesocosm study indicated that nitrate amendment may induce the release of bioavailable ferric iron or sulfate, which might be used as alternative TEAs after the depletion of nitrate and might be more favorable for anaerobic biodegradation of certain PHC contaminants.

In both mesocosm studies, the TEA utilization coupled to degradation of target PHC compounds only accounted for a small portion of the total TEA consumption. Under highly reduced conditions, especially at a site with long history of PHC contamination, the amended TEAs may be preferentially consumed by the oxidation of the more easily biodegradable organic compounds and the reducing byproducts. Therefore, the effective electron accepting capacity achieved after TEA amendment should be evaluated and used to estimate the enhancement effects. The effective amendment of TEAs and/or nutrients will rely on the knowledge and understanding of the site-specific geochemical processes and the influencing factors.

7.3 Recommendations

Due to lack of experience with the novel setup protocols, some variability was introduced during the mesocosm setup, such as losses of some volatile PHC compounds, the low concentrations of target organic compounds in the sterile controls, and the methanol added in some of the Site 1 mesocosms. Improvements on the setup and monitoring protocols should be further investigated, such as using argon instead of N_2 to fill the headspace and to obtain anerobic conditions, more frequent analysis of redox-sensitive species, as a few examples. For the preparation of the sterile controls, a stock solution with high PHC concentration could be prepared with the autoclaved groundwater samples and sterilized free products. The SCs could then be prepared by diluting the stock solution with autoclaved groundwater samples. To avoid of the loss of PHCs due to autoclaving, biocides could be used to prepare the SCs.

The weekly mixing conducted on each mesocosms may have increased the contact between microorganisms and the substrate and thus enhanced the biodegradation process. The frequency of mixing should be reduced to minimize the potential influence of mixing on the biodegradation process, to better simulate field conditions.

The sampling and analytical program could be better tuned to decrease the variability of the results. For instance, by sampling and analyzing specific biogeochemical byproducts, it will be possible to better understand the geochemical processes. The lack of MPN data in each non-sterile mesocosm made the comparison of MPN data less reliable due to the variability between individual mesocosms. Less frequent metabolite analysis also made the correlation between the metabolites and the biodegradation process more difficult. It is recommended that the MPN and metabolite analyses should be conducted in all testing mesocosms and be more frequent, when practical. Radio-labelled PHC compounds could be introduced into the mesocosms as an additional control for "quality control" purposes. Radio-labelled PHC compounds could also be used to investigate the biodegradation pathways and the signature metabolites.

The feasibility assessment and implementation of enhanced remediation by TEA and nutrient amendment require a profound understanding of the anaerobic biodegradation process at a specific PHC contaminated site. Further study is recommended to address some of the uncertainties and confounding factors in both mesocosm studies.

All occurring TEAPs and the shifts of the TEAPs after TEA amendment should be further studied. Iron reduction process was idenfied as an important TEAP at both contaminated sites. The feasibility of using chelating agents to stimulate iron reduction at the contaminated sites merits further investigation. It is also worthwhile to investigate the autotrophic nitrate reducing, sulfide oxidizing process induced after nitrate amendment. Sulfate produced from this process may serve as an alternative TEA after nitrate is depleted.

PHC contamination often involves a complex mixture of organic compounds. Substrate interactions between PHCs of interest under different TEAPs should be further investigated to identify the most recalcitrant compound(s) of concern at the contaminated site. The most recalcitrant compound(s) will determine the

selection of appropriate remedial options and the timeframe required to meet the remediation goals.

The presence of other easily biodegradable organic compounds and the reducing byproducts should be further studied to determine the effective TEA capacity (i.e. TEA consumption attributable to the biodegradation of target PHC compounds) and to select the required TEA amendment concentrations at the contaminated site.

Sulfate amendment showed enhancement effects, particularly on biodegradation of F1 and xylenes, in the mesocosm study. High sulfate concentrations were amended in the Site 3 mesocosm study due to the high background concentrations at the site. However, the potential toxicity of the sulfide produced from the sulfate reduction may decrease the anaerobic PHC biodegradation and limit the enhancement effects of sulfate amendment. The amendment sulfate concentrations should be carefully selected to prevent the potential toxicity of sulfide.

It is generally accepted that the biological reaction rate will double for every 10°C increase in temperature (Suthersan 1997). However, it has been found that the *in-situ* biodegradation rates were not reduced in cold environment (Bradley and Chapelle 1995). The temperature at which the laboratory mesocosm studies were conducted was 15 °C, approximately 10 °C higher than the temperature at the site. Temperature effects should be investigated to better estimate the biodegradation rates within the range of the site temperatures.

A variety of anaerobic PHC mebolites were identified from both mesocosm studies, indicating the occurrence of the anaerobic biodegradation processes. However, the detection of some metabolites appeared to be not correlated with the depletion of the parent compounds of interest. Furthermore, some of the metabolites may be only dead-end metabolites produced from cometabolic

processes rather than true intermediates of complete metabolic pathways. Whether these metabolites can be used as signature metabolites should be further studied.

7.4 References

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Appendices

Appendix A. Methods and Calibration Data

APPENDIX A1. MICRO GC FOR HEADSPACE GAS ANALYSES

Table A1-1. Micro-GC calibration curves for headspace gas analysis.

Gases	Retention Time (min)	Calibration levels (v %)				Calibration curve	r^2
		Level 1	Level 2	Level 3	Level 4		
O_2	0.53	30	15	5	1	Y=3.67(e+004) x	0.9998
N_2	0.56	100	75	50	25	Y=3.63(e+004) x	0.9999
CH_4	0.81	80	50	20	1	Y=3.58(e+004) x	0.9989
CO_2	0.44	20	10	5	1	Y=1.81(e+005) x	0.9986
C_2H_4	0.55	10	5	2	0.1	Y=1.95(e+005) x	0.9991
H_2S	1.3	5	3	1		Y=1.73(e+005) x	0.9934

APPENDIX A2. GC-FID FOR CCME F1 ANALYSES

Table A2-1. GC/FID calibration records and summary of average toluene response factors (RFs) used for F1 calculation.

Date	Average Toluene RFs	C (mg/L)	Hexane			Toluene			Decane		
			RT (min)	Area	RFs	RT (min)	Area	RFs	RT (min)	Area	RFs
		0.05	3.107	105.1	2102.0	7.794	115.2	2304.0	16.632	67.6	1352.0
		0.1	3.107	209.4	2094.0	7.793	235.1	2351.0	16.634	134.5	1345.0
		0.5	3.108	1119.0	2238.0	7.795	1216.9	2433.8	16.634	716.4	1432.8
		1	3.107	2376.9	2376.9	7.795	2689.2	2689.2	16.635	1591.2	1591.2
		5	3.107	14047.3	2809.5	7.808	15895.8	3179.2	16.641	7649.7	1529.9
19-Jul-04	2610.1	10	3.111	27162.5	2716.3	7.823	27902.5	2790.3	16.658	16001.8	1600.2
		0.05		99.1	1982.0		126.2	2524.0		34.1	682.0
		0.1		209.4	2094.0		288.7	2887.0		91.5	915.0
		0.5		1023.2	2046.4		1380.4	2760.8		604.3	1208.6
		1		2044.6	2044.6		2840.1	2840.1		1292.9	1292.9
28-Feb-05	2808.2	5		12560.4	2512.1		15146.2	3029.2		6988.1	1397.6
		0.05	3.112	87.5	1749.0	7.801	123.5	2469.1	16.638	31.3	626.6
		1	3.111	1393.1	1393.1	7.8	1910.5	1910.5	16.636	1025.6	1025.6
30-Aug-05	2406.6	5	3.111	10591.6	2118.3	7.812	14201.5	2840.3	16.645	7935.2	1587.0
		0.1	3.116	192.5	1925.0	7.806	244.3	2443.0	16.643	65.9	659.0
		1	3.118	1318.1	1318.1	7.809	1921.2	1921.2	16.644	656.7	656.7
08-Nov-05	2289.9	5	3.116	8471.4	1694.3	7.818	12527.0	2505.4	16.647	4745.4	949.1

RT = Retention time
C = Concentration

Table A2-1. (Continued).

Date	Average Toluene RFs	C (mg/L)	Hexane			Toluene			Decane		
			RT (min)	Area	RFs	RT (min)	Area	RFs	RT (min)	Area	RFs
		0.05	3.116	110.2	2204.0	7.805	144.3	2886.0	16.644	31.6	632.0
		0.5	3.115	1094.4	2188.8	7.805	1416.1	2832.2	16.64	746.0	1492.0
13-Dec-05	2998.6	5	3.117	13829.1	2765.8	7.823	16388.5	3277.7	16.651	8575.3	1715.1
		0.05	3.115	91.4	1828.0	7.805	108.8	2176.0	16.641	24.7	494.0
		0.5	3.115	779.2	1558.4	7.804	971.8	1943.6	16.639	480.1	960.2
08-Feb-06	2149.7	2.5	3.116	4681.5	1872.6	7.812	5823.4	2329.4	16.645	3243.1	1297.2
		0.05	3.115	63.5	1270.0	7.805	80.0	1600.0	16.641	20.5	410.0
		0.1	3.115	162.6	1626.0	7.804	200.1	2001.0	16.639	73.6	736.0
		0.5	3.116	1080.4	2160.8	7.812	1395.1	2790.2	16.645	677.2	1354.4
		1		2180.5	2180.5		2926.5	2926.5		1415.6	1415.6
05-Apr-06	2420.9	5		12262.1	2452.4		13934.4	2786.9		6572.3	1314.5
		0.1	3.111	189.5	1895.0	7.799	251.1	2511.0	16.634	71.1	711.0
		0.5	3.114	841.2	1682.4	7.803	1319.0	2638.0	16.638	511.5	1023.0
		0.5	3.113	783.4	1566.8	7.8	1346.4	2692.8	16.635	416.6	833.2
07-Jun-06	2644.2	5	3.112	11517.9	2303.6	7.812	13674.3	2734.9	16.641	7027.0	1405.4
		0.1	3.115	123.4	1234.0	7.804	237.5	2375.0	16.64	26.7	267.0
		0.1	3.114	1874.2	18742.0	7.804	2828.5	2763.7	16.637	1276.7	12767.0
		1	3.11	2147.7	2147.7	7.798	2888.3	2888.3	16.633	1533.6	1533.6
25-Aug-06	2733.9	5	3.111	11680.0	2336.0	7.811	14542.8	2908.6	16.643	7844.3	1568.9

RT = Retention time
C = Concentration

APPENDIX A3. IC FOR ANALYSES OF MAJOR IONS

Table A3-1. IC calibration standards (mg/L).

		STD1	STD2	STD3	STD4	STD5
Cations	Li^+	0.5	2.5	5	25	50
	Na^+	2	10	20	100	200
	NH_4^+	2.5	12.5	25	125	250
	K^+	5	25	50	250	500
	Mg^{2+}	2.5	12.5	25	125	250
	Ca^{2+}	5	25	50	250	500
Anions	F^-	1	2	4	10	20
	Cl^-	5	10	20	50	100
	Br^-	5	10	20	50	100
	NO_3^-	5	10	20	50	100
	NO_2^-	5	10	20	50	100
	SO_4^{2-}	5	10	20	50	100
	PO_4^{3-}	10	20	40	100	200

APPENDIX A4. MICROWAVE ACID DIGESTION

The procedures for Microwave Acid Digestion are described as follows,

- Unfreeze the centrifuged sediment samples at room temperature.
- Decant the water and scrape off the top layer
- Mix the sediment sample thoroughly
- Weigh ~0.5 g sediment sample into the tube (triplicates, from 3 centrifuge tubes)
- Add 5 mL DI water and 10 mL concentrated HNO_3 acid
- Cool the samples to room temperature after Microwave acid digestion
- Filter through 0.2 um syringe filter
- Store the digested samples at 4°C

The temperature program of Microwave Acid Digestion is as follows,

- Heat from ~20°C to 185°C in 10 min
- Hold the temperature for 15 min
- Vent the system for 5 min

Appendix B. Media for MPN Enumeration

APPENDIX B1. MEDIA FOR ENUMERATION OF SULFATE REDUCING BACTERIA (SRB) AND IRON REDUCING BACTERIA (IRB)

Table B1-1. SRB - modified API-RST medium (Tanner 1989)

Ingredients	Concentration (g/L)
K_2HPO_4	0.6
$CaSO_4$	0.04
$MgSO_4.7H_2O$	0.2
Sodium lactate (60% syrup)	4 (mL)
Pyruvic acid	1
Na Acetate	2
NaCl	1
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.3
Ascorbic acid	0.1
Yeast Extract	0.7
$(NH_4)_2SO_4$	0.3
Cysteine-HCl	0.2
Resazurin (0.1g/L)	10 (mL)
Trace Metals	5 (mL)
TES buffer	1.5

Note:
Adjust to pH 7.5 final. Before inoculation, add 0.1 mL Vitamin solution.
Distribute into Hungate tubes each containing 1 iron finishing nail. Nails may have to be solvent-washed to remove greasy coating.
Positive if there is black precipitate.

Method References:

Tanner, R. S. (1989). Monitoring sulfate-reducing bacteria: comparison of enumeration media. *Journal of Microbiological Methods*, 10(2), 83-90.

Table B1-2. IRB (Fered) – using Coates iron medium

Ingredients	Conc. (g/L)
NH_4Cl	0.25
NaH_2PO_4	0.60
$NaHCO_3$	2.50
KCl	0.1 g
Sodium Acetate	0.27
Sodium Nitrotriacetate	0.75
Wolfes mineral solution	10 mL
$Fe(OH)_3$ slurry	30 mL

Note:
Prepare 1 L of boiled distilled water. Add ingredients above.
Dispense into flushed hungate tubes, seal and autoclave.
Before inoculation, add 0.1 mL sterile, anoxic 250 mM $FeCl_2$ and 0.1 mL Wolfes vitamins solution per tube.
After 60 day incubation, the tubes are assayed for Fe^{2+} formation spectrophotmetrically using the ferrozine assay outlined by Lovely and Phillips (1986).

Method References:

Coates, J. D., Woodward, J., Allen, J., Philp, P., and Lovley, D. R. (1997). Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Applied and Environmental Microbioogy,* 63(9), 3589-3593.

Lovley, D. R., and Phillips, E. J. P. (1987). Competitive Mechanisms for Inhibition of Sulfate Reduction and Methane Production in the Zone of Ferric Iron Reduction in Sediments. *Applied and Environmental Microbioogy,* 53(11), 2636-2641.

Lovley, D. R., and Phillips, E. J. P. (1986). Availability of Ferric Iron for Microbial Reduction in Bottom Sediments of the Fresh-Water Tidal Potomac River. *Applied and Environmental Microbioogy,* 52(4), 751-757.

Lovley, D. R., and Phillips, E. J. P. (1986). Organic-Matter Mineralization with Reduction of Ferric Iron in Anaerobic Sediments. *Applied and Environmental Microbioogy,* 51(4), 683-689.

Table B1-3. Wolfes vitamins used for IRB and SRB API-RST additions (ATCC 1992)

Ingredients	Concentration (mg/100 mL)
Biotin	2
Folic Acid	2
Pyroxidine-HCl	10
Thiamine-HCl	5
Riboflavin	5
Nicotinic acid	5
Na-Pantothenate	5
B12 (Cyanocobalamine)	trace
p-Amionbenzoate	5
Thioctic acid	5

Note: Filter sterilize (0.2μm)

Table B1-4. Trace metals mineral solution for SRB API-RST media:

Ingredients	Concentration (g/L)
Nitrilotriacetic acid *	2
$MnSO_4.2H_2O$	1
$Fe(NH_4)_2(SO_4).6H_2O$	0.8
$CoCl_2.6H_2O$	0.2
$ZnSO_4.7H_2O$	0.2
$CuCl_2.6H_2O$	0.02
$NiCl_2.6H_2O$	0.02
$Na_2MoO_4.2H_2O$	0.02
Na_2SeO_4	0.02
Na_2WO_4	0.02

*: adjust to pH 6.0 with KOH.

Table B1-5. Wolfes mineral solution (ATCC 1992).

Ingredients	Concentration (g/100 mL)
Nitrilotriacetic acid	1.5
$MgSO_4.7H_2O$	3
$MnSO_4.H_2O$	0.5
NaCl	1
$FeSO_4.7H_2O$	0.1
$CoCl_2.6H_2O$	0.1
$CaCl_2$	0.1
$ZnSO_4.7H_2O$	0.1
$CuSO_4.5H_2O$	0.01
$AlK(SO_4)_2.12H_2O$	0.01
H_3BO_3	0.01
$Na_2MoO_4.2H_2O$	0.01
$NiCl_2.6H_2O$	0.025

APPENDIX B2. MEDIUM FOR ENUMERATION OF NITRATE REDUCING BACTERIA (NRB)

The medium used for heterotrophic NRB was a modified Tiedje medium, which contained half strength Nutrient Broth (4 g/L) and 5 g/L KNO_3. The medium was prepared in 16 mL hungate tubes as for aerobic media (not flushed with N_2).

Do spot test for nitrite to confirm activity.

Method References:

Tiedje, J.M., 1982. Denitrification. In: Page, A.L. (Ed.). Methods of Soil Analysis. Soil Science of America, Madison, WI, pp. 1011 – 1024.

APPENDIX B3. MEDIUM FOR ENUMERATION OF METHANOGENS

The recipe of the medium is presented below:

- 4g NaOH
- 1L dH_2O (boiled)
- Sparge with 30%CO_2, N_2 to pH 7.2-7.4 (about 30-45 min)
- 2 g Yeast extract
- 2 g trypticase peptones
- 0.5 g sodium 2-mercaptoethanesulfonate
- 6.8 g sodium acetate
- 14 mL mineral solution I (Fedorak and Hrudey 1984)
- 1.4 mL mineral solution II (Fedorak and Hrudey 1984)
- 14 mL resazurin

Add 9.0 mL per tube (flushing required).

After autoclaving, add 0.1mL NaS and 0.1 mL Vitamin B solution per tube.

Table B3-1. Mineral Solution I (Fedorak and Hrudey 1984)

Ingredients	Amount
NaCl	5.0 g
$CaCl_2.H_2O$	1.0 g
NH_4Cl	1.0 g
$MgCl_2.6H_2O$	1.0 g
0.01 M HCl	100 mL

Table B3-2. Mineral Solution II (Fedorak and Hrudey 1984)

Ingredients	Amount
$(NH_4)_6Mo_7O_24.2H_2O$	1.0 g
$ZnSO_4.7H_2O$	0.01 g
H_3BO_3	0.3 g
$FeCl_2.4H_2O$	0.15 g
$CoCl_2.6H_2O$	1.0 g
$MnCl_2.4H_2O$	0.003 g
$NiCl_2.6H_2O$	0.003 g
$AlK(SO_4)_2.12H_2O$	0.01 g
H_2O	100 mL

Table B3-3. Vitamin B solution (Fedorak and Hrudey 1984)

Ingredients	Amount/ 100 mL
Pyridoxine	0.025
Thiamine	0.005
Nicotinic acid	0.01
Pantotheinic acid	0.0025
B12 (Cyanobobalamine)	0.01
p-Aminobenzoic acid	0.005
dH2O	100 mL

Method References:

Fedorak, P.M., and Hrudey, S.E. (1984). The effects of phenol and some alkyl phenolics on batch methanogenesis. *Water Research*, 18, 361–367.

APPENDIX B4. MEDIUM FOR ENUMERATION OF NITRATE REDUCING SULFIDE OXIDIZING BACTERIA (NRSOB)

Table B4-1. NRSOB - CSB medium (Eckford and Fedorak 2002)

Ingredients	Concentrations (g/L)
NaCl	7
K_2HPO_4	0.027
$MgSO_4.7H_2O$	0.68
$CaCl_2.2H_2O$	0.24
NH_4Cl_2	0.02
$(NH4)_2SO_4$	0.13
Na HCO_3	1.9
KNO_3	1
$NaS\cdot 9H_2O$ (1M)	2.5 mL
Trace Mineral Solution #3	50 mL
rezazurine (0.1 g/L)	10 mL
dH_2O	1L

Note:
Media prepared anaerobically and 0.225 mL Na_2S was add just before inoculation.

Table B4-2. Trace Mineral Solution #3 for CSB media

Ingredients	Amount (g/L)
Nitrilotriacetic acid	2
$CaSO_4.2H_2O$	1.2
$FeCl_3$ (0.29g/L)	20 mL
$MgSO_4.7H_2O$	2
NaCl	0.16
Na_2HPO_4	1.4
KH_2PO_4	0.72
Micronutrients solution	10 mL

Note: adjust to pH 6.0 with KOH

Table B4-3. Micronutrient Solution.

Ingredients	Amount (g/L)
H_2SO_4	0.5 mL
$MnSO_4.2H_2O$	2.28
$ZnSO_4.7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.025
$CoCl_2.6H_2O$	0.045

Method References:

Eckford, R. E., and Fedorak, P. M. (2002). Planktonic nitrate-reducing bacteria and sulfate-reducing bacteria in some western Canadian oil field waters. *Journal of Industrial Microbiology & Biotechnology,* 29, 83-92.

Appendix C. Reproducibility Test

Due to limited sample sizes and due to limitations of the analytical instruments, some samples could not be analyzed in duplicate. The reproducibility test was conducted to determine the variability of the results, in particular of BTEX, F1, TEAs, and nutrients, caused by the instrumentation and the operation. Triplicate samples were taken from four Site 1 mesocosms and then analyzed for all chemical parameters. Considering the variations introduced during Site 1 mesocosm setup and amendment (i.e., the methanol addition), the mesocosms with no methanol addition were selected for this reproducibility test, which were Ctrl(#1), Ctrl+NP(#2), SO4(#5) and SO4+NP(#6).

The results of the reproducibility test were summarized in Tables C-1 to C-3.

Table C-1. Summary of the BTEX results in the reproducibility test (µg/L).

	Ctrl(#1)		Ctrl+NP(#2)		SO4(#5)		SO4+NP(#6)
Replicate	**Benzene**	**Ethylbenzene**	**Benzene**	**Ethylbenzene**	**Ethylbenzene**	**m-,p-Xylene**	**Benzene**
1	245.30	35.73	68.76	5.19	16.64	9.08	0.56
2	248.60	32.95	71.28	4.29	15.92	7.31	0.44
3	243.00	31.02	68.60	4.26	17.27	6.60	0.43
Average	245.63	33.23	69.55	4.58	16.61	7.66	0.48
Std Dev	2.81	2.37	1.50	0.53	0.68	1.27	0.07
% RSD	1.15	7.12	2.16	11.53	4.07	16.64	15.15

Table C-2. Summary of the F1 results in the reproducibility test (mg/L).

Replicate	**Ctrl(# 1)**	**Ctrl+NP(# 2)**	**SO4(#5)**	**SO4+NP(# 6)**
1	1.7	1.3	0.4	0.3
2	1.7	1.4	0.3	0.3
3	1.7	1.4	0.3	0.3
Average	1.73	1.36	0.34	0.28
Std. Dev.	0.00	0.01	0.02	0.02
%RSD	0.29	0.74	5.52	6.01

Table C-3. Summary of the TEA and nutrient results in the reproducibility test (mg/L).

	Ctrl+NP(#2)		SO4(#5)		SO4+NP(#6)	
Replicate	**NH_4^+**	**PO_4^{3-}**	**SO_4^{2-}**	**NH_4^+**	**SO_4^{2-}**	**PO_4^{3}-**
1	11.36	41.61	157.66	13.59	153.13	54.46
2	11.27	40.93	157.03	13.60	152.84	54.53
3	11.31	42.05	157.21	13.66	151.80	53.93
Average	11.32	41.53	157.30	13.61	152.59	54.31
Std Dev	0.04	0.57	0.32	0.04	0.70	0.33
% RSD	0.38	1.37	0.21	0.28	0.46	0.61

It can be seen that the variability of the results was very satisfying. The largest variability was associated with GC/MS. However, the relative standard deviations were still acceptable (within 15% with only a few exceptions). It can be concluded that the variability in lab results was negligible.

Appendix D. Raw Data

Table D-1. Summary of headspace gas data in Site 1 mesocosms (%).

	SC1				SC2			
Days	**O_2**	**N_2**	**CH_4**	**CO_2**	**O_2**	**N_2**	**CH_4**	**CO_2**
0	3.0	96.1	-	0.5	2.5	96.7	-	0.3
7	0.4	98.3	-	1.2	0.2	98.5	-	0.9
14	0.4	98.3	-	1.3	0.2	98.4	-	1.1
31	0.4	98.5	-	1.3	0.7	98.1	-	1.1
45	0.3	98.2	-	1.3	0.2	98.1	-	1.3
63	0.3	98.5	-	1.4	0.2	98.1	-	1.3
75	0.3	98.8	-	1.5	0.4	98.9	-	1.4
96	1.0	98.0	-	1.3	0.7	98.6	-	1.2
124	0.5	98.5	-	1.2	0.4	98.8	-	1.2
149	0.5	98.8	-	1.2	0.4	99.2	-	1.2
179	0.6	98.2	-	1.5	0.5	98.3	-	1.3
193	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
223	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
241	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
276	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
305	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
355	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
452	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
493	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
620	0.5	100.0	-	1.4	0.5	100.3	-	1.2

Table D-1. *(Continued).*

Days	Ctrl (# 1)				Ctrl (# 7)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	1.2	95.6	0.1	3.0	0.3	98.9	-	0.3
7	2.9	90.7	0.1	6.4	0.4	93.8	-	5.8
14	1.1	92.2	0.1	7.1	0.4	93.9	-	6.0
31	0.9	92.7	0.1	6.8	2.2	93.2	-	5.4
45	0.8	92.9	0.1	7.1	0.6	96.7	-	6.2
63	0.6	92.8	0.1	6.8	0.4	93.9	-	6.0
75	0.7	93.5	0.1	7.1	0.4	95.1	0.1	5.8
96	1.4	93.4	0.1	6.5	3.6	93.7	0.1	3.9
124	0.9	94.2	0.1	6.5	0.5	95.7	0.2	3.9
149	0.8	93.8	0.0	6.5	0.4	96.2	0.3	3.7
179	0.8	93.7	0.0	6.4	1.0	95.7	1.6	4.0
193	1.0	94.9	0.0	5.5	0.9	93.5	3.1	3.6
223	0.6	94.4	-	6.0	0.5	88.0	8.0	5.2
241	0.6	94.5	-	5.9	0.6	85.9	9.9	5.6
276	0.6	94.1	-	6.0	0.4	85.2	10.0	6.1
305	0.6	94.9	-	5.8	0.6	86.4	9.6	6.0
355	0.6	95.6	0.0	5.4	0.5	86.8	9.4	6.3
452	2.8	93.6	-	5.6	0.4	87.4	8.9	6.6
493	0.8	95.3	-	6.2	0.6	87.4	8.6	6.7
620	0.6	96.6	-	5.4	0.5	89.7	7.3	6.0

Table D-1. *(Continued).*

Days	Ctrl+NP(#2)				Ctrl+NP(#8)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	1.2	96.1	0.1	2.7	5.4	92.1	0.0	2.0
7	0.9	92.3	0.1	6.7	1.1	92.0	0.1	6.6
14	1.8	91.6	0.1	6.7	1.3	91.9	0.1	6.9
31	1.0	92.5	0.1	7.0	1.1	92.4	0.1	6.9
45	0.9	91.9	0.1	7.5	0.9	92.5	0.7	7.5
63	0.9	92.4	0.1	7.2	0.7	91.5	3.4	7.4
75	0.9	92.9	0.1	7.5	0.7	89.2	4.1	7.7
96	1.1	92.7	0.1	6.8	0.7	88.8	4.3	7.2
124	1.0	92.9	0.1	6.9	0.7	88.7	4.8	7.2
149	0.9	92.9	0.1	6.8	0.7	88.4	5.0	7.1
179	0.9	93.3	0.0	6.8	0.9	89.2	4.6	6.9
193	1.0	94.8	0.0	5.3	0.9	90.7	3.5	5.7
223	0.7	93.9	0.0	6.3	0.6	90.7	3.4	6.5
241	0.9	93.8	-	6.2	0.6	91.2	3.2	6.4
276	0.6	93.9	-	6.3	0.5	90.8	3.1	6.4
305	0.6	94.4	-	6.2	0.5	91.3	2.9	6.3
355	0.6	94.6	0.0	6.3	0.6	91.9	2.9	6.4
452	0.6	95.5	-	6.2	0.7	93.2	4.5	6.6
493	0.8	95.3	-	6.3	0.7	92.9	2.8	6.7
620	0.5	96.5	-	5.6	0.6	94.1	2.3	5.9

Table D-1. *(Continued).*

Days	NO3(#3)				NO3(#9)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	1.1	95.5	0.1	3.0	2.6	94.0	0.1	2.9
7	0.8	92.0	0.1	7.2	0.9	91.3	0.1	7.7
14	0.8	91.8	0.1	7.3	1.2	91.3	0.1	7.7
31	1.0	92.4	0.1	7.1	0.8	92.7	0.1	7.6
45	0.8	91.5	0.1	7.7	0.8	92.7	1.2	8.1
63	0.7	92.3	0.1	7.5	0.7	92.4	0.5	8.2
75	0.7	92.4	0.1	8.1	0.8	92.0	0.2	8.4
96	1.1	91.9	0.1	7.8	0.8	92.3	0.0	7.6
124	1.2	92.8	0.0	8.2	1.5	91.9	0.1	7.3
149	0.9	91.6	0.0	8.8	0.8	92.1	0.0	7.6
179	0.7	91.2	0.0	8.9	1.8	91.8	0.0	7.3
193	0.9	92.7	0.0	7.5	1.2	93.6	0.0	6.5
223	0.6	92.0	-	8.5	0.8	93.0	0.0	7.3
241	0.5	92.2	-	8.4	0.7	93.2	-	7.2
276	0.6	91.8	-	8.6	0.6	92.9	-	7.4
305	0.6	92.1	-	8.4	0.7	93.8	-	7.2
355	0.5	92.8	-	8.6	0.6	93.8	-	7.5
452	1.8	91.7	-	8.5	1.7	94.1	-	7.5
493	0.8	92.6	-	9.1	0.8	93.6	-	7.9
620	0.6	93.9	-	8.1	0.7	94.9	-	7.0

Table D-1. *(Continued).*

Days	NO3+NP(#4)				NO3+NP(#10)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	2.6	94.7	0.0	2.2	1.1	96.0	0.0	2.6
7	0.7	92.2	0.1	7.0	0.9	92.3	0.1	7.0
14	1.3	91.6	0.1	7.2	1.7	91.7	0.1	7.0
31	0.7	92.0	0.2	8.5	2.5	90.3	0.1	7.7
45	0.5	91.7	0.1	8.5	0.6	91.3	0.1	9.1
63	0.6	91.2	0.2	8.3	0.7	90.6	0.1	8.8
75	0.7	92.0	0.1	8.4	0.7	91.9	0.1	9.3
96	3.4	90.9	0.0	6.8	0.8	92.4	0.1	8.3
124	1.7	91.4	0.0	7.4	0.7	92.3	0.0	7.9
149	0.9	92.3	0.0	7.7	0.6	92.4	0.0	7.8
179	1.6	91.8	0.0	7.6	1.2	91.9	0.0	7.6
193	1.4	92.9	-	6.8	0.8	93.6	0.0	6.6
223	1.1	92.7	-	7.6	0.7	92.9	-	7.5
241	0.8	93.0	-	7.5	0.8	93.1	-	7.3
276	1.1	91.9	-	7.6	0.6	92.5	-	7.6
305	1.3	96.4	-	7.5	0.5	93.4	-	7.4
355	0.8	92.8	-	7.7	0.6	93.8	-	7.6
452	1.1	93.6	-	7.9	0.6	93.8	-	7.8
493	0.7	93.7	-	8.1	0.6	93.8	-	7.9
620	1.8	93.5	-	7.0	0.5	94.7	-	7.1

Table D-1. *(Continued).*

Days	SO4(#5)				SO4(#11)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	1.5	95.7	0.1	3.5	1.7	95.1	0.1	3.0
7	0.9	91.7	0.1	7.6	0.9	91.1	0.1	7.8
14	1.4	91.3	0.1	7.4	1.0	91.4	0.1	7.8
31	0.9	92.3	0.1	7.2	1.1	91.8	0.1	7.4
45	0.8	92.9	0.1	7.6	0.8	91.3	0.4	7.7
63	0.7	92.4	0.1	7.2	0.8	91.3	1.6	7.8
75	0.8	93.5	0.1	7.6	0.8	90.3	1.8	8.3
96	1.4	92.6	0.1	7.2	0.9	90.3	2.2	7.9
124	0.8	92.5	0.1	7.5	0.8	89.9	2.7	8.1
149	0.7	92.7	0.0	7.6	0.7	90.0	2.5	8.2
179	1.0	92.5	0.0	7.4	1.3	89.9	2.2	7.9
193	1.0	93.6	0.0	6.5	1.0	91.8	1.8	6.6
223	0.8	93.8	0.0	7.2	0.7	91.2	1.7	7.6
241	0.8	93.3	-	7.0	0.8	91.6	1.7	7.4
276	0.7	93.1	-	7.1	0.6	91.1	1.6	7.7
305	0.6	93.3	-	7.0	0.6	92.6	1.6	7.7
355	0.6	93.6	0.0	7.2	0.8	91.9	1.5	8.0
452	0.5	94.5	-	7.3	0.8	93.1	1.5	8.3
493	0.7	94.5	-	7.3	1.0	91.9	1.4	8.4
620	0.5	95.3	-	6.4	0.5	93.6	1.2	7.3

Table D-1. *(Continued).*

Days	SO4+NP(#6)				SO4+NP(#12)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	1.5	95.9	0.0	2.4	1.6	95.5	0.0	2.5
7	0.7	92.8	0.1	6.3	0.9	92.4	0.1	6.7
14	1.0	92.6	0.1	6.6	0.8	92.3	0.1	7.0
31	0.7	93.3	0.1	6.5	0.7	92.4	0.1	7.0
45	0.7	92.2	0.1	7.0	0.7	90.4	2.0	7.5
63	0.6	93.0	0.0	6.9	0.7	91.4	2.7	7.5
75	0.7	93.2	0.1	7.5	0.7	89.6	3.1	8.2
96	0.7	92.8	0.0	7.4	1.1	89.3	3.1	7.8
124	0.8	92.7	0.0	7.5	0.8	89.2	2.9	8.0
149	0.7	92.5	0.0	7.5	0.7	90.2	2.7	8.1
179	1.6	92.3	0.0	7.0	1.7	89.9	2.4	7.6
193	1.1	93.7	0.0	6.3	2.5	89.8	2.0	6.8
223	0.7	93.3	-	7.1	0.7	91.1	1.7	7.9
241	0.8	93.5	-	7.0	0.7	91.4	1.6	7.7
276	0.5	93.4	-	6.9	0.7	92.5	1.8	7.8
305	0.5	93.6	-	7.0	0.6	91.4	1.5	7.7
355	0.5	93.6	-	7.2	0.6	91.5	1.4	7.9
452	0.4	94.3	-	7.2	0.7	92.0	1.4	8.1
493	0.6	94.6	-	7.2	0.9	93.5	1.4	8.2
620	0.5	96.0	-	6.1	0.6	93.9	1.1	7.0

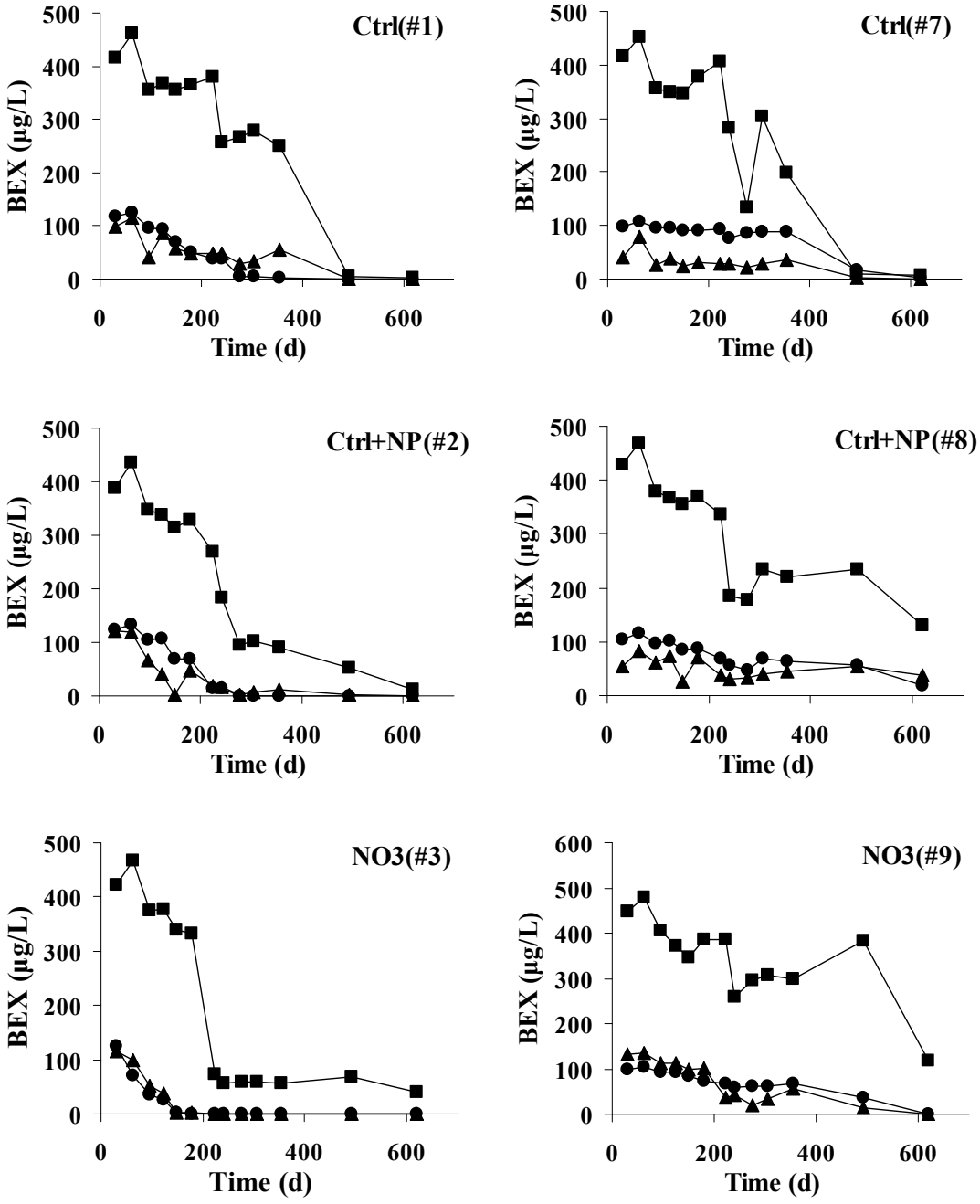

Figure D-1. Depletion of benzene (■), ethylbenzene (▲), and m-, p-xylenes (●) over time in Site 1 non-sterile mesocosms.

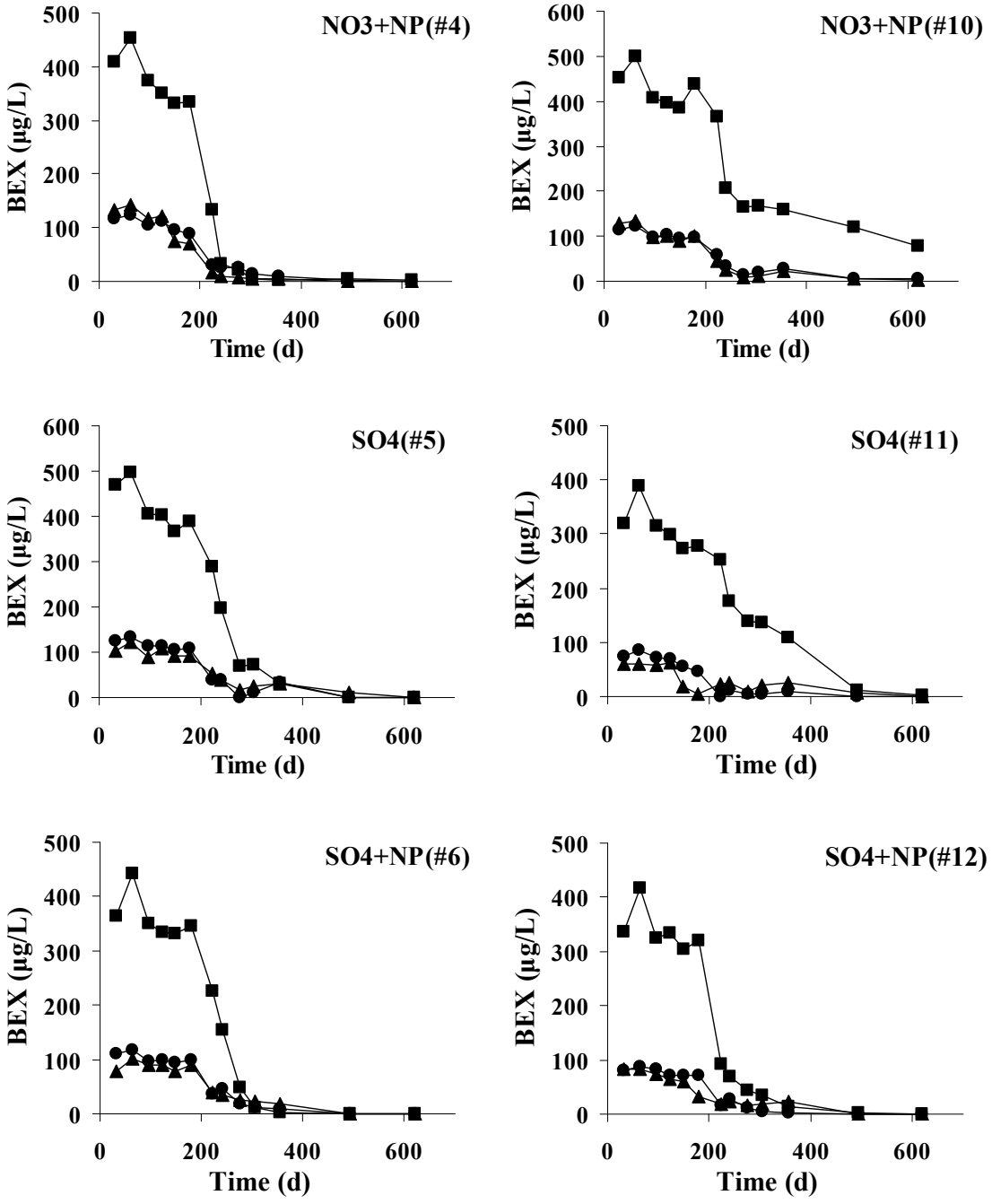

Figure D-1. *(Continued).*

Table D-2. Summary of BTEX concentrations in Site 1 mesocosms (µg/L).

	SC1					SC2				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0										
14										
31	5	1	8	9	1	2	1	4	4	0
63	5	0	9	9	1	2	0	4	4	0
96	4	1	8	8	1	1	1	2	1	0
124	4	0	7	8	1	2	0	3	2	0
149	3	0	7	8	0	1	0	2	1	0
179	5	0	6	7	0	0	0	0	0	0
223	0	0	0	0	0	0	0	0	0	0
241	0	0	1	1	0	0	0	0	0	0
276	0	0	0	0	0	1	0	0	0	0
305	0	0	0	0	0	0	0	0	0	0
355	1	0	0	0	0	1	0	0	0	0
493	1	0	0	0	0	1	0	0	0	0
620	0	0	0	0	0	0	0	0	0	0

Table D-2. *(Continued).*

	Ctrl(#1)					Ctrl(#7)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0										
14										
31	415	1	99	117	3	417	2	42	98	3
63	460	1	115	126	2	452	0	79	108	3
96	357	0	41	96	1	357	0	27	95	2
124	367	0	87	94	1	350	0	37	95	2
149	356	0	59	71	0	347	1	24	90	2
179	364	0	48	49	0	379	0	30	90	2
223	380	0	48	39	0	407	0	29	94	0
241	256	0	48	39	0	283	0	29	76	2
276	266	0	29	4	0	134	0	22	86	2
305	279	0	33	5	0	304	0	28	89	2
355	249	0	54	1	0	200	0	35	89	2
493	4	0	0	0	0	9	0	3	16	1
620	1	0	0	0	0	6	0	0	1	0

Table D-2. *(Continued).*

	Ctrl+NP(#2)					Ctrl+NP(#8)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0										
14										
31	389	1	121	124	3	429	1	55	105	3
63	437	1	119	134	2	469	1	82	117	3
96	348	0	67	104	1	379	0	61	97	2
124	338	0	41	108	1	367	0	73	102	2
149	315	0	3	70	0	355	0	25	86	2
179	328	0	47	69	0	369	0	70	87	1
223	270	0	19	14	0	336	0	37	68	0
241	184	0	17	15	0	185	0	31	56	1
276	94	0	3	1	0	178	0	32	47	1
305	101	0	7	0	0	235	0	41	68	1
355	90	0	12	0	0	221	0	44	65	1
493	52	0	2	0	0	235	0	54	57	1
620	11	0	0	0	0	130	0	38	19	1

Table D-2. *(Continued).*

	NO3(#3)					NO3(#9)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0										
14										
31	423	1	115	125	4	448	1	131	99	4
63	468	1	99	70	4	479	0	134	105	4
96	375	0	52	36	3	405	0	113	92	4
124	376	0	39	26	3	372	0	114	94	4
149	338	0	3	2	2	345	0	100	86	3
179	333	0	2	1	2	386	0	102	72	3
223	74	0	0	0	0	386	0	38	68	0
241	56	0	1	0	1	258	0	42	60	3
276	60	0	1	0	1	297	0	19	62	3
305	58	0	1	0	1	307	0	34	61	3
355	56	0	1	1	1	298	0	56	69	4
493	68	0	1	1	2	384	0	15	36	4
620	40	0	0	0	0	119	0	1	0	1

Table D-2. *(Continued).*

	NO3+NP(#4)					NO3+NP(#10)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0										
14										
31	409	1	133	117	4	452	1	127	113	5
63	453	0	142	125	4	500	0	133	122	5
96	374	0	117	106	3	407	0	98	97	4
124	351	0	122	113	4	398	0	101	103	4
149	332	0	75	95	3	384	0	90	94	3
179	335	0	69	88	3	439	0	100	98	4
223	133	0	17	31	0	365	0	45	59	0
241	32	0	9	25	2	206	0	26	35	3
276	20	0	8	27	2	164	0	9	13	2
305	6	0	4	14	1	169	0	10	20	2
355	3	0	4	10	1	158	0	21	29	2
493	4	0	1	3	1	121	0	4	7	2
620	2	0	0	1	0	78	0	3	5	1

Table D-2. *(Continued).*

	SO4(#5)					SO4(#11)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0										
14										
31	469	1	104	126	4	318	1	1	75	2
63	498	1	123	133	3	388	0	61	85	2
96	405	0	90	115	2	315	0	57	71	1
124	403	0	108	113	2	298	0	63	70	1
149	367	1	93	105	2	273	0	19	55	1
179	388	1	93	110	2	278	0	5	45	1
223	289	0	54	38	0	253	0	24	0	0
241	198	0	38	38	1	175	0	25	12	0
276	71	0	15	0	0	139	0	9	5	0
305	73	0	25	11	0	136	0	22	5	0
355	31	0	31	34	0	109	0	25	9	0
493	0	0	11	1	0	11	0	7	0	0
620	0	0	0	0	0	2	0	0	0	0

Table D-2. *(Continued).*

	SO4+NP(#6)					SO4+NP(#12)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0										
14										
31	363	1	78	112	3	336	2	17	81	3
63	442	0	102	117	2	417	0	83	88	2
96	350	0	91	97	1	324	0	73	82	2
124	333	1	90	100	1	335	1	65	71	1
149	331	1	79	94	1	304	1	60	73	1
179	346	1	91	99	1	321	1	32	71	1
223	225	0	40	38	0	93	0	19	17	0
241	155	0	36	46	0	70	0	23	28	0
276	48	0	24	20	0	45	0	16	11	0
305	12	0	23	11	0	33	0	19	5	0
355	1	0	18	9	0	13	0	23	1	0
493	0	0	0	0	0	2	0	0	0	0
620	0	0	0	0	0	1	0	0	0	0

Table D-3. Summary of CCME F1 hydrocarbon concentrations in Site1 mesocosms (mg/L).

Day	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
0	0.4	0.2	3.2	3.6	3.3	2.7	3.4	2.9	3.0	3.2	3.4	2.7	2.9	2.6
14	0.2	0.2	2.7	2.2	3.0	2.6	2.7	2.2	2.8	2.7	2.8	1.8	2.1	1.7
31	0.4	0.2	2.6	2.7	3.1	3.3	3.4	3.1	3.0	3.0	3.1	2.6	2.9	2.6
63	0.4	0.2	3.0	2.6	3.1	2.7	3.1	2.9	3.0	2.8	3.0	2.5	2.3	2.1
96	0.4	0.2	3.1	2.7	3.2	3.1	3.0	3.2	3.2	3.2	1.7	1.5	1.3	1.2
124	0.4	0.2	2.9	2.4	2.8	2.6	2.5	2.8	3.1	2.9	1.5	1.2	1.3	1.1
149	0.4	0.2	2.9	2.7	2.8	2.9	2.5	3.0	3.1	3.1	1.4	1.1	1.2	1.0
179	0.3	0.1	2.5	2.5	2.5	2.5	2.3	2.7	2.8	3.1	1.2	0.8	1.1	0.9
223	0.2	0.1	2.3	2.1	2.2	2.4	1.8	2.5	2.3	2.6	0.8	0.7	0.7	0.5
241	0.2	0.1	2.4	2.1	2.1	2.3	1.7	2.5	2.2	2.3	0.8	0.7	0.7	0.5
276	0.0	0.1	1.4	1.5	1.2	2.0	1.5	2.2	1.9	1.7	0.4	0.4	0.3	0.3
305	0.0	0.0	1.9	1.7	1.6	2.0	1.6	2.2	1.9	1.9	0.4	0.5	0.4	0.3
355	0.2	0.1	1.9	1.9	1.6	2.1	1.6	2.3	1.9	2.0	0.5	0.6	0.4	0.3
493	0.1	0.1	1.3	1.3	1.3	2.0	1.4	2.3	1.8	1.8	0.3	0.3	0.3	0.2
620	0.1	0.1	1.3	1.1	1.2	1.9	1.2	2.0	1.5	1.8	0.1	0.3	0.2	0.1

Table D-4. Summary of TEA and nutrient data in the respective amended Site 1 mesocosms (mg/L).

	SC1					SC2					Ctrl+NP(#2)		Ctrl+NP(#8)	
Day	NO_3^-	NO_2^-	NH_4^+	SO_4^{2-}	PO_4^{3-}	NO_3^-	NO_2^-	NH_4^+	SO_4^{2-}	PO_4^{3-}	NH_4^+	PO_4^{3-}	NH_4^+	PO_4^{3-}
0	131.7	n.a.	27.8	234.2	253.7	128.7	N.D.	29.5	224.4	260.5	35.5	275.9	36.0	277.8
14	104.7	10.3	22.6	222.4	185.8	124.5	N.D.	22.5	224.2	190.1	20.8	191.6	21.2	206.5
31	104.7	7.2	20.5	221.6	154.9	116.5	N.D.	19.9	223.4	151.0	17.0	164.7	17.1	170.4
63	105.9	5.3	18.8	221.3	121.4	113.9	1.3	18.6	220.2	120.5	14.0	130.2	13.6	128.3
96	105.8	3.4	18.3	219.7	107.5	112.3	0.6	18.3	218.2	107.0	13.2	113.6	12.9	99.6
124	105.0	2.4	18.3	219.3	99.3	111.7	0.7	18.4	222.6	102.0	12.8	107.0	12.4	81.7
149	100.8	2.1	18.1	214.7	90.4	107.1	0.6	18.0	216.7	92.8	12.6	95.7	12.3	68.5
179	102.6	1.8	18.5	222.5	86.9	108.4	0.6	17.1	222.4	88.2	12.8	89.5	12.5	60.2
223	94.6	1.5	17.9	215.9	79.2	102.3	0.8	18.0	216.6	81.3	12.0	82.4	11.9	51.7
241	95.1	1.2	18.3	213.1	75.1	103.1	1.1	18.3	214.1	79.3	12.2	75.1	11.8	48.2
276	95.7	0.5	18.0	219.4	72.9	103.8	0.6	18.3	219.7	77.3	11.7	72.2	11.8	45.6
305	96.7	0.6	17.5	214.3	71.4	105.2	0.5	17.6	215.3	75.4	11.3	66.1	11.1	43.7
355	95.9	0.4	17.9	213.8	66.1	103.6	0.5	18.0	215.0	69.3	11.4	55.3	11.4	39.1
493	93.9	0.2	18.0	217.0	63.1	99.9	0.4	18.3	217.9	66.8	11.4	37.9	11.6	36.9
620	87.2	0.2	17.3	215.2	57.5	96.4	n.a.	17.5	217.2	60.5	10.7	28.2	11.0	34.1

N.D.: Not detected.

Table D-4. *(Continued).*

	NO3(#3)			NO3(#9)			NO3+NP(#4)				NO3+NP(#11)			
Day	NO_3^-	NO_2^-	NH_4^+	NO_3^-	NO_2^-	NH_4^+	NO_3^-	NO_2^-	NH_4^+	PO_4^{3-}	NO_3^-	NO_2^-	NH_4^+	PO_4^{3-}
0	133.9	N.D.	0.0	130.3	N.D.	0.0	129.0	n.a.	36.7	267.7	130.8	N.D.	35.2	269.1
14	112.9	4.3	0.0	98.9	8.1	0.0	108.2	3.4	22.6	193.7	105.8	N.D.	23.6	200.9
31	108.4	3.3	0.0	16.3	29.9	N.D.	18.2	30.7	17.9	126.0	27.3	N.D.	18.8	155.8
63	85.3	1.7	N.D.	13.9	0.4	0.1	N.D.	1.2	14.6	79.9	0.6	1.2	15.6	118.4
96	47.5	2.0	N.D.	0.8	N.D.	N.D.	N.D.	N.D.	13.6	72.9	N.D.	N.D.	14.7	104.2
124	123.2	2.9	0.2	121.9	N.D.	0.1	116.4	N.D.	14.6	75.7	121.9	N.D.	15.1	93.8
149	92.2	1.1	0.1	84.6	N.D.	0.1	89.5	N.D.	14.7	69.0	88.1	N.D.	15.4	78.1
179	84.0	1.7	0.3	73.0	0.9	0.1	60.0	0.5	15.6	55.9	79.8	0.6	15.8	66.6
223	59.6	1.9	0.2	61.9	1.8	0.2	45.8	2.3	13.9	43.6	64.8	0.8	14.3	53.2
241	53.6	1.3	0.2	59.1	1.9	0.2	43.2	2.6	14.6	38.9	59.9	0.8	14.9	48.3
265														
276	42.3	1.2	0.2	52.4	2.5	0.2	36.6	2.5		33.7	52.4	0.7	14.8	41.7
305	34.1	0.6	0.3	49.2	1.7	0.2	32.2	2.4	13.6	29.3	47.3	0.8	14.0	38.5
355	17.2	0.7	0.2	39.5	1.7	0.2	24.3	1.7	13.6	25.5	37.1	0.8	14.0	31.6
493	N.D.	N.D.	0.4	14.7	2.0	0.4	3.9	2.4	13.7	20.5	14.2	0.8	13.9	25.1
497	124.7			139.8			122.6				139.0			
620	56.1	0.5	0.5	92.3	1.6	0.5	79.4	2.4	14.2	9.2	102.3	0.8	14.8	16.1

N.D.: Not detected.

Table D-4. *(Continued).*

Day	SO4(#5)	SO4(#11)	SO4+NP(#6)			SO4+NP(#12)		
	SO_4^{2-}	SO_4^{2-}	SO_4^{2-}	NH_4^+	PO_4^{3-}	SO_4^{2-}	NH_4^+	PO_4^{3-}
0	225.6	234.6	222.2	34.5	259.3	225.6	35.6	265.2
14	218.6	225.7	216.1	23.6	202.6	219.8	23.5	201.2
31	214.0	220.3	213.5	20.0	162.4	214.9	18.7	160.9
63	197.2	165.6	177.8	15.6	134.3	157.0	14.9	131.4
96	141.7	103.9	104.7	14.3	135.2	64.4	13.3	126.9
124	101.9	46.5	83.1	13.7	126.9	42.2	13.0	118.9
149	85.1	24.8	69.6	13.3	115.8	30.5	12.7	108.0
179	72.1	11.7	59.7	13.2	105.7	23.6	12.8	98.3
223	57.2	1.4	51.5	12.5	94.4	13.7	11.9	87.4
241	52.3	0.2	46.9	12.6	87.9	9.6	12.1	81.3
265	190.6	175.4	192.2			171.2		
276	189.6	176.7	193.4	13.9	77.4	176.0	13.6	73.9
305	179.4	152.8	181.6	13.9	71.3	160.4	13.6	66.9
355	168.4	129.2	166.0	13.7	60.5	148.5	13.9	56.2
493	147.2	100.6	149.4	14.2	50.6	129.7	14.2	46.5
620	136.9	78.0	147.3	13.3	44.4	111.8	13.3	41.0

Table D-5. Summary of other major ion data in Site 1 mesocosms (mg/L).

Day	SC1					SC2				
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-
0	118	44	9	32	12	115	47	9	31	6
14	107	32	10	37	7	107	32	11	38	7
31	99	27	11	36	6	98	25	11	36	6
63	99	25	10	35	6	97	24	10	33	7
96	100	24	11	33	6	100	23	11	34	6
124	98	23	10	33	4	98	23	10	33	4
149	98	23	10	32	5	98	22	10	33	6
179	98	23	8	32	6	98	21	8	32	6
223	98	22	9	31	6	98	22	9	31	6
241	100	23	8	31	6	100	22	8	32	6
276	97	21	8	30	4	97	22	8	31	5
305	93	21	9	29	6	93	20	9	29	6
355	96	21	7	29	4	95	20	8	30	4
493	95	21	9	29	4	95	21	9	30	4
620	92	20	7	27	4	92	20	8	28	4

Table D-5. *(Continued).*

	NO3(#3)					NO3(#9)					NO3+NP(#4)					NO3+NP(#10)				
Day	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**
0	6	68	9	31	6	6	64	9	32	6	31	69	9	29	6	31	64	10	32	6
14	7	35	11	40	6	7	35	11	40	6	29	36	12	42	6	30	38	12	42	6
31	7	23	12	42	6	7	24	12	40	6	26	25	11	35	6	27	27	13	40	6
63	8	18	12	40	6	8	19	11	37	6	26	20	9	28	6	27	22	11	34	6
96	8	16	12	37	5	7	17	11	36	5	26	18	9	29	5	27	20	11	33	5
124	6	59	12	38	4	6	78	12	39	4	25	62	12	37	4	26	75	12	37	4
149	8	35	12	39	5	8	45	14	44	5	27	44	13	39	5	28	47	13	40	6
179	8	30	10	38	5	8	39	12	45	5	27	38	10	36	5	27	41	10	38	5
223	8	26	10	35	6	8	35	12	43	6	26	33	9	31	6	27	35	10	34	6
241	8	26	9	35	6	8	35	11	44	6	27	34	8	31	6	27	36	9	33	6
276	7	24	8	31	4	7	33	10	41	4	25	32	8	28	4	25	34	8	30	4
305	7	22	9	28	5	8	31	11	38	5	24	30	8	27	5	25	32	9	28	5
355	7	22	7	26	3	7	31	9	37	3	24	30	7	26	3	25	31	7	27	3
493	7	22	8	27	4	7	32	10	35	4	24	30	7	23	4	24	31	7	23	4
620	8	35	9	34	4	8	47	11	41	3	25	42	8	28	4	25	44	8	29	4

Table D-5. *(Continued).*

	Ctrl(#1)					Ctrl(#7)					Ctrl+NP(#2)					Ctrl+NP(#8)				
Day	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**
0	5	1	8	27	6	5	1	8	26	6	32	2	9	28	6	32	2	8	27	6
14	6	1	8	28	5	7	1	8	27	6	29	3	9	32	6	30	3	10	34	7
31	6	1	9	29	6	6	1	8	28	6	26	3	11	35	6	26	3	11	35	6
63	7	1	9	31	6	7	1	10	34	6	26	3	11	33	6	26	3	11	34	6
96	6	1	10	33	5	7	1	16	51	6	26	3	11	34	5	26	3	12	36	5
124	5	1	10	33	4	5	1	18	58	4	24	3	10	33	4	25	2	11	35	4
149	6	1	10	32	5	7	1	20	62	6	26	3	11	33	5	26	3	12	36	5
179	6	1	8	32	5	6	1	16	62	6	25	3	9	33	5	25	3	9	35	5
223	6	1	9	32	6	6	1	14	51	6	26	3	9	30	6	25	3	9	31	5
241	6	1	8	33	6	6	1	12	48	6	26	3	8	31	6	26	3	9	32	6
276	5	1	8	31	4	5	1	11	42	4	24	2	8	29	4	24	3	8	31	4
305	5	1	9	30	5	6	1	12	41	5	23	3	9	30	4	23	3	9	30	5
355	5	1	8	33	4	5	1	10	41	3	24	3	8	32	4	23	3	8	31	3
493	5	1	11	37	4	5	1	12	40	4	24	3	9	31	4	24	3	9	31	3
620	5	1	10	36	4	5	1	10	37	4	23	3	8	27	4	24	3	9	30	4

Table D-5. *(Continued).*

	SO4(#5)					SO4(#11)					SO4+NP(#6)					SO4+NP(#12)				
Day	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**
0	97	1	8	29	6	100	1	8	28	6	120	2	9	30	6	121	2	9	28	6
14	85	2	10	36	6	87	2	10	37	6	105	3	12	41	6	106	3	12	40	6
31	76	2	11	39	6	76	2	12	40	6	94	2	13	42	6	95	3	13	43	6
63	74	2	12	40	6	75	2	12	41	6	91	3	13	40	6	92	3	13	40	6
96	74	2	12	38	5	75	2	12	39	5	92	3	12	38	5	92	3	12	35	5
124	71	2	11	36	4	72	2	11	34	4	90	3	11	35	4	90	3	11	33	4
149	71	2	12	35	5	72	2	11	33	5	89	3	12	35	5	90	3	11	32	5
179	71	2	9	34	5	71	2	8	31	5	87	3	9	33	5	89	3	8	30	5
223	71	2	9	33	6	71	2	9	31	6	88	3	9	30	6	88	3	8	27	6
241	72	2	9	34	6	72	2	8	31	6	89	3	8	31	6	89	3	8	29	6
276	117	2	10	38	4	131	2	10	39	4	134	3	9	34	4	143	3	9	33	4
305	109	2	11	38	5	119	2	11	38	5	124	4	11	35	5	131	3	10	34	4
355	108	2	10	40	4	120	2	10	40	3	124	3	9	35	3	134	3	9	35	3
493	110	3	11	39	4	120	3	11	38	4	125	4	10	35	4	134	4	10	35	4
620	106	2	10	36	4	116	2	9	34	4	122	3	9	32	4	129	3	9	31	4

Table D-6. Summary of pH data in Site 1 mesocosms.

Day	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
0	6.5	6.6	6.3	6.3	6.4	6.3	6.2	6.2	6.4	6.3	6.2	6.2	6.4	6.3
96	6.6	6.4	6.2	5.5	6.3	6.3	6.2	6.4	6.4	6.4	6.4	6.4	6.4	6.4
124	6.3	6.2	6.1	5.6	6.2	6.2	6.2	6.2	6.2	6.2	6.3	6.4	6.3	6.4
149	6.1	6.1	6.0	5.5	6.2	6.2	6.1	6.1	6.1	6.1	6.1	6.3	6.2	6.3
179	6.1	6.2	6.2	5.8	6.0	6.2	6.1	6.2	6.3	6.3	6.4	6.3	6.3	6.4
223	6.1	6.1	6.3	6.2	6.1	6.2	6.1	6.1	6.2	6.2	6.3	6.3	6.3	6.3
241	6.1	6.1	6.2	6.2	6.1	6.2	6.1	6.2	6.2	6.2	6.3	6.3	6.3	6.3
305	6.3	6.3	6.4	6.4	6.3	6.4	6.3	6.4	6.4	6.4	6.6	6.5	6.5	6.5
493	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.5	6.6	6.7	6.7	6.7
620	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.5	6.4	6.5	6.6	6.6	6.6

Table D-7. Summary of alkalinity data in Site 1 mesocosms (as mg/L $CaCO_3$).

Day	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
0	68	74	102	98	154	150	98	96	151	147	99	99	144	146
96	42	40	122	119	126	134	110	142	149	158	154	178	163	189
124	43	40	128	156	139	146	114	144	150	155	179	221	186	213
149	40	37	129	162	136	148	109	145	154	156	189	236	191	219
179	40	36	122	162	126	145	108	148	166	155	193	234	187	214
223	41	37	118	162	126	141	106	142	158	147	205	245	189	218
241	40	36	123	176	133	155	108	144	157	151	210	247	199	234
305	42	36	120	145	133	142	108	142	157	151	203	245	193	224
620	41	36	137	141	149	151	125	139	156	145	216	277	217	265

Table D-8. Groundwater chemical data measured during the Site 1 mesocosm decommissioning.

Parameters	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
DO (mg/L)	0.17	0.12	0.25	0.37	0.17	0.21	0.18	0.13	0.22	0.13	0.20	0.11	0.25	0.14
Temp (°C)	19.7	19.7	17.2	19.8	19.0	19.5	17.7	19.7	19.4	19.8	19.5	20.0	19.9	20.0
Dissolved S^{2-} (ug/L)	11	11	-	45	42	42	-	18	24	17	52	124	52	85
Dissolved Fe^{2+} (mg/L)	1.2	1.4	15.0	18.3	14.3	15.5	2.8	3.5	2.2	3.0	13.5	14.8	13.8	13.3

Table D-9. Summary of groundwater DOC data in Site 1 mesocosms (analyzed by Limnology Lab, University of Alberta).

Date	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
18-Oct-05	13.52	16.47	10.17	10.22	14.38	13.45	5.20	6.27	10.97	10.85	13.41	14.45	16.35	16.67
6-May-06	12.88	12.44	13.72	14.82	16.41	14.36	6.22	6.63	10.74	9.93	16.88	16.27	17.07	17.08

Table D-10. Summary of sediment characterization data in Site 1 mesocosms at the end of incubation (mg/g sediment).

	Na	Mg	Al	P	K	Ca	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	As	Rb	Sr	Cs	Ba	Pb	U
SC1	0.4	6.4	23.9	0.7	3.5	4.4	0.0	0.0	0.2	20.6	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
SC2	0.4	6.0	22.3	0.6	3.3	4.0	0.0	0.0	0.2	19.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
Ctrl(#1)	0.2	6.6	24.9	0.5	3.0	4.6	0.0	0.0	0.3	23.5	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
Ctrl(#7)	0.2	7.3	29.5	0.5	4.2	5.0	0.0	0.0	0.3	24.2	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
Ctrl+NP(#2)	0.2	6.3	23.7	0.7	2.9	4.2	0.0	0.0	0.3	21.7	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
Ctrl+NP(#8)	0.3	7.1	28.5	0.8	3.9	4.8	0.0	0.0	0.3	24.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
NO3(#3)	0.2	6.4	23.4	0.5	3.5	4.5	0.0	0.0	0.2	20.9	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
NO3(#9)	0.2	6.5	24.9	0.5	4.0	4.4	0.0	0.0	0.3	21.8	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
NO3+NP(#4)	0.2	6.4	24.0	0.7	3.7	4.1	0.0	0.0	0.2	21.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
NO3+NP(#10)	0.2	6.0	23.8	0.7	4.0	3.8	0.0	0.0	0.2	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.5	0.0	0.0
SO4(#5)	0.5	6.1	24.6	0.4	3.3	4.3	0.0	0.0	0.2	20.2	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.5	0.0	0.0
SO4(#11)	0.5	6.1	24.6	0.4	3.3	4.3	0.0	0.0	0.2	20.3	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
SO4+NP(#6)	0.5	6.4	25.4	0.8	3.7	4.5	0.0	0.0	0.3	21.8	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0
SO4+NP(#12)	0.5	6.3	24.4	0.7	3.2	4.1	0.0	0.0	0.3	21.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0	0.6	0.0	0.0

Table D-11. Summary results of headspace gases in Site 3 mesocosms (%).

	SC1				SC2			
Day	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	1.1	98.7	-	0.1	1.6	98.2	-	0.1
10	0.5	99.0	-	0.1	0.9	98.9	-	0.1
16	0.7	99.0	-	0.1	1.0	98.6	-	0.1
30	0.7	99.5	-	0.0	0.8	99.4	-	0.0
46	0.4	99.6	-	0.1	0.4	100.0	-	0.1
63	0.4	99.2	-	0.1	0.4	99.2	-	0.1
91	0.4	99.6	-	0.1	0.5	94.1	-	0.1
127	0.5	100.0	-	0.1	2.2	98.3	-	0.1
171	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
197	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
224	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
253	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
317	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
351	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
420	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
456	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
512	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
730	0.4	101.0	0.0	0.0	0.6	100.8	0.0	0.0

Table D-11. *(Continued).*

Day	Ctrl (# 1)				Ctrl (# 7)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	0.6	97.9	0.7	0.8	0.7	97.8	0.7	0.7
10	0.7	96.2	0.9	2.2	0.6	95.6	0.9	2.4
16	0.8	96.1	0.8	2.1	0.8	95.4	0.9	2.4
30	3.1	94.1	0.7	2.2	1.4	95.5	0.8	2.5
46	0.5	95.9	0.8	2.6	0.5	95.6	0.8	2.7
63	0.6	96.5	0.7	2.1	0.7	95.9	0.8	2.5
91	0.6	96.5	0.7	2.2	0.8	96.1	0.7	2.5
127	0.8	96.7	0.6	2.0	0.7	96.6	0.7	2.5
171	0.6	96.7	0.6	2.1	0.7	96.3	0.7	2.4
197	0.8	97.4	0.5	1.9	1.2	96.9	0.5	2.0
224	0.5	97.7	0.4	1.8	0.6	96.9	0.5	2.4
253	0.5	98.2	0.4	1.8	0.5	97.1	0.5	2.4
317	0.5	97.9	0.4	1.9	0.6	97.1	0.4	2.4
351	0.6	97.4	0.4	2.2	0.7	96.9	0.4	2.4
420	0.5	98.6	0.4	2.4	0.6	98.1	0.4	2.5
456	0.5	98.3	0.4	2.4	0.5	98.2	0.4	2.5
512	0.5	98.8	0.4	2.4	0.6	98.5	0.4	2.5
730	0.5	98.7	0.3	2.0	0.5	98.5	0.3	2.1

Table D-11. *(Continued).*

Day	Ctrl+NP(#2)				Ctrl+NP(#8)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	1.0	97.0	0.8	1.2	4.5	93.2	0.8	1.2
10	0.6	94.0	1.1	3.9	0.7	93.6	1.0	4.1
16	0.8	94.3	1.0	3.7	1.0	94.1	0.9	3.9
30	1.6	94.3	0.9	3.6	1.0	94.3	0.9	4.0
46	0.6	94.7	0.9	3.9	0.6	94.0	0.9	4.3
63	0.6	94.9	0.9	3.7	0.8	94.5	0.8	3.9
91	0.6	94.9	0.8	3.6	0.7	94.8	0.8	3.8
127	0.7	95.6	0.7	3.5	0.8	95.3	0.7	3.8
171	0.6	95.5	0.7	3.5	0.7	95.1	0.7	3.7
197	1.3	95.8	0.5	2.9	0.8	96.4	0.5	3.0
224	0.6	96.2	0.5	3.3	0.6	95.7	0.5	3.5
253	0.6	96.7	0.5	3.2	0.5	96.3	0.5	3.5
317	0.6	96.3	0.5	3.2	0.6	96.0	0.4	3.4
351	0.7	96.3	0.4	3.3	0.7	95.9	0.4	3.4
420	0.6	97.5	0.4	3.4	0.6	97.1	0.4	3.5
456	0.5	97.4	0.4	3.4	0.6	98.2	0.4	3.5
512	0.6	97.8	0.4	3.3	0.6	97.4	0.4	3.4
730	0.5	97.8	0.3	2.7	0.4	98.0	0.3	2.7

Table D-11. *(Continued).*

Day	NO3(#3)				NO3(#9)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	5.9	91.5	0.8	1.2	2.3	95.8	0.6	1.0
10	2.5	93.0	1.1	2.8	0.6	95.4	0.8	2.4
16	2.0	93.9	1.0	2.5	0.7	96.2	0.8	2.2
30	1.2	95.2	0.9	2.4	1.1	96.9	0.7	2.0
46	0.7	95.8	0.9	2.4	0.5	96.8	0.7	2.1
63	0.8	95.9	0.8	2.1	0.6	96.7	0.7	1.9
91	0.9	95.6	0.8	2.1	0.7	96.5	0.6	1.8
127	0.8	96.7	0.7	2.0	0.9	97.3	0.6	1.7
171	0.7	96.6	0.7	2.0	0.6	97.3	0.5	1.7
197	1.1	97.6	0.5	1.3	1.5	97.3	0.4	1.3
224	1.1	96.7	0.5	2.1	0.6	97.5	0.4	1.8
253	1.1	97.1	0.4	2.1	0.6	97.8	0.4	1.7
317	0.8	97.5	0.4	2.2	0.6	97.9	0.4	1.7
351	0.8	97.1	0.4	2.2	0.7	97.7	0.3	1.7
420	0.7	98.3	0.4	2.3	0.7	99.0	0.3	1.8
456	0.7	98.2	0.4	2.2	0.6	98.8	0.3	1.8
512	0.8	98.6	0.3	2.2	0.6	99.0	0.3	1.8
730	0.6	98.6	0.3	1.8	0.5	99.0	0.2	1.5

Table D-11. *(Continued).*

Day	NO3+NP(#4)				NO3+NP(#10)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	0.5	97.2	0.7	1.4	1.1	97.0	0.6	1.1
10	0.5	94.3	0.9	3.4	0.6	94.8	0.8	3.4
16	0.7	95.0	0.9	3.1	0.7	94.9	0.8	3.1
30	1.3	94.9	0.8	3.1	1.3	94.8	0.7	3.0
46	0.5	96.0	0.8	3.2	0.6	95.6	0.8	3.0
63	0.6	95.7	0.7	2.7	0.9	95.7	0.7	2.6
91	0.7	95.8	0.7	2.5	1.0	95.7	0.7	2.4
127	0.7	96.6	0.6	2.3	0.8	96.6	0.6	2.1
171	0.6	96.7	0.6	2.2	0.6	96.9	0.6	2.0
197	1.0	97.4	0.5	1.7	1.1	98.0	0.4	1.6
224	0.6	97.2	0.4	2.1	0.6	97.3	0.4	1.9
253	0.5	97.8	0.4	2.0	0.5	97.7	0.4	1.8
317	0.6	97.6	0.4	2.0	0.5	97.7	0.4	1.8
351	0.7	97.7	0.4	1.9	0.7	97.7	0.3	1.8
420	0.7	98.9	0.4	2.0	0.6	98.7	0.4	1.9
456	0.6	98.7	0.4	2.0	0.6	98.6	0.3	1.9
512	0.6	99.1	0.3	2.0	0.5	98.7	0.3	1.9
730	0.5	98.9	0.3	1.7	0.5	99.0	0.2	1.6

Table D-11. *(Continued).*

Day	SO4(#5)				SO4(#11)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	0.6	97.8	0.7	0.8	0.7	97.6	0.7	0.8
10	0.5	95.9	0.9	2.2	0.5	95.2	0.9	2.2
16	0.9	95.7	0.9	2.3	0.8	96.0	0.9	2.1
30	1.5	95.1	0.8	2.3	1.5	95.1	0.8	2.2
46	0.6	95.9	0.9	2.6	0.6	96.3	0.8	2.4
63	0.6	96.7	0.8	2.2	0.8	96.4	0.7	2.1
91	0.7	96.1	0.8	2.1	0.8	95.9	0.7	2.0
127	0.8	97.0	0.7	2.0	0.6	96.9	0.7	1.9
171	0.6	97.3	0.7	1.9	0.7	96.8	0.6	1.8
197	1.1	97.9	0.5	1.5	5.8	92.3	0.5	1.5
224	0.6	97.6	0.5	1.7	0.9	97.1	0.4	1.8
253	0.6	98.4	0.5	1.6	0.8	97.4	0.4	2.1
317	0.5	98.1	0.4	1.5	0.8	97.3	0.4	2.1
351	0.7	98.6	0.4	1.4	0.9	97.4	0.4	2.1
420	0.6	99.8	0.4	1.3	0.8	98.3	0.4	2.1
456	0.7	100.0	0.4	1.3	0.8	98.6	0.4	2.1
512	0.6	100.2	0.4	1.2	0.8	98.6	0.4	1.9
730	0.5	100.2	0.3	0.8	0.6	99.3	0.3	1.1

Table D-11. *(Continued).*

Day	SO4+NP(#6)				SO4+NP(#12)			
	O_2	N_2	CH_4	CO_2	O_2	N_2	CH_4	CO_2
0	0.8	96.7	0.7	1.1	0.7	97.4	0.7	1.2
10	0.6	94.2	1.0	3.7	0.5	94.7	0.9	3.6
16	0.9	94.2	0.9	3.5	1.1	94.2	0.8	3.4
30	1.7	93.6	0.8	3.6	1.5	94.2	0.8	3.5
46	0.5	94.7	0.9	3.8	1.3	94.5	0.8	3.6
63	0.7	95.4	0.8	3.3	0.9	94.7	0.7	3.2
91	0.8	94.8	0.8	3.1	0.7	95.4	0.7	3.1
127	0.7	95.7	0.7	3.1	0.7	96.1	0.6	3.0
171	0.7	95.8	0.7	3.0	0.6	96.4	0.6	2.9
197	0.9	97.1	0.5	2.2	1.5	96.6	0.4	2.2
224	0.6	96.3	0.5	2.7	0.6	96.8	0.4	2.5
253	0.6	97.4	0.5	2.5	0.6	97.3	0.4	2.4
317	0.6	97.3	0.4	2.3	0.6	97.3	0.4	2.3
351	0.6	97.5	0.4	2.1	0.6	97.6	0.4	2.1
420	0.6	99.1	0.4	1.9	0.6	98.6	0.4	2.0
456	0.5	98.8	0.4	1.8	0.4	99.0	0.4	1.8
512	0.5	99.2	0.4	1.6	0.5	99.3	0.3	1.6
730	0.4	99.7	0.3	0.9	0.5	99.6	0.3	1.0

Table D-12. Summary of BTEX concentrations in Site 3 mesocosms (µg/L).

	SC1					SC2				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0	10	9	0	3	1	6	7	0	3	1
16	10	12	0	4	1	5	5	0	2	1
30	11	12	0	4	1	5	4	0	2	1
63	27	11	1	7	1	20	3	0	5	1
91	11	8	0	4	1	5	4	0	2	0
127	35	10	1	6	1	24	3	0	4	1
197	56	10	2	13	2	32	1	1	9	2
224	26	11	0	6	1	15	1	0	4	1
253	49	11	1	8	1	29	0	0	5	1
286	48	10	1	8	1	25	0	0	5	1
317	40	9	1	7	1	31	0	0	5	1
351	39	9	1	6	1	26	0	0	3	1
420	10	3	0	2	1	1	18	0	0	2
630	41	9	1	7	1	24	0	0	4	1
722	29	9	0	7	1	21	0	0	4	1

Table D-12. *(Continued).*

	Ctrl(#1)					Ctrl(#7)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0	6580	2406	538	5111	1235	6740	2832	374	5320	1232
16	5657	0	699	4995	1086	5722	0	600	5015	1072
30	5815	0	684	5025	899	5743	0	576	4847	569
63	5726	0	745	5668	736	6076	0	607	5352	101
91	5962	0	726	5201	704	6015	0	614	4982	100
127	5659	0	742	5403	745	5565	0	560	4611	104
197	5058	0	630	4940	654	5205	0	555	5237	126
224	5532	0	339	5474	714	5671	0	341	5702	137
253	5577	0	419	5249	692	5752	0	413	5370	128
286	5531	0	510	5021	630	5553	0	402	5092	118
317	5052	0	414	4464	616	5318	0	328	4960	125
351	5203	0	474	4719	644	5244	0	362	4609	115
420	5266	0	572	5616	740	5469	0	378	5034	121
456	5416	0	478	4974	655	5525	0	401	5326	125
512	4572	0	307	4472	607	4586	0	220	4529	113
630	4346	0	339	4061	537	4577	0	264	4434	109
722	4747	0	426	5665	777	4670	0	300	5207	132

Table D-12. *(Continued).*

	Ctrl+NP (#2)					Ctrl+NP (#8)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0	6783	2403	730	5231	1216	6916	2751	758	5254	1259
16	5884	0	682	5161	1129	5567	0	603	4855	1098
30	5920	0	685	5088	819	5667	0	573	4614	443
63	5923	0	675	5319	633	5717	0	630	4978	0
91	5925	0	655	4941	627	5766	0	643	4964	60
127	5596	0	602	4472	582	5244	0	522	4072	50
197	5061	0	610	4930	577	4873	0	543	4723	54
224	5179	0	324	4690	679	5278	0	276	4695	64
253	5398	0	488	5930	711	5488	0	464	5389	62
286	5379	0	471	5045	588	5237	0	475	4980	55
317	4855	0	360	4428	545	5244	0	416	5134	60
351	5179	0	467	4955	622	5174	0	427	4664	53
420	5238	0	521	6011	723	5225	0	506	5222	66
456	5078	0	426	4877	575	5159	0	502	5459	72
512	4365	0	343	4360	532	4983	0	382	5233	62
630	4359	0	366	4227	511	4434	0	347	4239	49
722	4332	33	448	5378	658	4464	0	366	4840	58

Table D-12. *(Continued).*

	NO3(#3)					NO3(#9)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0	6852	2315	726	5270	1259	7201	2970	690	5250	1241
16	5796	221	198	5170	1136	5764	57	499	4932	1120
30	5906	212	160	5199	1130	5966	58	541	5140	1126
63	5598	207	171	5166	1106	5965	54	583	5813	1216
91	5588	62	168	4948	1092	5593	48	502	4803	1052
127	5291	0	164	4910	1065	5617	60	497	4724	1036
197	4911	0	99	5390	1058	5185	0	538	5632	1123
224	4651	0	0	5495	1097	5248	0	73	6210	1253
253	4256	0	0	5486	1098	5539	0	206	7538	1443
286	3882	0	0	4740	905	5416	0	146	5791	1123
317	3434	0	0	4122	850	4999	0	132	5585	1142
351	3438	0	0	4696	958	4948	0	126	5141	1071
420	3691	0	0	5852	1190	5183	0	149	6510	1286
456	3614	0	0	4698	929	5179	0	124	6598	1288
512	2964	0	0	4112	833	4664	0	80	6085	1204
630	2993	0	0	4477	888	4269	0	56	5312	1073
722	2945	0	0	5155	1062	4235	0	30	5625	1182

Table D-12. *(Continued).*

	NO3+NP(#4)					NO3+NP(#10)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0	7040	2713	785	5536	1302	6969	2927	702	5130	1192
16	6054	0	749	5237	1154	6092	0	656	4897	1084
30	6026	0	744	5274	1161	6006	0	660	5092	1098
63	6189	0	844	6201	1269	6222	0	774	5980	1173
91	6008	0	800	5658	1183	5832	0	707	5276	1062
127	5673	0	799	5716	1195	5904	0	751	5666	1139
197	5326	0	831	6289	1233	5214	0	612	5377	993
224	5934	0	829	8221	1586	5840	0	577	8278	1517
253	6356	0	1041	10220	1899	6093	0	600	7284	1346
286	5722	0	634	5938	1118	5703	0	545	6499	1158
317	5703	0	687	7305	1443	5577	0	443	5536	1086
351	5629	0	636	6119	1214	5339	0	488	5855	1126
420	5818	0	744	6946	1349	5587	0	545	6759	1281
456	5607	0	672	6606	1248	5530	0	471	5906	1092
512	4949	0	516	5203	1035	4892	0	419	5510	1033
630	4973	0	570	5780	1116	4690	0	426	5499	1025
722	4938	0	678	8595	1672	4738	0	305	6760	1288

Table D-12. *(Continued).*

	SO4(#5)					SO4(#11)				
Day	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0	6558	2321	754	5282	1192	7246	2912	703	5103	1208
16	5856	0	705	4977	961	5946	0	606	4452	930
30	5944	0	717	4895	72	6047	0	644	4708	229
63	5832	0	740	5173	0	6164	0	710	5225	0
91	6027	0	748	4409	0	5930	0	642	3865	0
127	5217	0	636	1559	0	5374	0	567	1515	0
197	5050	0	708	1476	0	5340	0	651	1376	0
224	5725	0	811	1681	0	5617	0	699	1562	0
253	5783	0	802	1659	0	5537	0	746	1657	0
286	5833	0	770	1568	0	5579	0	674	1449	0
317	5484	0	751	1445	0	5312	0	677	1427	0
351	5519	0	715	1463	0	5197	0	596	1300	0
420	5548	0	876	1844	0	5156	0	698	1523	0
456	5457	0	670	1569	0	5241	0	599	1417	0
512	4761	0	551	1408	0	4577	0	414	1295	0
630	4691	0	430	1300	0	4438	0	297	1339	0
722	4857	0	490	1687	0	4653	0	258	1488	0

Table D-12. *(Continued).*

Day	SO4+NP (#6)					SO4+NP (#12)				
	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene	Benzene	Toluene	Ethylbenzene	m-,p-Xylene	o-Xylene
0	6641	2738	637	4833	1166	7145	2675	674	5094	1190
16	5945	0	700	5345	964	5651	0	589	4496	924
30	5946	0	641	4809	0	6153	0	669	5077	0
63	5708	0	702	4870	0	5966	0	687	4991	0
91	6050	0	739	2216	0	5585	0	617	2164	0
127	5602	0	687	1391	0	5325	0	607	1261	0
197	5054	0	617	1432	0	5419	0	679	1520	0
224	5467	0	701	1681	0	5823	0	744	1695	0
253	5761	0	672	1708	0	5703	0	729	1675	0
286	5612	0	557	1463	0	5602	0	651	1575	0
317	5616	0	576	1494	0	5642	0	624	1561	0
351	5134	0	502	1351	0	5625	0	534	1474	0
420	5405	0	657	1694	0	5532	0	571	1520	0
456	5380	0	582	1525	0	5618	0	551	1597	0
512	4844	0	505	1390	0	5175	0	482	1458	0
630	4793	0	497	1365	0	4875	0	441	1394	0
722	4822	0	578	1681	0	4896	0	473	1603	0

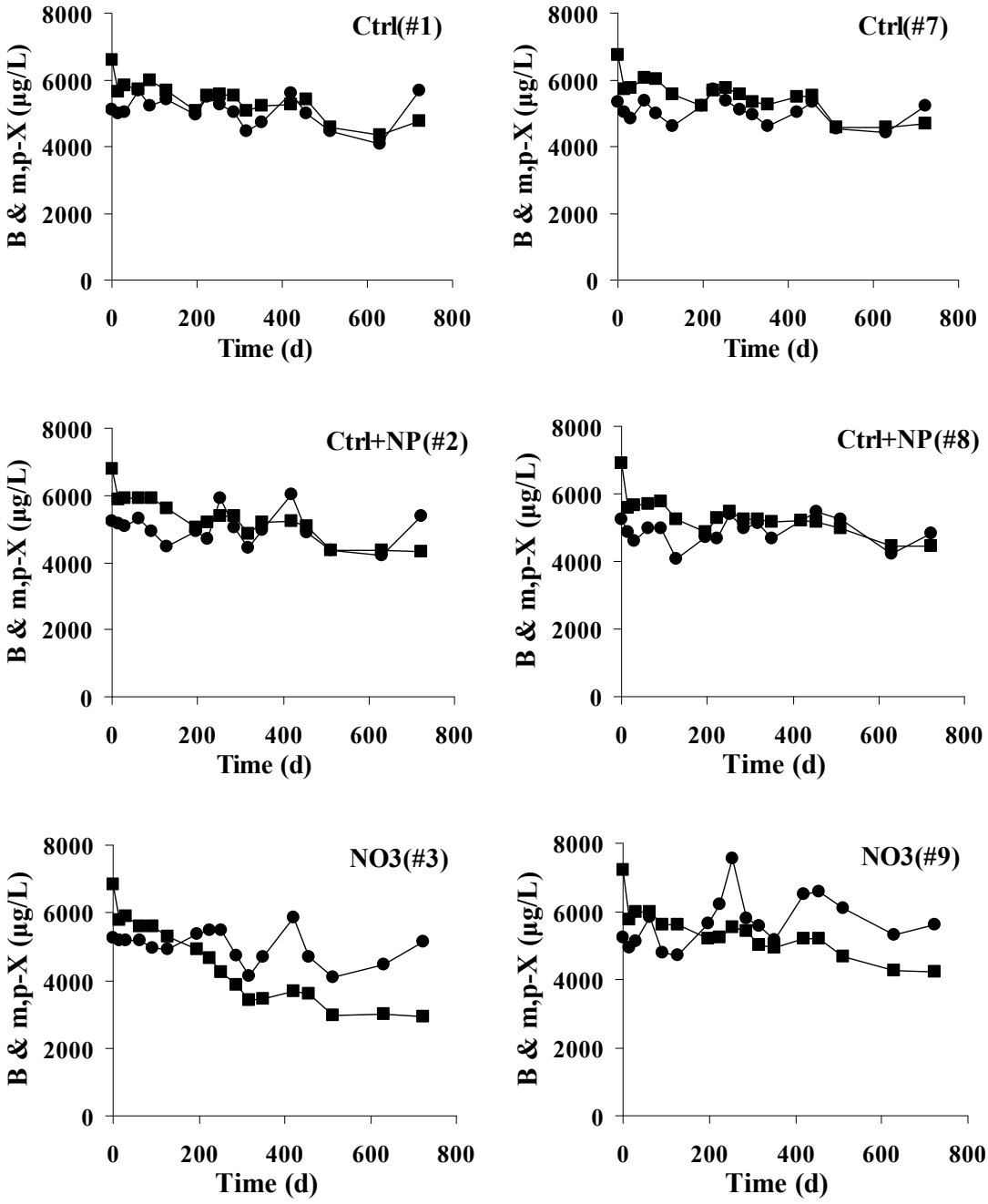

Figure D-2. Depletion of benzene (■) and m-, p-xylenes (●) with time in Site 3 non-sterile mesocosms.

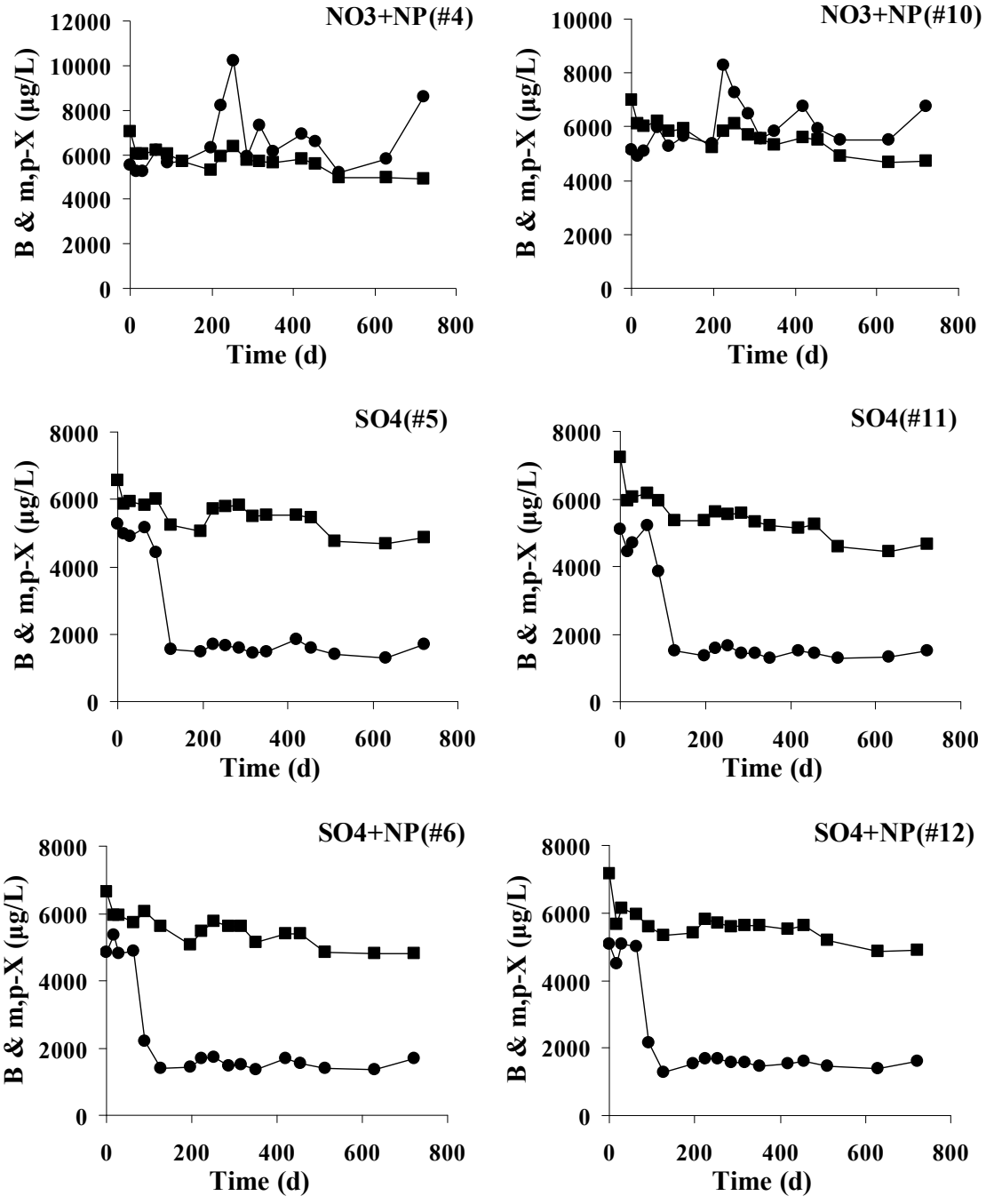

Figure D-2. *(Continued).*

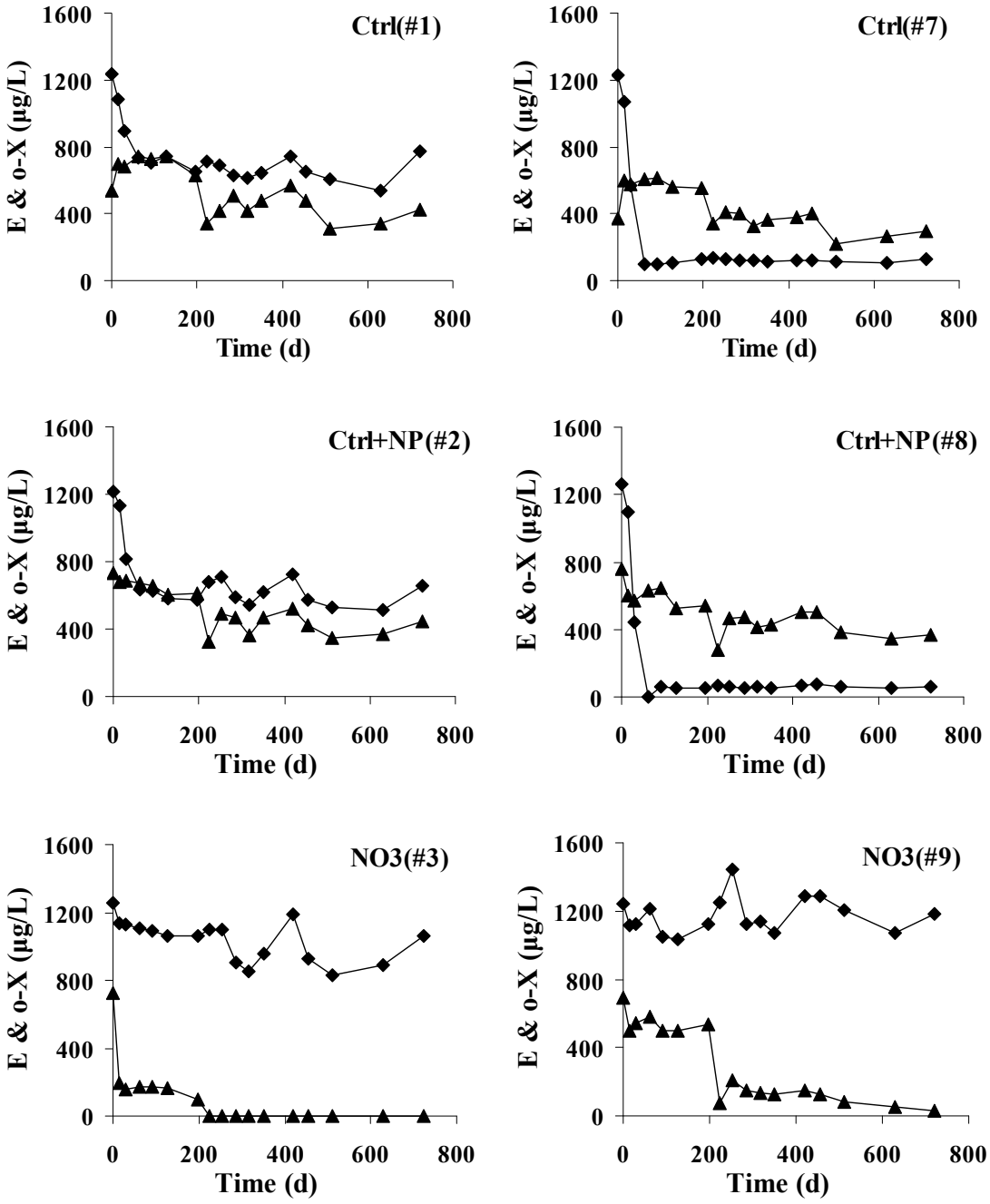

Figure D-3. Depletion of ethylbenzene (▲) and o-xylene (♦) with time in Site 3 non-sterile mesocosms.

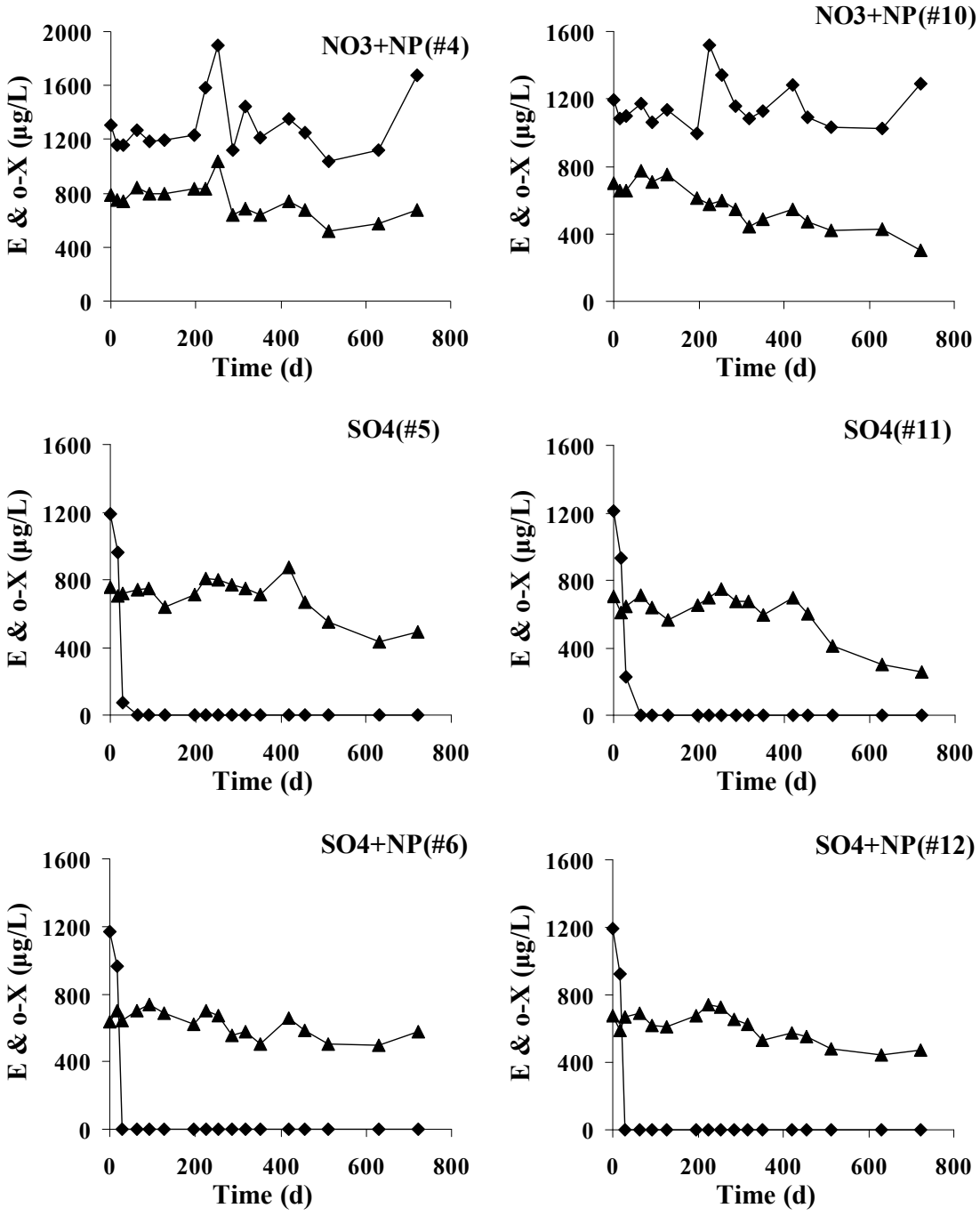

Figure D-3. *(Continued).*

Table D-13. Summary of CCME F1 hydrocarbon concentrations in Site 3 mesocosms (mg/L).

Day	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
0	0.1	0.1	19.3	20.2	19.0	21.6	22.8	19.9	22.2	19.6	21.9	22.5	23.4	20.9
16	0.1	0.1	18.6	17.9	17.8	18.6	17.4	18.7	21.3	19.5	17.7	17.8	20.3	22.1
30	0.1	0.0	19.8	17.4	17.7	16.6	19.8	20.8	19.5	18.3	15.7	17.2	17.4	17.0
63	0.1	0.1	16.7	17.0	17.1	17.7	18.1	22.4	19.5	19.5	16.7	16.0	17.6	16.9
91	0.1	0.1	17.4	17.2	18.1	16.9	17.9	20.0	21.0	19.9	15.5	15.5	13.2	13.2
127	0.1	0.1	18.1	17.4	18.1	17.1	16.5	19.1	19.7	21.9	12.7	13.4	12.7	12.1
171	0.1	0.1	17.2	14.2	17.2	14.7	14.4	18.1	19.4	18.5	11.7	11.9	11.9	9.6
197	0.1	0.1	16.5	15.6	17.6	14.9	17.7	17.8	20.0	17.8	11.9	11.7	11.2	1.5
224	0.1	0.1	15.6	12.9	15.6	13.4	15.0	13.0	18.3	12.1	11.7	10.8	11.3	10.5
253	0.0	0.0	15.7	15.3	16.6	15.7	15.5	19.4	21.1	13.9	12.1	11.3	11.3	9.9
286	0.0	0.1	16.4	14.4	16.9	15.9	14.3	16.8	20.7	19.7	12.8	11.5	11.5	11.6
317	0.1	0.0	15.7	15.4	15.4	17.3	13.1	19.1	22.1	17.8	11.6	11.0	10.7	11.2
351	0.1	0.2	16.6	13.9	15.0	15.6	14.5	17.9	20.6	18.6	12.1	11.0	11.1	10.9
420	0.1	0.0	16.7	15.2	15.9	14.3	14.4	16.2	19.4	19.7	11.1	10.3	11.1	10.7
456			16.2	15.2	16.2	16.3	14.2	18.4	20.9	22.2	10.7	10.1	10.6	10.8
512			16.2	13.9	14.6	14.5	17.0	15.5	18.1	17.9	10.7	10.1	10.6	10.9
630	0.1	0.1	15.4	15.0	16.0	14.9	13.7	16.1	19.3	17.5	10.9	10.2	11.1	10.9
710	0.0	0.0	15.6	15.1	14.9	12.4	13.3	15.5	17.1	19.3	10.4	9.6	11.6	10.7

Table D-14. Summary of TEA and nutrient concentrations in the respective amended Site 3 mesocosms (mg/L).

	SC1					SC2					Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)			Ctrl+NP(#2)		
Day	**NO_3^-**	**NO_2^-**	**NH_4^+**	**SO_4^{2-}**	**PO_4^{3-}**	**NO_3^-**	**NO_2^-**	**NH_4^+**	**SO_4^{2-}**	**PO_4^{3-}**	**SO_4^{2-}**	**SO_4^{2-}**	**NH_4^+**	**SO_4^{2-}**	**PO_4^{3-}**	**NH_4^+**	**SO_4^{2-}**	**PO_4^{3-}**
0	106.0	n.a.	40.7	888.0	222.5	108.0	n.a.	40.5	945.8	234.8	65.6	111.7	41.7	68.8	209.4	37.5	77.9	172.6
16	80.6	15.0	36.4	904.6	204.0	79.6	13.7	37.4	945.5	214.7	20.8	39.1	31.1	24.0	177.2	32.0	41.7	179.6
30	79.6	17.5	36.8	925.4	204.3	80.8	11.9	37.3	956.6	207.5	6.4	15.8	30.2	7.3	160.3	31.5	19.4	163.9
63	74.6	20.8	36.8	921.8	196.7	76.7	11.0	37.1	954.5	197.5	N.D.	N.D.	30.5	N.D.	131.2	31.5	N.D.	136.7
91	73.1	21.4	37.1	911.5	195.3	76.6	12.6	37.6	997.9	192.3	N.D.	N.D.	30.6	N.D.	112.5	31.8	N.D.	118.1
127	71.9	22.8	37.1	925.5	194.2	75.3	12.1	37.8	956.4	191.9	N.D.	N.D.	30.8	N.D.	96.7	31.1	N.D.	103.3
171	67.7	23.9	35.8	885.8	183.2	68.7	14.0	36.7	927.8	179.5	0.0	0.0	30.6	0.0	81.4	30.8	0.0	85.3
197	66.9	21.8	40.0	838.6	183.3	67.2	12.1	40.2	894.3	185.0	N.D.	N.D.	30.5	N.D.	75.7	31.5	N.D.	76.3
224	67.1	23.1	42.9	902.4	184.5	68.7	11.8	46.7	957.1	183.3	N.D.	0.3	31.3	N.D.	70.5	32.3	0.3	77.4
253	63.6	24.8	44.4	874.5	187.4	70.2	13.7	44.1	922.2	183.6	0.3	0.8	31.5	N.D.	65.2	31.9	0.8	68.5
286	62.2	26.2	39.9	900.9	192.3	71.3	14.6	40.1	945.9	184.3	N.D.	0.5	30.7	N.D.	61.9	32.0	N.D.	62.7
317	61.2	25.6	41.1	906.5	189.2	70.8	15.0	41.4	971.3	183.0	N.D.	N.D.	31.0	0.1	54.5	32.4	0.6	56.7
351	58.8	18.1	44.5	916.0	184.5	65.7	9.8	46.4	940.1	180.4	0.2	N.D.	31.9	N.D.	46.3	32.9	N.D.	49.6
420	57.2	25.6	44.5	894.3	184.7	63.1	16.2	47.4	943.9	181.8	0.2	2.9	33.2	0.4	38.5	34.7	0.7	40.3
456	53.7	25.8	49.7	906.9	187.0	62.4	16.9	50.6	944.7	181.7	N.D.	N.D.	33.4	N.D.	37.0	35.0	N.D.	37.9
512	51.6	25.5	43.2	911.0	183.4	70.3	20.0	46.2	942.0	176.4	N.D.	0.2	31.1	N.D.	32.5	32.8	N.D.	33.9
630	46.7	24.1	61.3	863.0	174.7	54.7	18.9	62.4	882.8	179.0	N.D.	N.D.	42.4	N.D.	24.2	49.5	N.D.	25.4
710	56.3	30.7	59.8	1093.3	207.7	52.3	26.0	62.3	961.5	190.2	0.3	0.6	28.2	N.D.	22.7	30.9	0.4	29.0
722	50.1	24.1	55.7	1113.8	176.9	56.2	25.9	59.0	1099.7	199.2	0.3	2.3	29.4	N.D.	26.5	30.4	0.5	33.6

N.D.: Not detected.

Table D-14. *(Continued).*

Day	NO3(#3)			NO3(#9)			NO3+NP(#4)					NO3+NP(#4)				
	NO_3^-	NO_2^-	SO_4^{2-}	NO_3^-	NO_2^-	SO_4^{2-}	NO_3^-	NO_2^-	NH_4^+	SO_4^{2-}	PO_4^{3-}	NO_3^-	NO_2^-	NH4+	SO_4^{2-}	PO_4^{3-}
0	93.8	7.8	70.3	106.0	N.D.	76.5	102.4	7.2	37.3	72.8	168.3	111.6	4.1	39.9	75.7	184.3
16	34.4	38.4	69.8	41.9	34.6	74.2	44.0	14.5	37.9	65.7	160.4	71.3	N.D.	37.2	72.4	168.2
30	24.1	43.1	72.1	35.7	36.5	76.3	6.8	37.2	38.4	68.4	137.9	0.2	33.4	39.8	71.0	143.6
63	18.0	33.9	82.7	32.4	33.3	76.4	1.7	35.0	38.8	71.3	115.9	N.D.	21.2	40.5	72.2	116.6
91	193.3	26.3	92.3	131.7	29.4	79.7	118.6	27.5	39.9	72.3	100.7	123.0	14.6	41.7	75.6	102.1
127	104.2	17.9	104.3	122.2	19.3	91.5	119.9	18.7	40.3	81.0	89.0	99.3	9.5	42.1	88.6	86.4
171	93.7	12.9	111.0	111.3	12.1	98.1	99.7	10.3	39.4	93.7	77.5	87.4	6.1	41.4	94.4	74.2
197	89.2	8.4	115.7	108.9	6.7	102.0	96.9	5.8	38.8	97.8	74.1	83.2	2.3	41.2	102.0	75.8
224	75.0	14.0	125.6	96.5	9.8	113.1	89.9	7.7	41.4	107.8	70.5	77.3	3.0	43.5	110.9	68.4
253	59.6	17.0	132.2	88.7	8.4	120.6	85.9	5.4	40.5	111.7	66.1	71.8	3.0	43.1	115.1	64.8
286	47.6	17.3	143.3	86.2	4.1	134.1	82.4	2.3	40.7	120.3	61.8	65.5	1.9	42.6	124.0	60.2
317	33.2	19.6	149.7	76.9	2.3	139.0	75.6	1.8	41.0	126.7	58.4	58.3	1.7	43.0	129.6	57.5
351	24.4	20.3	152.8	68.2	2.7	145.4	66.9	1.8	42.3	133.2	53.9	50.5	2.3	43.9	134.6	54.0
420	17.1	19.5	161.1	54.8	4.3	159.5	61.4	3.0	43.7	144.4	50.5	42.0	3.2	46.1	144.7	51.7
456	13.8	19.1	161.6	45.5	6.4	162.1	51.8	3.7	44.4	147.7	51.3	35.5	3.4	46.3	147.1	50.2
512	9.7	17.7	160.6	35.6	8.6	167.6	44.9	4.6	41.8	149.7	49.4	27.4	5.7	43.7	153.9	50.4
526	145.8	18.2	160.4	158.6	8.7	166.8	129.5	5.8		151.4		155.1	6.7		153.3	
630	121.8	16.2	160.1	121.1	9.9	167.7	119.0	7.9	74.2	152.6	46.4	115.4	8.5	62.4	153.3	45.5
710	127.7	17.9	167.7	144.8	13.5	205.2	134.0	11.8	42.0	183.1	56.1	135.3	14.8	45.8	198.0	57.8
722	175.6	22.3	230.6	165.6	14.3	235.4	170.5	12.7	40.6	233.9	71.7	135.3	13.5	44.6	203.8	60.8

N.D.: Not detected.

Table D-14. *(Continued).*

Day	SO4(#5)	SO4(#11)	SO4+NP(#6)			SO4+NP(#12)		
	SO_4^{2-}	SO_4^{2-}	SO_4^{2-}	NH_4^+	PO_4^{3-}	NH_4^+	SO_4^{2-}	PO_4^{3-}
0	1584.0	947.6	950.6	43.0	186.4	43.3	905.0	210.8
16	791.8	862.6	834.6	35.5	177.6	35.7	854.1	190.1
30	700.8	819.6	759.5	35.2	180.5	35.3	778.2	192.1
63	539.0	674.0	492.3	33.6	193.6	33.4	509.3	196.5
91	444.2	566.7	408.9	33.2	191.8	33.5	421.8	195.6
127	367.2	468.8	324.3	32.8	188.6	32.3	311.0	190.5
171	281.2	371.3	192.1	31.8	172.9	31.5	196.2	177.8
197	251.4	342.3	170.4	37.1	191.1	36.9	195.4	184.7
224	261.8	371.7	151.0	35.2	191.2	36.8	172.9	194.4
253	243.6	353.9	111.6	36.7	176.1	36.0	131.8	175.7
286	217.6	324.8	90.4	34.2	178.9	33.4	84.8	186.1
317	218.1	385.7	70.8	33.6	190.4	33.8	47.3	166.9
351	176.7	324.9	57.1	39.1	187.3	37.9	12.6	190.9
399	99.8	243.3	37.3				3.7	
407	1028.3	1041.3	1125.1				1154.2	
420	977.8	964.4	1051.1	42.7	178.7	62.3	1078.1	183.5
456	908.4	861.7	995.7	61.8	180.5	62.7	1005.7	187.8
512	874.7	844.8	953.3	57.4	177.5	56.2	955.8	183.9
630	705.7	636.3	864.3	73.7	167.6	67.2	853.8	162.0
710	823.1	783.7	1019.0	52.0	163.1	52.1	1030.4	155.9
722	859.0	838.4	1227.8	54.1	151.1	55.6	974.4	177.6

Table D-15. Summary of other major ion concentrations in Site 3 mesocosms (mg/L).

Day	SC1					SC2				
	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-
0	852	50	2	10	195	873	46	2	10	190
16	836	33	2	9	191	841	36	2	10	187
30	862	32	3	8	193	866	34	3	9	188
63	860	33	3	8	194	867	35	3	9	188
91	870	34	3	8	194	865	35	3	9	187
127	859	33	3	8	194	863	35	3	9	187
171	857	33	3	8	183	858	35	3	9	177
197	868	39	3	8	184	867	40	4	9	176
224	873	37	3	8	187	871	40	3	8	181
253	832	35	3	8	190	837	38	4	9	182
286	843	33	3	8	189	837	38	3	8	182
317	853	34	3	8	187	857	38	4	8	181
351	866	35	3	8	181	867	38	3	9	176
420	868	36	3	7	189	871	42	4	9	169
456	889	38	4	8	190	891	42	5	9	183
512	878	37	4	9	190	883	39	4	9	184
630	1094	45	4	9	185	1095	47	5	10	189
710	946	33	2	7	218	962	36	3	8	199
722	895	36	3	6	196	907	40	3	7	221

Table D-15. *(Continued).*

	Ctrl(#1)					Ctrl(#7)					Ctrl+NP(#2)					Ctrl+NP(#8)				
Day	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**
0	395	4	81	59	137	387	4	82	58	136	417	5	83	50	135	411	6	82	44	137
16	377	4	79	51	136	376	4	82	51	134	395	6	83	48	134	396	6	84	47	135
30	390	3	81	41	138	387	3	84	51	136	408	5	80	36	136	408	5	83	45	135
63	392	3	80	51	136	390	3	82	51	137	408	6	76	48	135	408	6	76	47	135
91	392	4	82	52	137	392	4	85	52	138	411	5	76	48	137	412	6	77	47	136
127	387	3	67	53	137	388	4	72	53	137	410	6	62	48	135	406	5	63	47	134
171	393	3	72	49	130	386	3	74	50	129	409	5	64	47	128	404	5	63	44	127
197	397	4	69	55	133	396	3	74	54	134	415	6	61	49	129	411	6	62	48	124
224	395	4	70	55	133	395	4	73	55	131	411	6	60	48	131	412	6	60	48	130
253	381	4	77	52	132	376	4	80	52	132	394	6	66	46	130	395	6	66	46	130
286	379	4	66	53	134	379	4	71	53	133	396	6	56	47	133	395	6	58	47	131
317	389	4	72	55	133	388	4	77	55	133	401	6	61	48	132	402	6	62	48	132
351	386	4	70	54	130	389	4	74	54	129	403	6	58	47	129	404	6	58	45	129
420	396	4	73	54	134	394	4	78	55	133	409	7	61	49	133	410	7	61	48	131
456	402	5	72	55	133	403	4	79	55	132	411	7	59	48	132	415	7	62	48	131
512	397	4	71	55	130	401	4	79	55	132	408	7	58	48	130	414	7	61	48	131
630	439	5	88	59	127	484	5	100	61	130	466	7	70	50	127	526	8	78	56	123
710	419	2	70	55	118	425	3	71	54	149	439	5	53	47	142	455	5	53	47	155
722	399	4	73	55	143	400	4	75	55	189	417	6	57	47	152	419	7	57	47	188

Table D-15. *(Continued).*

	NO3(#3)					NO3(#9)					NO3+NP(#4)					NO3+NP(#10)				
Day	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**
0	394	46	88	59	133	391	51	87	58	135	410	55	84	48	134	413	62	83	49	134
16	382	42	88	57	133	382	41	90	55	132	399	43	88	51	132	397	43	87	49	134
30	395	43	92	59	134	393	41	93	39	134	410	43	87	47	133	411	43	87	45	134
63	393	44	89	58	133	391	42	90	56	133	409	45	82	49	134	412	44	80	47	132
91	396	136	93	61	133	395	85	96	59	133	413	99	87	51	131	415	107	84	50	132
127	391	85	79	63	133	387	76	82	60	132	410	92	73	51	128	410	93	72	50	131
171	396	83	83	58	126	390	76	84	56	125	407	89	73	45	124	407	92	72	43	125
197	400	83	81	65	126	404	76	86	64	124	415	87	74	51	124	419	90	73	51	123
224	399	84	81	66	129	398	77	82	64	127	405	89	71	49	128	417	92	70	50	128
253	383	80	89	62	128	384	74	92	62	128	388	84	78	47	128	397	88	78	47	127
286	384	81	78	63	131	383	74	82	62	131	396	87	70	48	130	397	89	69	47	129
317	394	83	84	66	130	393	76	88	64	130	404	89	75	48	129	404	91	74	48	128
351	394	83	82	65	127	392	76	84	63	127	406	89	73	47	128	406	91	71	47	127
420	401	88	85	66	131	399	79	89	65	130	410	93	76	48	131	412	94	75	48	128
456	406	87	85	66	132	408	80	92	63	130	419	91	76	47	131	419	94	77	47	129
512	407	84	84	66	129	405	77	90	65	129	416	88	77	46	128	417	90	77	46	128
630	467	158	110	75	127	470	138	113	72	123	592	175	120	60	124	464	147	94	46	122
710	439	150	84	63	135	453	134	86	65	153	443	130	74	45	149	460	146	74	49	156
722	409	141	88	71	186	405	123	89	68	176	417	124	80	45	191	428	138	81	48	161

Table D-15. *(Continued).*

	SO4(#5)					SO4(#11)					SO4+NP(#6)					SO4+NP(#12)				
Day	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**	**Na^+**	**K^+**	**Mg^{2+}**	**Ca^{2+}**	**Cl^-**
0	1024	6	91	65	133	787	5	85	59	135	794	7	83	50	134	787	6	86	51	134
16	696	5	87	56	132	735	5	87	55	131	736	7	90	54	133	744	7	92	54	131
30	710	4	88	34	135	755	4	88	41	133	768	6	86	31	135	766	6	88	34	133
63	707	4	81	52	134	754	4	81	52	133	760	7	83	47	133	761	7	82	41	131
91	712	5	82	51	135	755	5	81	50	135	768	7	82	37	133	767	7	85	46	132
127	704	4	68	50	134	751	5	68	50	133	767	7	73	51	135	758	7	72	48	130
171	704	4	69	37	127	747	4	69	37	126	757	6	69	26	128	753	6	69	27	125
197	718	5	68	50	128	756	5	68	49	123	774	9	74	51	136	763	9	71	36	125
224	713	6	68	51	131	760	7	68	52	129	765	10	71	46	131	767	10	70	44	128
253	680	6	73	48	131	728	6	75	49	129	736	9	78	47	129	731	9	77	46	128
286	690	5	65	49	133	733	5	67	50	131	736	7	68	47	131	736	7	69	46	129
317	699	5	68	49	132	748	6	71	51	133	749	8	72	48	132	750	8	73	45	130
351	702	5	66	48	130	752	6	68	50	128	753	8	68	39	130	758	8	68	39	129
420	1086	8	80	58	132	1079	8	81	59	130	1173	11	85	58	132	1183	11	86	56	130
456	1111	10	81	58	131	1103	10	85	59	131	1229	13	86	53	129	1250	13	91	57	129
512	1107	10	80	58	130	1092	10	82	58	131	1223	13	86	56	131	1233	14	87	48	128
630	1194	10	90	56	124	1224	8	93	58	145	1450	13	103	59	133	1335	11	95	53	122
710	1086	3	65	48	135	1182	3	70	55	150	1156	5	65	39	150	1341	6	72	39	147
722	1075	8	72	51	145	1081	9	73	54	145	1197	11	76	51	149	1230	12	77	50	160

Table D-16. Summary of pH data in Site 3 mesocosms.

Day	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
0	9.4	9.3	7.5	7.3	7.3	7.3	7.5	7.4	7.3	7.3	7.5	7.5	7.3	7.3
16														
30	9.3	9.2	8.1	8.0	8.0	7.9	8.2	8.0	7.8	7.7	8.0	7.9	8.0	7.6
63	9.4	9.3	7.7	7.7	7.8	7.5	7.8	7.8	7.7	7.7	7.9	7.9	7.8	7.7
91	9.2	9.2	7.6	7.6	7.4	7.3	7.6	7.6	7.6	7.6	7.8	7.7	7.6	7.6
127	9.3	9.3	7.6	7.5	7.4	7.3	7.6	7.7	7.6	7.5	7.8	7.7	7.6	7.6
171	9.3	9.2	7.5	7.5	7.4	7.4	7.6	7.7	7.6	7.7	7.9	7.9	7.7	7.7
197	9.3	9.3	7.7	7.7	7.6	7.5	7.8	7.9	7.7	7.8	8.0	8.0	7.8	7.8
253	9.3	9.2	7.7	7.7	7.5	7.6	7.7	7.8	7.8	7.8	8.1	8.0	7.9	7.9
420	9.3	9.3	7.7	7.6	7.6	7.6	7.8	7.9	7.9	7.9	8.3	8.2	8.1	8.2
512	9.3	9.3	7.7	7.7	7.7	7.6	7.8	7.9	7.8	7.9	8.3	8.1	8.1	8.1
630	9.3	9.3	7.6	7.7	7.6	7.6	7.7	7.8	7.8	7.8	8.2	8.1	8.2	8.2
710	9.4	9.3	7.8	7.8	7.7	7.7	7.9	8.0	7.9	8.0	8.3	8.2	8.2	8.2

Table D-17. Summary of alkalinity data in Site 3 mesocosms (as mg/L $CaCO_3$).

Day	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
0	644	646	1030	780	1065	1054	1026	995	1054	1025	1005	998	1046	1045
30	656	650	1097.5	1090	1122.5	1117.5	1072	1066	1108.5	1149	1120.5	1093.5	1190	1171.5
63	665	662.5	1030	1110	1095	1132	1082.5	1075	1145.5	1169	1220	1216	1384	1372
91	651	651	1063	1105	1125	1129	1080	1069	1148	1160	1290	1275	1470	1458
127	720	670	1103	1103	1120	1120	1080	1068	1141	1151	1370	1350	1570	1555
171	655	650	1080	1088	1098	1105	1059	1056	1125	1140	1405	1400	1605	1588
197	655	659	1098	1097	1109	1110	1075	1064	1135	1150	1440	1450	1690	1665
253	643	610	1093	1093	1104	1100	1078	1048	1133	1128	1468	1425	1728	1660
420	628	624	1013	1008	1021	1028	1015	999	1045	1068	1495	1498	1623	1649
512	655	654	1059	1055	1068	1070	1055	1040	1095	1108	1659	1694	1763	1804
630	670	660	1078	1084	1095	1095	1085	1059	1120	1134	1793	1815	1850	1905
710	673	660	1080	1080	1093	1094	1090	1065	1125	1138	1721	1755	1805	1840

Table D-18. Groundwater chemical data measured during the Site 3 mesocosm decommissioning.

Parameters	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
DO (mg/L)	0.17	0.12	0.25	0.37	0.17	0.21	0.18	0.13	0.22	0.13	0.20	0.11	0.25	0.14
Temp (°C)	19.7	19.7	17.2	19.8	19.0	19.5	17.7	19.7	19.4	19.8	19.5	20.0	19.9	20.0
Dissolved S^{2-} (ug/L)	11	11	-	45	42	42	-	18	24	17	52	124	52	85
Dissolved Fe^{2+} (mg/L)	1.2	1.4	15.0	18.3	14.3	15.5	2.8	3.5	2.2	3.0	13.5	14.8	13.8	13.3

Table D-19. Summary of groundwater DOC data in Site 3 mesocosms (analyzed by Limnology Lab, University of Alberta).

Date	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3(#3)	NO3(#9)	NO3+NP (#4)	NO3+NP (#10)	SO4(#5)	SO4(#11)	SO4+NP (#6)	SO4+NP (#12)
26-Oct-05	38.17	37.74	39.98	39.32	34.93	33.83	35.15	34.52	41.20	41.96	54.17	49.90	48.01	45.95
30-Aug-06	43.90	42.49	44.23	41.97	35.55	34.46	34.90	34.88	49.06	48.35	50.62	47.60	47.33	45.60

Table D-20. Summary of sediment characterization data in Site 3 mesocosms at the end of incubation (mg/g sediment).

	B	Na	Mg	Al	Si	P	K	Ca	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	As	Rb	Sr	Cs	Ba	Pb	U
SC1	0.0	0.7	5.4	5.7	0.1	0.4	1.3	14.4	0.0	0.0	0.2	5.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
SC2	0.0	0.8	5.6	6.5	0.1	0.4	1.4	14.8	0.0	0.0	0.2	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Ctrl(#1)	0.0	0.4	6.1	10.5	0.1	0.4	1.9	16.5	0.0	0.0	0.2	9.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Ctrl(#7)	0.0	0.3	5.7	7.9	0.1	0.4	1.6	15.4	0.0	0.0	0.2	7.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Ctrl+NP (#2)	0.0	0.4	6.3	8.4	0.1	0.7	1.6	16.8	0.0	0.0	0.2	7.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Ctrl+NP (#8)	0.0	0.3	6.0	8.5	0.1	0.6	1.7	15.7	0.0	0.0	0.2	7.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
NO3(#3)	0.0	0.3	6.2	8.7	0.0	0.4	2.0	16.8	0.0	0.0	0.2	8.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
NO3(#9)	0.0	0.2	5.5	7.5	0.3	0.4	1.8	15.1	0.0	0.0	0.2	6.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
NO3+NP (#4)	0.0	0.3	5.8	6.8	0.1	0.6	1.6	15.4	0.0	0.0	0.2	6.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
NO3+NP (#10)	0.0	0.3	6.0	8.3	0.1	0.6	2.0	16.3	0.0	0.0	0.2	7.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
SO4(#5)	0.0	0.8	5.9	9.1	0.2	0.4	2.0	16.0	0.0	0.0	0.2	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
SO4(#11)	0.0	0.8	5.9	9.0	0.1	0.4	1.9	15.6	0.0	0.0	0.2	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
SO4+NP (#6)	0.0	0.9	6.2	9.2	0.1	0.5	1.7	16.5	0.0	0.0	0.2	8.4	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
SO4+NP (#12)	0.0	0.8	6.0	8.8	0.1	0.5	1.7	16.5	0.0	0.0	0.2	8.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0

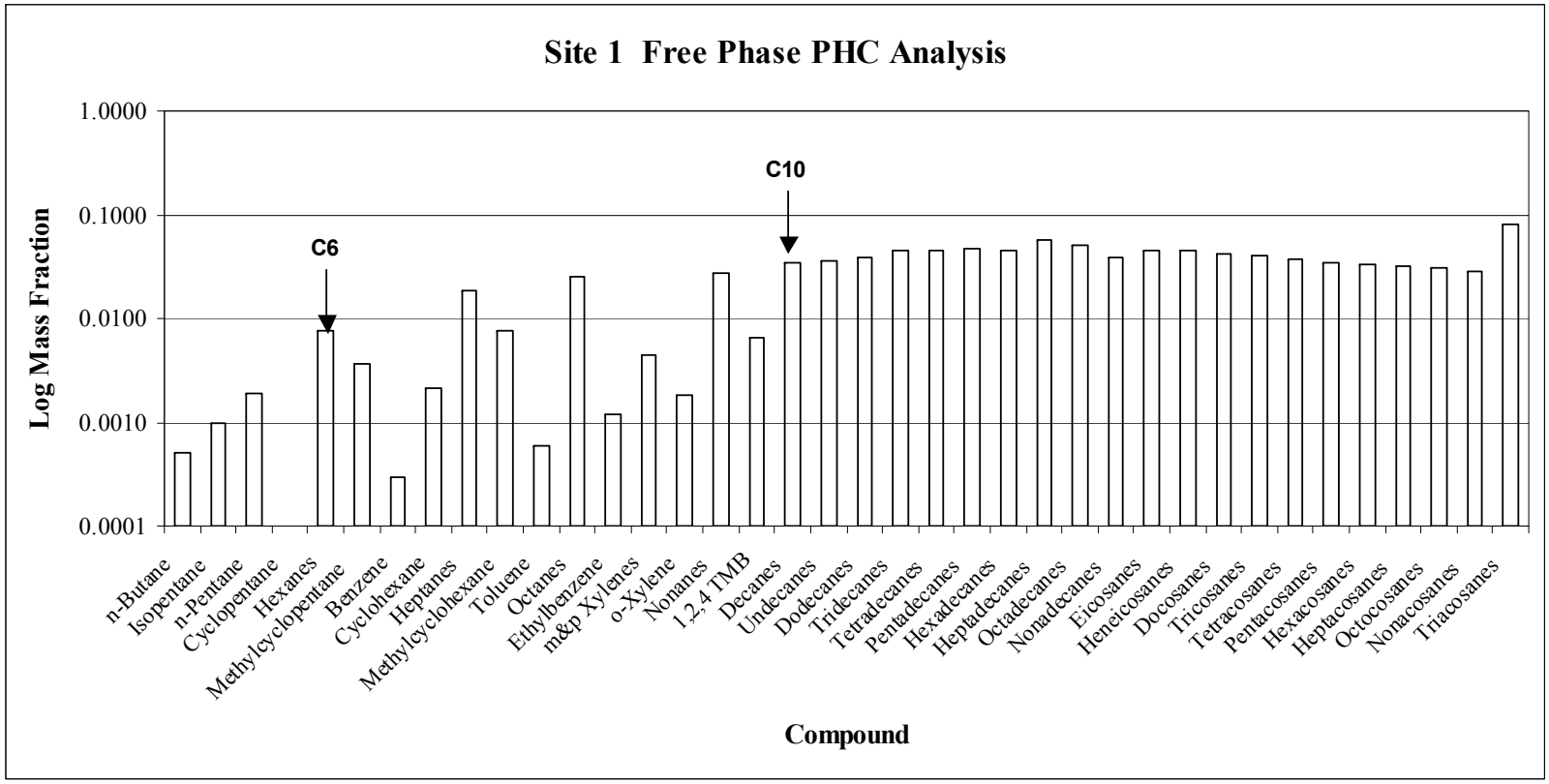

Figure D-4. Analysis of free product recovered from Site 1.

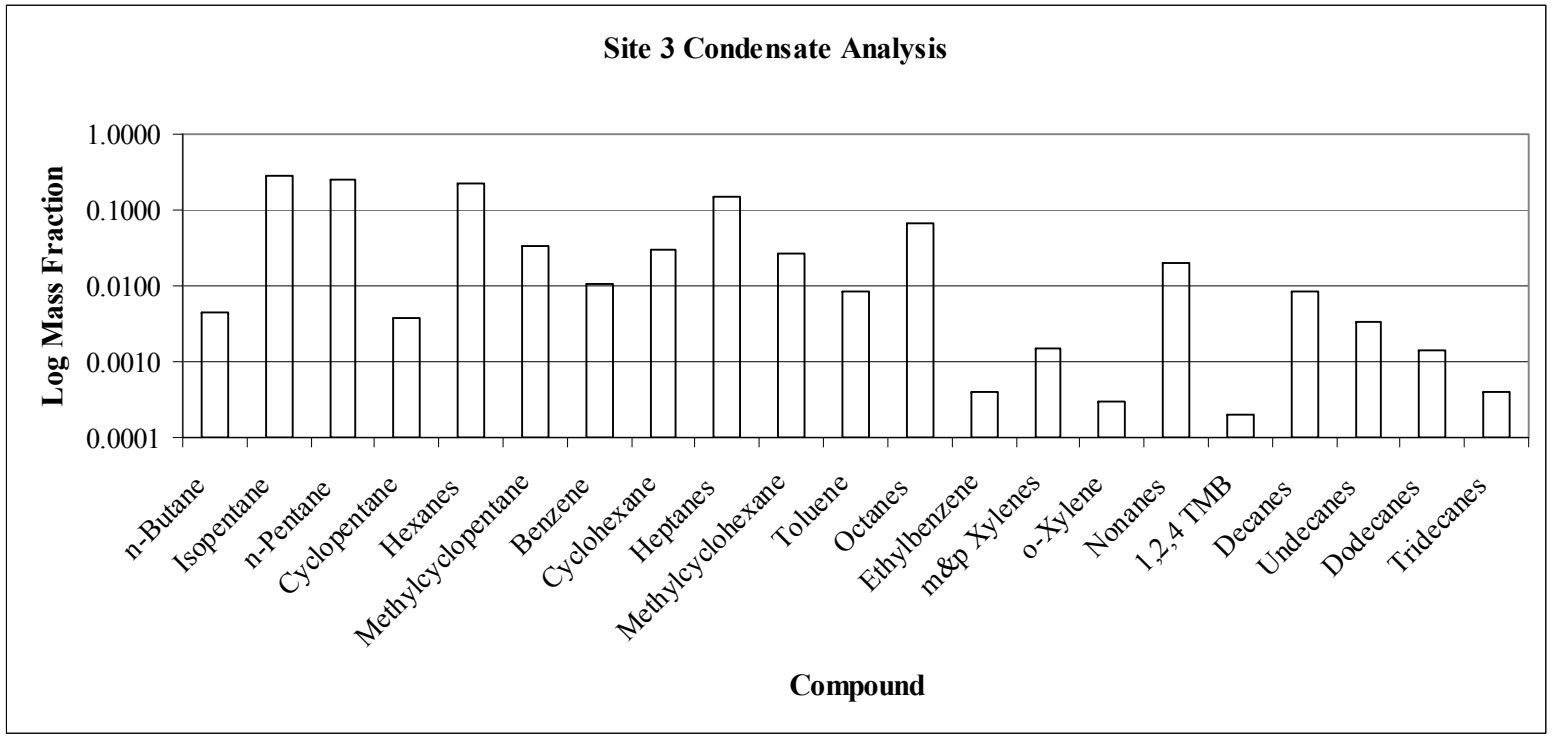

Figure D-5. Analysis of free product recovered from Site 3.

Appendix E. Calculations and Results

APPENDIX E1. CALCULATIONS OF THE CUMULATIVE AMOUNTS OF HEADSPACE CO_2 IN THE MESOCOSMS

The amount of headspace CO_2 in each mesocosm was calculated using the ideal gas law

$$\text{n (mmole)} = \frac{PV}{RT} \times 1000$$

where

P = CO_2 partial pressure (assuming the headspace gas pressure was 1 atm)

V = the headspace volume (L);

R = gas law constant (0.082 atm.L/K.mol)

T = room temperature (288K).

The calculated cumulative amounts of headspace CO_2 in Site 1 and Site 3 mesocosms are summarized in Tables E1-1 and E1-2.

Table E1-1. Cumulative amounts of headspace CO_2 in Site 1 mesocosms (mmole).

Days	V (L)	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3 (#3)	NO3 (#9)	NO3+NP (#4)	NO3+NP (#10)	SO4 (#5)	SO4 (#11)	SO4+NP (#6)	SO4+NP (#12)
0	2.5	0.5	0.4	3.2	0.3	2.8	2.1	3.2	3.1	2.3	2.8	3.7	3.2	2.5	2.6
7	2.5	1.2	1.0	6.7	6.2	7.1	7.0	7.6	8.1	7.4	7.4	8.0	8.3	6.7	7.1
14	2.7	1.5	1.3	8.1	6.8	7.7	7.9	8.4	8.8	8.2	8.1	8.5	9.0	7.5	8.0
31	2.9	1.5	1.4	8.4	6.7	8.6	8.5	8.7	9.3	10.5	9.4	8.9	9.1	8.0	8.6
45	2.9	1.5	1.5	8.8	7.6	9.2	9.2	9.4	10.0	10.4	11.2	9.3	9.5	8.6	9.2
63	3.1	1.7	1.7	9.0	7.8	9.4	9.7	9.9	10.7	10.8	11.6	9.5	10.3	9.1	9.9
75	3.1	2.0	1.8	9.3	7.6	9.8	10.1	10.6	11.1	11.0	12.1	10.0	10.9	9.9	10.7
96	3.4	1.8	1.7	9.4	5.6	9.8	10.4	11.2	10.9	9.8	12.0	10.4	11.3	10.6	11.2
124	3.7	1.8	1.9	10.2	6.1	10.8	11.2	12.9	11.5	11.7	12.4	11.8	12.7	11.7	12.5
149	4	2.0	2.0	11.1	6.3	11.5	12.0	14.9	12.9	13.1	13.1	12.8	13.8	12.7	13.7
179	4.3	2.7	2.4	11.7	7.3	12.4	12.6	16.2	13.2	13.8	13.9	13.5	14.4	12.7	13.8
193	5.3	3.0	2.5	12.2	8.0	11.8	12.8	16.8	14.5	15.2	14.8	14.7	14.8	14.2	15.3
223	5.5	3.2	2.8	14.1	12.0	14.8	15.1	19.8	17.0	17.6	17.4	16.7	17.8	16.5	18.4
241	5.8	2.8	2.6	14.6	13.8	15.3	15.6	20.5	17.7	18.3	18.0	17.3	18.1	17.1	18.9
276	6	4.1	3.3	15.3	15.5	16.0	16.3	21.7	18.8	19.2	19.2	18.2	19.6	17.6	19.8
305	6.3	3.9	3.2	15.3	16.1	16.5	16.7	22.5	19.2	20.0	19.7	18.7	20.4	18.6	20.4
355	6.5	4.6	3.6	14.8	17.3	17.4	17.6	23.8	20.6	21.2	20.9	19.9	22.0	19.8	21.6
452	6.5	4.4	3.6	15.4	18.0	17.1	18.2	23.4	20.7	21.8	21.5	20.1	22.8	19.8	22.3
493	6.7	4.6	3.8	17.6	19.0	17.8	19.1	25.7	22.5	23.0	22.5	20.8	23.7	20.3	23.1
620	8.2	4.9	4.2	18.6	20.9	19.3	20.4	28.3	24.2	24.2	24.5	22.4	25.5	21.1	24.4

Table E1-2. Cumulative amounts of headspace CO_2 in Site 3 mesocosms (mmole).

Days	V (L)	SC1	SC2	Ctrl(#1)	Ctrl(#7)	Ctrl+NP (#2)	Ctrl+NP (#8)	NO3 (#3)	NO3 (#9)	NO3+NP (#4)	NO3+NP (#10)	SO4 (#5)	SO4 (#11)	SO4+NP (#6)	SO4+NP (#12)
0	2.5	0.9	0.6	8.3	7.9	12.3	12.5	12.6	10.7	14.4	11.9	8.1	8.0	12.0	12.2
10	2.5	1.4	0.9	23.3	25.4	41.4	43.5	29.8	25.6	35.9	36.1	23.4	23.4	38.9	37.9
16	2.7	1.5	1.1	24.4	27.3	42.6	44.2	28.5	25.0	35.7	35.8	26.1	24.6	40.1	38.5
30	2.9	0.6	0.4	26.6	30.4	44.8	48.6	28.9	24.5	37.7	36.7	28.6	27.4	43.9	43.4
46	2.9	1.3	0.8	32.4	33.6	48.3	52.6	29.8	25.8	39.0	37.2	31.6	30.1	46.8	44.1
63	3.2	1.7	1.1	28.4	33.7	49.6	52.6	28.4	25.5	37.2	35.7	29.8	28.6	44.3	42.8
91	3.4	1.7	1.1	31.1	35.5	52.2	55.2	29.9	25.6	36.1	34.7	29.8	28.1	45.3	44.6
127	3.6	2.0	1.4	31.1	37.5	53.7	57.2	30.6	25.3	35.5	32.5	30.8	28.9	47.8	46.4
171	3.8	2.3	1.4	33.8	38.5	56.1	59.7	31.7	26.7	34.8	31.8	30.8	29.6	47.8	46.1
197	5.0	2.8	1.9	40.4	41.4	61.1	63.9	27.9	28.2	36.1	33.8	31.7	31.5	47.2	47.4
224	5.2	2.8	2.0	40.5	53.4	72.6	77.5	45.4	38.8	46.1	41.7	37.5	40.3	59.5	54.2
253	5.4	3.0	2.2	41.5	54.5	73.7	79.3	48.2	39.0	45.5	41.5	37.2	47.7	57.7	55.4
317	5.8	3.4	2.8	45.4	59.2	78.3	84.2	54.8	41.1	47.9	44.2	37.0	51.8	55.6	55.9
351	6.0	1.9	1.4	55.9	61.6	83.7	86.6	55.6	42.7	49.0	45.4	35.6	52.5	53.4	54.0
420	6.2	0.6	0.6	62.7	66.2	89.5	91.8	59.1	45.9	52.5	50.1	34.4	55.0	49.9	51.2
456	6.3	0.6	0.6	63.8	67.2	90.2	93.9	59.5	47.5	53.4	49.9	33.3	55.9	47.5	49.1
512	6.4	0.7	0.6	65.0	68.4	89.8	92.4	59.9	48.4	54.3	50.5	31.7	52.4	42.8	44.2
730	8.0	0.8	0.7	66.7	71.9	90.1	93.0	62.3	51.8	56.6	53.9	25.7	35.6	31.7	33.5

APPENDIX E2. CALCULATIONS OF THE CORRECTED BTEX CONCENTRATIONS IN THE MESOCOSMS

Concentrations of dissolved BEX should be corrected for partitioning into the headspace. The measured BEX concentrations were corrected based on changes of the headspace volume and the respective Henry's Law constant. The calculation is discussed as follows,

Assuming initially

C_L = the dissolved concentration of one specific compound (mg/L);

C_G = the headspace concentration of this specific compound (mg/L);

V_L = the groundwater volume in the mesocosm (L);

V_G = the headspace volume in the mesocosm (L);

ΔV = the volume of water sample taken from the mesocosm; and

H = Henry's Law constant of this compound (unitless).

The total mass of this specific compound is

$M_T = C_G \times V_G + C_L \times V_L$

After the water sample ΔV has been taken, the volumes of water and headspace become $(V_L - \Delta V)$ and $(V_G + \Delta V)$.

Assuming the new concentrations are C_L' and C_G', respectively.

The new mass of this compound $M = M_T - C_L \times \Delta V$

Thus $C_G \times V_G + C_L \times V_L - C_L \times \Delta V = C_G' \times (V_G + \Delta V) + C_L' \times (V_L - \Delta V)$ **(Eq. E-1)**

Since $H = C_G/C_L = C_G'/C_L'$,

$C_G = H \times C_L$ and $C_G' = H \times C_L'$

Substituting C_G and C_G' into Eq. D-1 and rearranging the equation,

The dissolved concentration after the sampling can be calculated as follows,

$$C_L' = \frac{C_L \times (H \times V_G + V_L - \Delta V)}{H \times V_G + H \times \Delta V + V_L - \Delta V} \quad \textbf{(Eq. E-2)}$$

The unitless Henry's Law constants used in the calculation are 0.23 for benzene, 0.35 for ethylbenzene, and 0.32 for xylenes.

The corrected BEX concentrations in both mesocosm studies are tabulated in Tables E2-1 to E2-7.

Table E2-1. Dissolved benzene concentrations corrected for partitioning into the headspace in Site 1 mesocosms (µg/L).

Time (d)	Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)	Ctrl+NP(#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)	SO4(#5)
0									
14									
31	324	321	299	330	325	344	315	348	360
63	362	350	338	363	362	370	350	387	385
96	283	278	271	295	292	315	291	316	315
124	293	275	265	288	295	292	275	312	316
149	287	275	249	281	268	274	263	304	290
179	297	303	263	295	266	309	268	351	310
223	321	329	218	271	60	312	107	295	233
241	218	240	156	157	47	219	27	175	168
276	229	116	81	153	52	255	17	141	61
305	242	263	88	204	51	266	5	146	63
355	219	175	79	194	49	261	3	139	27
493	4	8	46	208	60	340	3	107	0
620	1	5	10	116	35	106	2	69	0

Table E2-2. Dissolved ethylbenzene concentrations corrected for partitioning into the headspace in Site 1 mesocosms (µg/L).

Time (d)	Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)	Ctrl+NP(#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)	SO4(#5)
0									
14									
31	71	30	87	40	82	94	96	91	74
63	84	57	86	59	72	97	103	96	89
96	30	20	49	44	38	83	86	72	65
124	64	28	30	54	29	84	90	74	80
149	44	18	2	19	2	75	56	67	69
179	36	23	36	53	2	77	53	76	70
223	37	22	15	28	0	29	13	35	42
241	40	24	14	26	1	35	8	21	31
276	24	19	3	27	1	15	7	7	13
305	28	24	6	35	1	29	3	9	21
355	47	31	11	38	1	48	3	18	27
493	0	2	2	47	1	13	1	4	9
620	0	0	0	33	0	1	0	2	0

Table E2-3. Dissolved m-, and p-xylene concentrations corrected for partitioning into the headspace in Site 1 mesocosms (µg/L).

Time (d)	Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)	Ctrl+NP(#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)	SO4(#5)
0									
14									
31	87	72	91	76	91	72	85	82	92
63	94	79	98	86	52	77	92	90	97
96	72	70	77	72	27	68	78	72	85
124	72	71	81	76	19	70	85	77	85
149	55	68	53	65	2	65	72	71	80
179	39	69	53	67	0	55	67	75	84
223	32	73	11	53	0	53	24	46	30
241	33	63	12	47	0	50	21	29	32
276	3	73	1	39	0	52	22	11	0
305	4	76	0	58	0	52	12	17	9
355	1	77	0	56	1	59	8	25	29
493	0	14	0	50	1	32	3	6	1
620	0	1	0	17	0	0	1	5	0

Table E2-4. Dissolved benzene concentrations corrected for partitioning into the headspace in Site 3 mesocosms (µg/L).

Time (d)	Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)	Ctrl+NP(#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)
0	4915	5034	5066	5166	5118	5378	5258	5205
16	4427	4478	4604	4356	4536	4510	4737	4767
30	4576	4519	4658	4459	4647	4694	4742	4726
63	4531	4807	4687	4523	4429	4720	4897	4923
91	4759	4801	4729	4602	4460	4464	4795	4655
127	4543	4468	4492	4210	4248	4509	4554	4740
197	4109	4229	4112	3959	3990	4213	4328	4236
224	4716	4834	4414	4499	3965	4473	5058	4978
253	4787	4938	4634	4711	3654	4755	5457	5230
286	4783	4802	4651	4528	3356	4683	4948	4931
317	4401	4633	4230	4569	2991	4355	4968	4859
351	4568	4604	4546	4542	3018	4344	4942	4686
420	4659	4838	4634	4622	3265	4585	5147	4943
456	4830	4927	4529	4601	3223	4619	5000	4932
512	4094	4106	3909	4462	2654	4177	4432	4380
630	3907	4115	3919	3987	2691	3838	4472	4217
722	4304	4234	3928	4048	2671	3840	4478	4296

Table E2-5. Dissolved ethylbenzene concentrations corrected for partitioning into the headspace in Site 3 mesocosms (µg/L).

Time (d)	Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)	Ctrl+NP(#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)
0	366	254	496	516	494	469	534	478
16	509	437	496	439	144	363	545	478
30	502	422	503	420	118	397	546	484
63	551	449	499	466	126	432	624	572
91	544	460	491	482	126	376	600	530
127	561	423	455	395	124	376	604	567
197	484	427	469	418	76	414	639	471
224	278	280	266	226	0	60	681	474
253	347	343	405	385	0	171	863	497
286	427	337	394	397	0	123	531	457
317	350	277	304	352	0	112	581	375
351	405	309	399	365	0	108	544	417
420	494	327	450	437	0	129	643	471
456	417	350	372	438	0	109	587	411
512	270	193	302	335	0	70	453	368
630	300	233	324	306	0	49	503	376
722	380	267	400	327	0	27	606	272

Table E2-6. Dissolved m-, and p-xylene concentrations corrected for partitioning into the headspace in Site 3 mesocosms (µg/L).

Time (d)	Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)	Ctrl+NP(#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)
0	3550	3695	3634	3649	3661	3647	3846	3564
16	3694	3709	3817	3591	3824	3648	3873	3622
30	3744	3612	3791	3438	3874	3830	3930	3794
63	4255	4018	3993	3738	3879	4364	4656	4490
91	3951	3784	3753	3771	3759	3648	4298	4008
127	4137	3530	3424	3117	3759	3617	4376	4338
197	3843	4074	3836	3675	4193	4382	4893	4184
224	4529	4718	3880	3885	4547	5138	6802	6849
253	4383	4485	4952	4501	4582	6295	8535	6083
286	4232	4293	4253	4198	3996	4882	5006	5478
317	3800	4221	3769	4370	3508	4753	6217	4712
351	4056	3961	4259	4009	4036	4419	5260	5032
420	4875	4370	5219	4534	5081	5652	6030	5868
456	4362	4671	4278	4788	4121	5787	5794	5180
512	3943	3993	3844	4613	3625	5364	4586	4858
630	3599	3929	3745	3756	3967	4707	5122	4873
722	5074	4663	4816	4335	4617	5038	7698	6055

Table E2-7. Dissolved o-xylene concentrations corrected for partitioning into the headspace in Site 3 mesocosms (µg/L).

Time (d)	Ctrl(#1)	Ctrl(#7)	Ctrl+NP(#2)	Ctrl+NP(#8)	NO3(#3)	NO3(#9)	NO3+NP(#4)	NO3+NP(#10)
0	933	930	918	950	950	937	983	901
16	857	846	891	867	897	884	911	856
30	713	452	650	352	897	893	922	871
63	587	80	505	48	882	970	1012	936
91	566	81	504	48	878	846	952	854
127	603	84	471	40	862	838	967	921
197	535	103	472	44	866	919	1008	812
224	612	117	582	55	939	1073	1358	1299
253	597	110	613	53	946	1244	1638	1160
286	547	102	510	48	786	975	970	1005
317	538	109	477	53	744	999	1262	950
351	567	101	548	46	844	943	1069	992
420	657	107	642	59	1056	1141	1197	1137
456	586	112	514	64	831	1152	1116	976
512	545	101	477	55	748	1081	930	928
630	484	99	460	44	800	967	1006	924
722	706	120	598	52	965	1074	1519	1171

Appendix F. MPN Results and Statistical Analyses

APPENDIX F1: MPN RESULTS IN SITE 1 AND SITE 3 MESOCOSMS

Table F1-1. Summary of MPN results of SRB, NRB, and IRB in Site 1 mesocosms at Time 0, on Day 193 and Day 620 (MPN/mL). MPN results on Day 193 were obtained after 1-year incubation, whereas results at Time 0 and on Day 620 were counted after 6-month incubation.

Time (d) / Mesocosms	SRB			NRB			IRB		
	0	**193**	**620**	**0**	**193**	**620**	**0**	**193**	**620**
Ctrl (#1)	NA	NA	4.3E+00	NA	NA	1.1E+06	NA	NA	1.5E+04
Ctrl (#7)	7.0E-01	1.1E+03	3.9E+00	2.4E+05	1.5E+06	2.4E+06	4.3E+04	2.8E+03	9.3E+04
Ctrl+NP (#2)	9.3E+01	1.5E+02	3.9E+00	2.4E+05	4.6E+06	1.1E+06	9.3E+04	7.5E+03	2.4E+05
Ctrl+NP (#8)	NA	2.8E+03	2.1E+02	NA	4.3E+05	4.3E+05	NA	2.1E+04	1.5E+04
NO3 (#3)	1.5E+00	7.5E+00	1.5E+01	2.1E+04	2.3E+05	3.9E+04	1.5E+05	1.5E+05	3.9E+04
NO3 (#9)	NA	NA	3.0E-01	NA	NA	4.3E+04	NA	NA	4.3E+04
NO3+NP (#4)	NA	NA	7.0E-01	NA	NA	2.4E+05	NA	NA	1.5E+05
NO3+NP (#10)	NA	2.3E+00	4.0E-01	NA	1.5E+05	9.3E+04	NA	2.4E+05	4.6E+05
SO4 (#5)	NA	NA	2.1E+01	NA	NA	9.3E+05	NA	NA	1.5E+04
SO4 (#11)	1.5E+00	4.3E+03	1.5E+03	2.4E+05	9.3E+04	9.3E+04	4.3E+05	2.4E+04	2.3E+02
SO4+NP (#6)	NA	1.5E+02	1.5E+02	NA	4.3E+04	4.3E+04	NA	2.3E+03	7.5E+04
SO4+NP (#12)	NA	NA	2.1E+03	NA	NA	1.5E+05	NA	NA	1.2E+05

NA = Not analyzed.

Table F1-2. Summary of MPN results of methanogens and fermenters in Site 1 mesocosms at Time 0, on Day 193, and Day 620 (MPN/mL). MPN results on Day 193 were obtained after 1-year incubation, whereas results at Time 0 and on Day 620 were counted after 6-month of incubation.

Time (d) / Mesocosms	Methanogens			Fermenters		
	0	193	620	0	193	620
Ctrl (#1)	NA	NA	NA	NA	NA	1.5E+03
Ctrl (#7)	BDL	7.0E-01	NA	NA	NA	9.3E+02
Ctrl+NP (#2)	BDL	BDL	NA	NA	NA	3.9E+03
Ctrl+NP (#8)	NA	BDL	NA	NA	NA	2.1E+02
NO3 (#3)	BDL	BDL	NA	NA	NA	3.9E+03
NO3 (#9)	NA	NA	NA	NA	NA	2.4E+05
NO3+NP (#4)	NA	NA	NA	NA	NA	1.1E+06
NO3+NP (#10)	NA	BDL	NA	NA	NA	2.4E+05
SO4 (#5)	NA	NA	NA	NA	NA	2.8E+02
SO4 (#11)	BDL	BDL	NA	NA	NA	9.3E+03
SO4+NP (#6)	NA	BDL	NA	NA	NA	9.3E+02
SO4+NP (#12)	NA	NA	NA	NA	NA	2.4E+03

NA = Not analyzed
BDL = Below detection limit

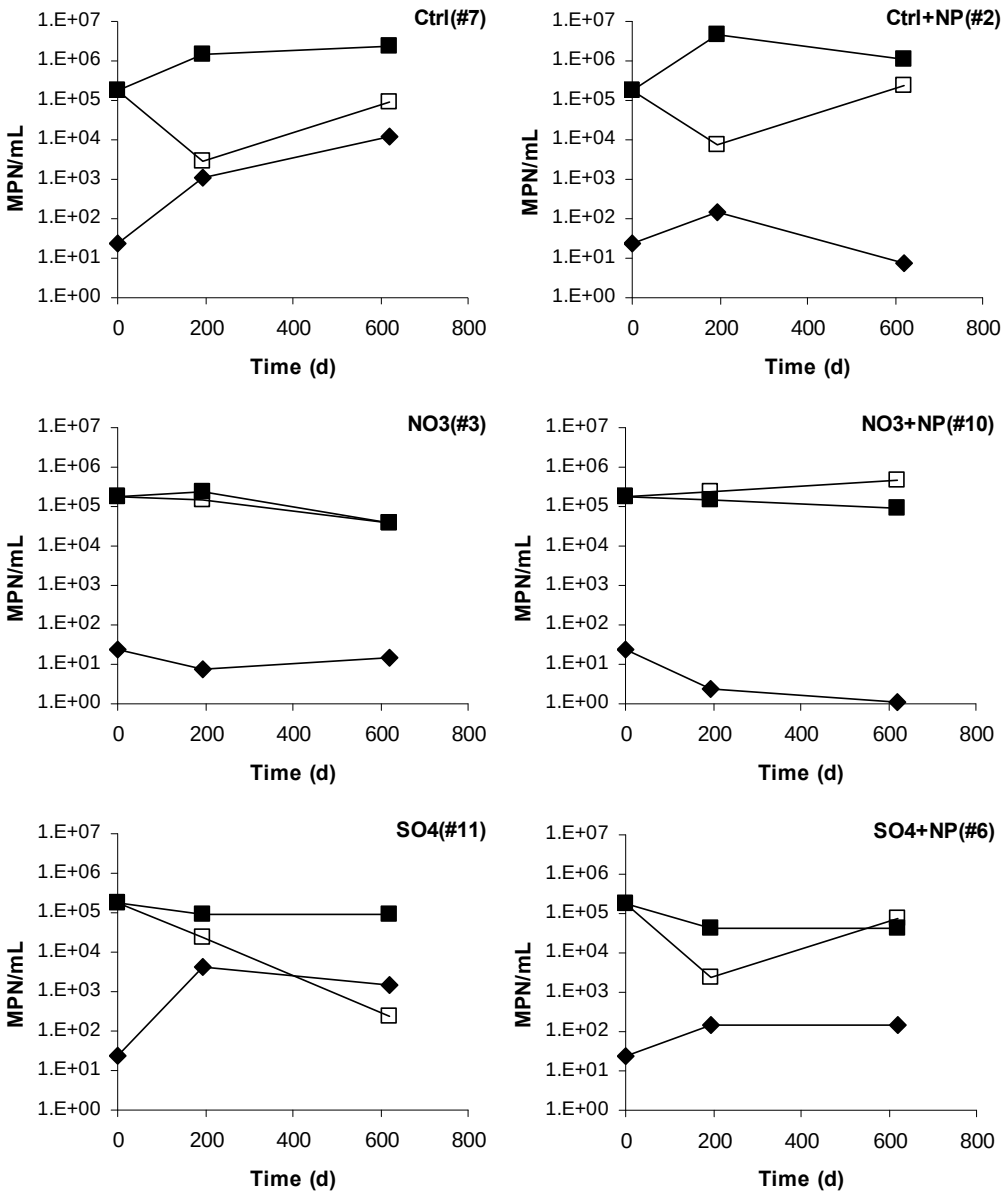

Figure F1-1. MPNs of NRB (■), IRB (□), and SRB (♦) on Day 0, Day 193, and Day 620 in representative mesocosms.

Table F1-3. Summary of MPN results of SRB, NRB, and IRB in Site 3 mesocosms at Time 0, on Day 197*, and at the end of incubation (Day 722) (MPN/mL).

Time (d) / Mesocosms	SRB			NRB			IRB		
	0	**197**	**722**	**0**	**197**	**722**	**0**	**197**	**722**
Ctrl (#1)	NA	NA	4.3E+01	NA	NA	1.5E+03	NA	NA	4.3E+01
Ctrl (#7)	4.6E+02	7.0E+01	1.5E+02	4.3E+04	2.3E+03	9.3E+03	2.8E+04	2.3E+02	9.3E+00
Ctrl+NP (#2)	NA	NA	9.3E+01	NA	NA	4.3E+03	NA	NA	4.3E+01
Ctrl+NP (#8)	NA	2.0E+01	1.1E+01	NA	2.3E+03	4.3E+03	NA	2.3E+02	9.3E+00
NO3 (#3)	2.4E+03	1.1E+00	2.8E+00	2.4E+04	2.0E+03	2.3E+03	2.1E+04	1.4E+03	9.3E+02
NO3 (#9)	NA	NA	1.5E+01	NA	NA	7.5E+03	NA	NA	9.3E+02
NO3+NP (#4)	NA	NA	9.3E+00	NA	NA	2.3E+03	NA	NA	1.5E+03
NO3+NP (#10)	NA	2.3E+01	7.0E-01	NA	9.0E+02	4.3E+04	NA	9.3E+02	9.3E+03
SO4 (#5)	NA	NA	6.4E+01	NA	NA	4.3E+02	NA	NA	2.3E+01
SO4 (#11)	NA	3.0E+01	6.4E+01	NA	4.3E+03	9.0E+02	NA	1.5E+01	4.3E+01
SO4+NP (#6)	1.1E+02	9.3E+01	3.9E+01	9.3E+03	9.3E+03	4.3E+03	1.5E+04	2.3E+01	2.3E+01
SO4+NP (#12)	NA	NA	4.3E+01	NA	NA	9.3E+03	NA	NA	2.3E+01

*: The incubation temperature for the Day 197 MPN analysis was 10 °C instead of 15 °C for all other MPN analyses

NA = Not analyzed.

Table F1-4. Summary of MPN results of methanogens, fermenters, and NRSOB in Site 3 mesocosms at Time 0, after 197 days of incubation, and at the end of incubation (MPN/mL).

Time (d) / Mesocosms	Methanogens			Fermenters			NRSOB		
	0	**197**	**722**	**0**	**197**	**722**	**0**	**197**	**722**
Ctrl (#1)	NA	NA	1.5E+03	NA	NA	4.3E+03	NA	NA	4.0E-01
Ctrl (#7)	2.3E+03	2.3E+01	4.3E+03	NA	NA	4.6E+04	NA	NA	2.1E+00
Ctrl+NP (#2)	NA	NA	1.5E+03	NA	NA	7.5E+03	NA	NA	2.3E+00
Ctrl+NP (#8)	NA	9.3E+01	1.5E+03	NA	NA	2.3E+03	NA	NA	2.1E+00
NO3 (#3)	4.3E+03	BDL	3.0E-02	NA	NA	2.4E+02	NA	NA	3.0E-02
NO3 (#9)	NA	NA	3.0E-02	NA	NA	4.3E+03	NA	NA	3.0E-02
NO3+NP (#4)	NA	NA	3.0E-02	NA	NA	2.3E+03	NA	NA	3.0E-02
NO3+NP (#10)	NA	BDL	3.0E-02	NA	NA	3.9E+03	NA	NA	3.0E-02
SO4 (#5)	NA	NA	2.3E+02	NA	NA	4.3E+03	NA	NA	1.5E+01
SO4 (#11)	NA	9.3E+00	2.3E+03	NA	NA	4.3E+02	NA	NA	1.1E+00
SO4+NP (#6)	9.3E+03	7.5E+01	2.0E+02	NA	NA	1.5E+03	NA	NA	1.2E+01
SO4+NP (#12)	NA	NA	2.3E+03	NA	NA	9.3E+03	NA	NA	2.9E+00

NA = Not analyzed.

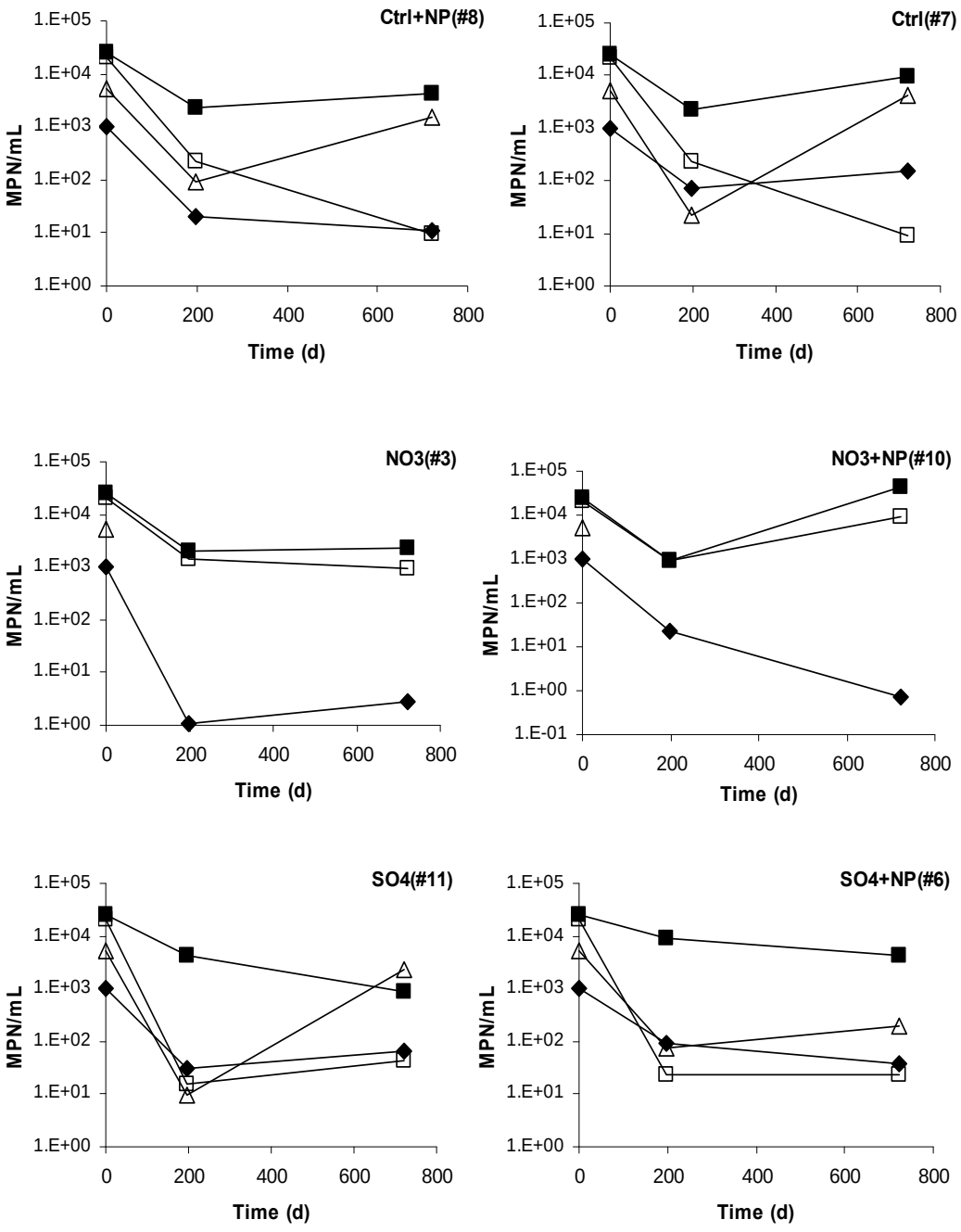

Figure F1-2. MPNs of NRB (■), IRB (□), SRB (♦), and methanogens (Δ) in representative mesocosms. (MPNs on Day 197 were incubated at 10ºC, rather than 15ºC).

APPENDIX F2: STATISTIC COMPARISONS OF MPN RESULTS

Pairs of MPNs can be compared using the method described in Cochran (1950). The equation for calculating the test statistic Z is given below.

$$Z = \frac{|\log MPN_1 - \log MPN_2|}{c\sqrt{\frac{\log a_1}{n_1} + \frac{\log a_2}{n_2}}}$$

where c is a constant

a_i is the dilution ratio (I = 1,2)

n_i is the number of samples per dilution (I = 1,2)

In the case of serial 10-fold dilutions and 3 tube MPN method,

c = 0.55;

$a_1 = a_2 = 10$; and

$n_1 = n_2 = 3$

Referring to the normal probability table, at P = 0.05 (the 95% confidence level), two MPN values are significantly different if the test statistic Z is > 1.96. Or, at P = 0.05, two MPN values are significantly different if the ratio of the larger MPN/smaller MPN is > 7.6 (based on 3-tube method with 10-fold dilution ratio).

Time 0 MPN results of each redox-specific bacterium were paired and the test statistic (Z) was calculated for each pair using this equation. The calculated Z values are tabulated in Tables F2-1 and F2-2.

Table F2-1. Calculated Z values for MPN results in Site 1 mesocosms at Time 0.

Mesocosm #	SRB	NRB	IRB
#2 vs. #3	3.99	2.36	0.46
#2 vs. #7	4.73	0	0.75
#2 vs. #11	3.99	0	1.48
#3 vs. #7	0.74	2.36	1.21
#3 vs. #11	0	2.36	1.02
#7 vs. #11	0.74	0	2.23

Table F2-2. Calculated Z values for MPN results in Site 3 mesocosms at Time 0.

Mesocosm #	SRB	NRB	IRB	Methanogen
#3 vs. #6	2.98	0.92	0.33	0.75
#3 vs. #7	1.60	0.56	0.28	0.61
#6 vs. #7	1.38	1.48	0.60	1.35

The redox-specific bacteria MPNs were compared between the different treatments after 6 months of incubation and at the end of incubation for each mesocosm study. The results are summarized in the following tables.

Table F2-3. Summary of average MPN results used for statistical analyses in Site 1 mesocosms after 193 days of incubation and at the end of incubation (MPN/mL).

Time (d)	193			620			
Bacteria	SRB	NRB	IRB	SRB	NRB	IRB	Fermenter
Ctrl	1.35E+03	2.18E+06	1.04E+04	5.55E+01	1.26E+06	9.08E+04	1.64E+03
NO3	4.90E+00	1.90E+05	1.95E+05	4.10E+00	1.04E+05	1.73E+05	3.96E+05
SO4	2.23E+03	6.80E+04	1.32E+04	2.30E+02	3.04E+05	5.26E+04	3.23E+03

Table F2-4. Statistics for comparisons of redox-specific bacteria between different treatments in Site 1 mesocosms after 193 days of incubation. Shaded numbers indicate that the MPNs are statistically significant between the compared treatments.

SRB		NRB		IRB	
SO4/Ctrl	1.6	Ctrl/NO3	11.5	NO3/Ctrl	18.7
SO4/NO3	454.1	Ctrl/SO4	32.0	NO3/SO4	14.8
Ctrl/NO3	275.5	NO3/SO4	2.8	SO4/Ctrl	1.3

Table F2-5. Statistics for comparisons of redox-specific bacteria between different treatments in Site 1 mesocosms at the end of incubation. Shaded numbers indicate that the MPNs are statistically significant between the compared treatments.

SRB		NRB		IRB		Fermenters	
SO4/Ctrl	4.1	Ctrl/NO3	12.1	NO3/Ctrl	1.9	NO3/Ctrl	242.2
SO4/NO3	56.0	Ctrl/SO4	4.1	NO3/SO4	3.3	NO3/SO4	122.7
Ctrl/NO3	13.5	SO4/NO3	2.9	Ctrl/SO4	1.7	SO4/Ctrl	2.0

Table F2-6. Summary of average MPN results used for statistical analyses in Site 3 mesocosms after 197 days of incubation (MPN/mL).

Bacteria	SRB	NRB	IRB	Methanogens
Ctrl	4.50E+01	2.30E+03	2.30E+02	5.80E+01
NO3	1.21E+01	1.45E+03	1.17E+03	
SO4	6.15E+01	6.80E+03	1.90E+01	4.22E+01

Table F2-7. Statistics for comparisons of redox-specific bacteria between different treatments in Site 3 mesocosms after 197 days of incubation. Shaded numbers indicate that the MPNs are statistically significant between the compared treatments.

SRB		NRB		IRB		Methanogen	
SO4/Ctrl	1.4	SO4/Ctrl	3.0	NO3/Ctrl	5.1	Ctrl/SO4	1.4
SO4/NO3	5.1	SO4/NO3	4.7	NO3/SO4	61.3		
Ctrl/NO3	3.7	Ctrl/NO3	1.6	Ctrl/SO4	12.1		

Table F2-8. Summary of average MPN results used for statistical analyses in Site 3 mesocosms at the end of incubation (MPN/mL).

Bacteria	SRB	NRB	IRB	Fermenter	NRSOB	Methanogen
Ctrl	7.43E+01	4.85E+03	2.62E+01	1.50E+04	1.73E+00	2.20E+03
NO3	6.95E+00	1.38E+04	3.17E+03	2.69E+03	3.00E-02	3.00E-02
SO4	5.25E+01	3.73E+03	2.80E+01	3.88E+03	7.75E+00	1.26E+03

Table F2-9. Statistics for comparisons of redox-specific bacteria between different treatments in Site 3 mesocosms at the end of incubation. Shaded numbers indicate that the MPNs are statistically significant between the compared treatments.

SRB		NRB		IRB	
Ctrl/NO3	10.7	NO3/Ctrl	2.8	NO3/Ctrl	121.0
Ctrl/SO4	1.4	NO3/SO4	3.7	NO3/SO4	113.0
SO4/NO3	7.6	Ctrl/SO4	1.3	SO4/Ctrl	1.1
Fermenter		**NRSOB**		**Methanogen**	
Ctrl/NO3	5.6	SO4/Ctrl	4.5	Ctrl/NO3	73333.3
Ctrl/SO4	3.9	SO4/NO3	258.3	Ctrl/SO4	1.7
SO4/NO3	1.4	Ctrl/NO3	57.5	SO4/NO3	41916.7

Reference:

Cochran, W.G. (1950.) Estimation of bacterial densities by means of the "Most Probable Number". *Biometrics*, 6, 105-116.

Appendix G. Plots of Metabolite Results with Time

APPENDIX G1: PLOTS OF IDENTIFIED METABOLITES IN SITE 1 MESOCOSMS

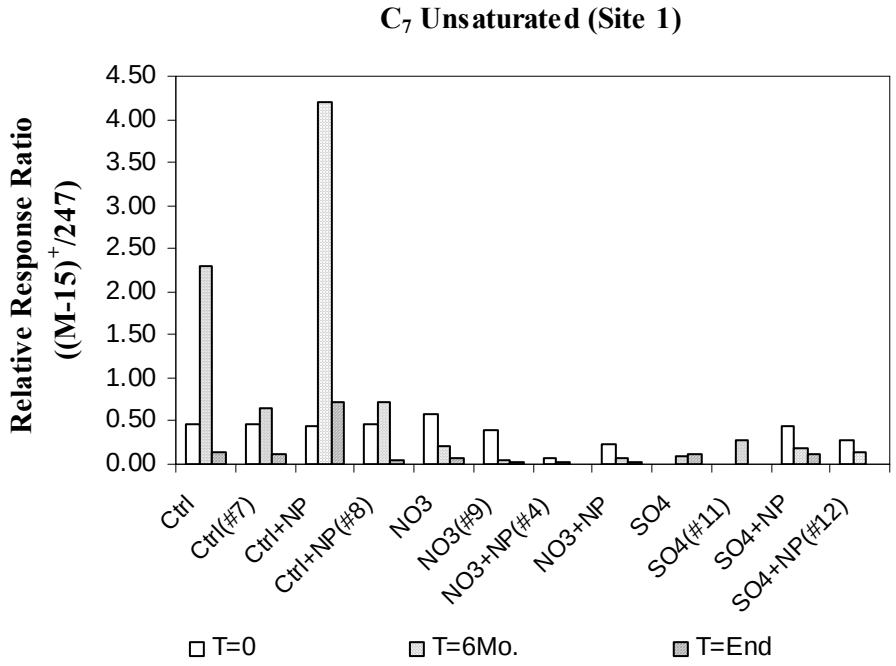

Figure G1-1. Changes in the abundance of C_7 unsaturated metabolite (as relative response ratio) over time in Site 1 mesocosms.

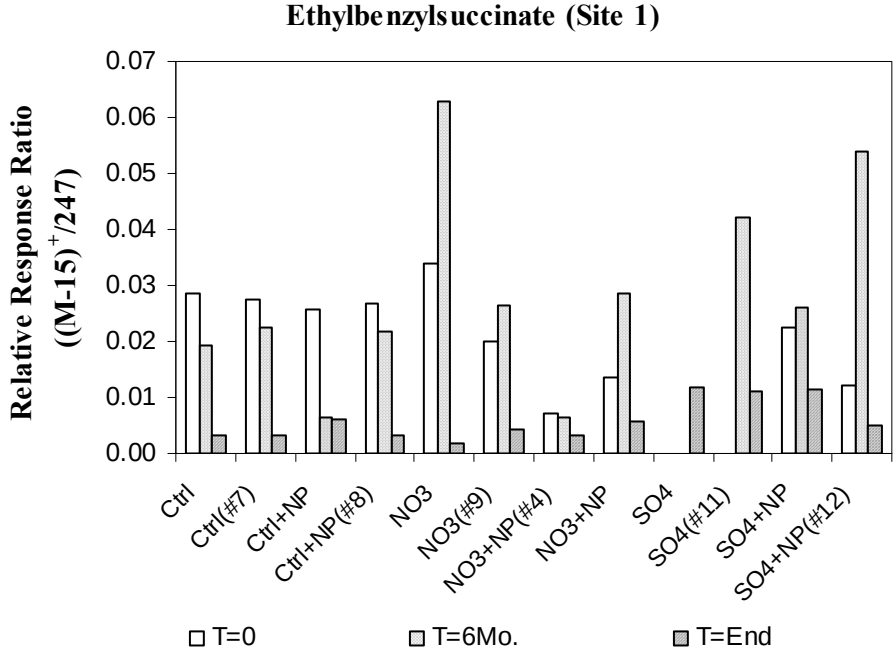

Figure G1-2. Changes in the abundance of ethylbenzylsuccinate (as relative response ratio) over time in Site 1 mesocosms.

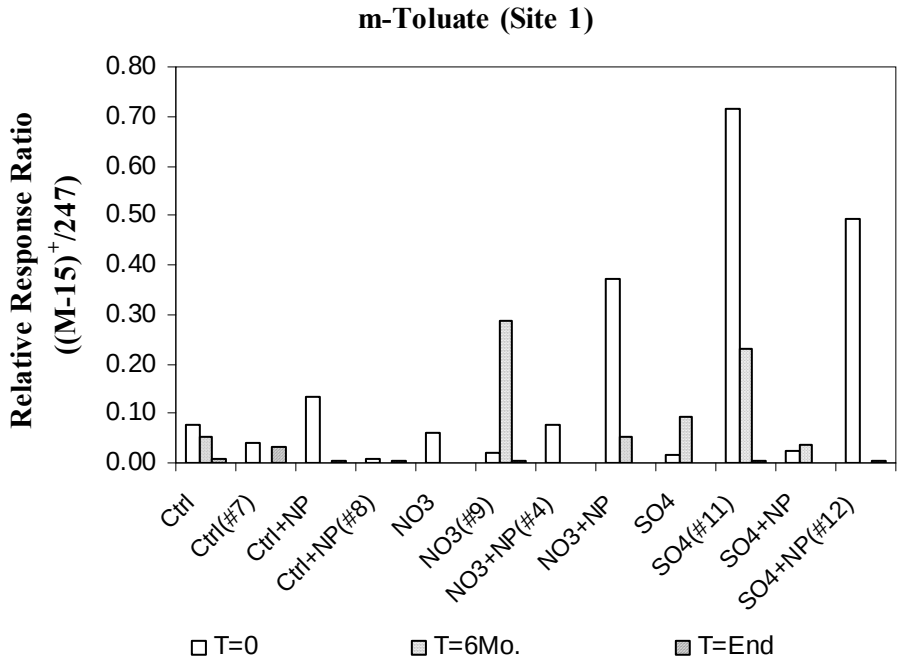

Figure G1-3. Changes in the abundance of m-toluate (as relative response ratio) over time in Site 1 mesocosms.

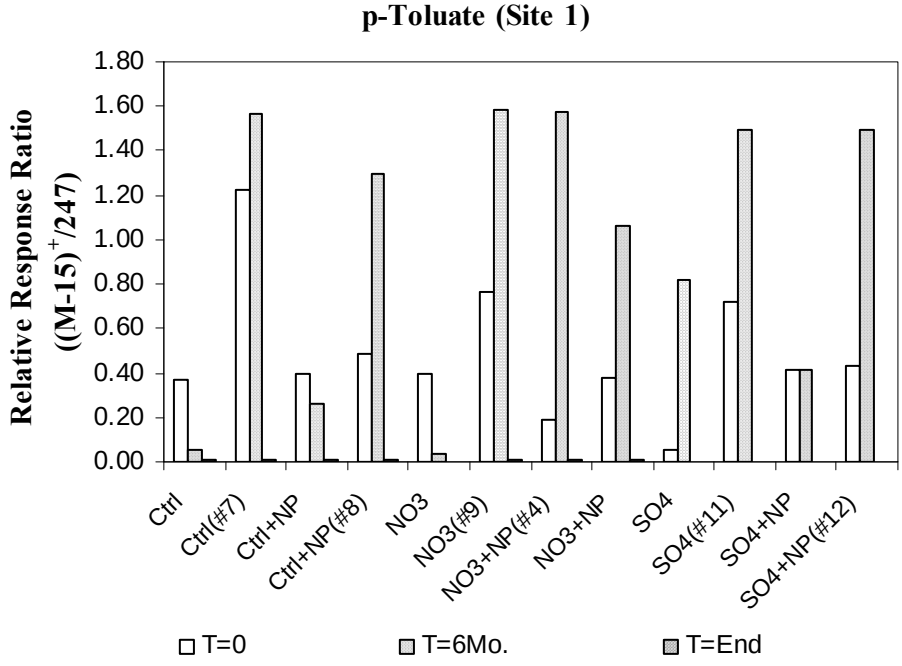

Figure G1-4. Changes in the abundance of p-toluate (as relative response ratio) over time in Site 1 mesocosms.

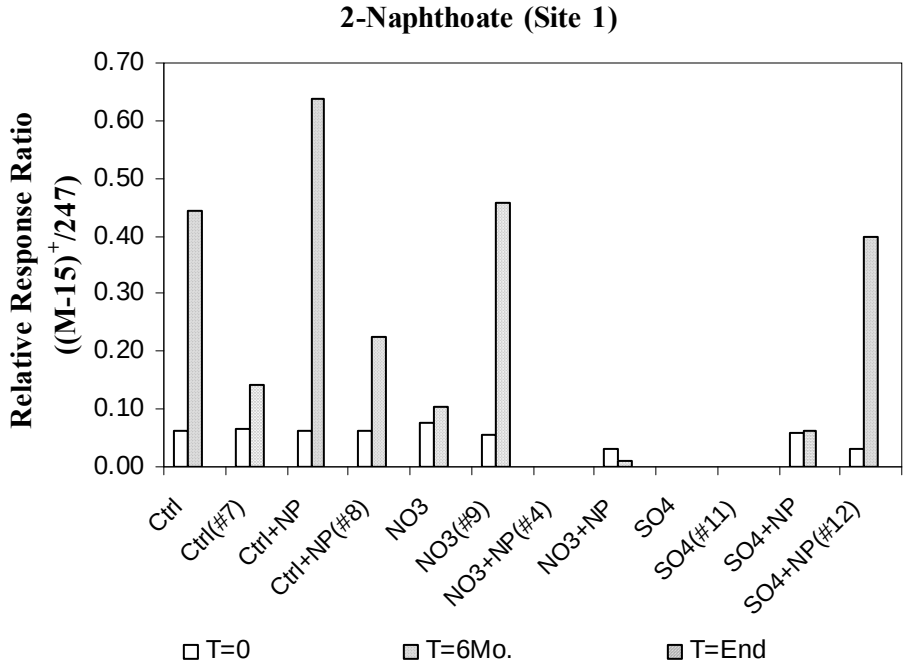

Figure G1-5. Changes in the abundance of 2-naphthoate (as relative response ratio) over time in Site 1 mesocosms.

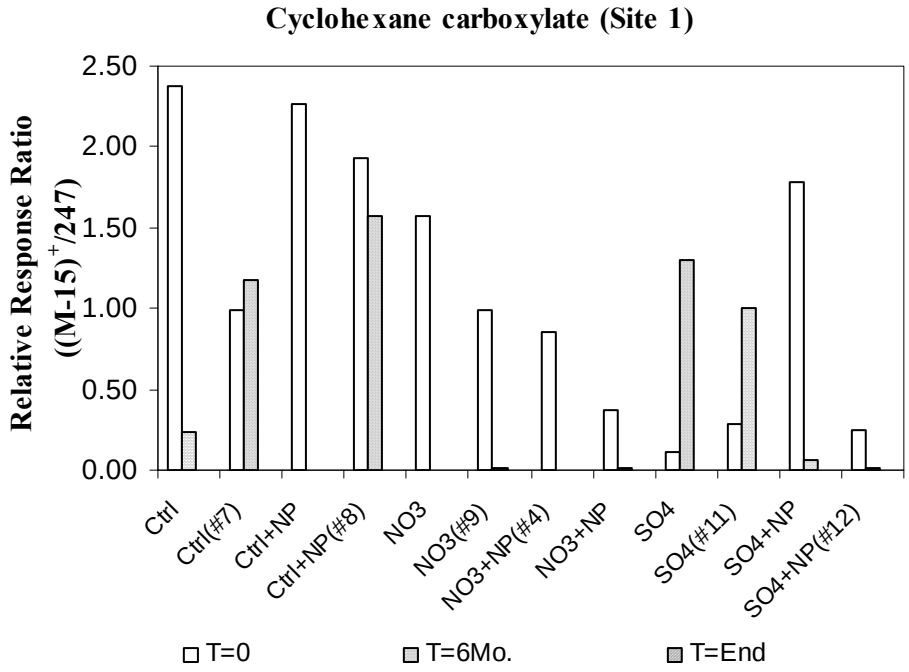

Figure G1-6. Changes in the abundance of cyclohexane carboxylate (as relative response ratio) over time in Site 1 mesocosms.

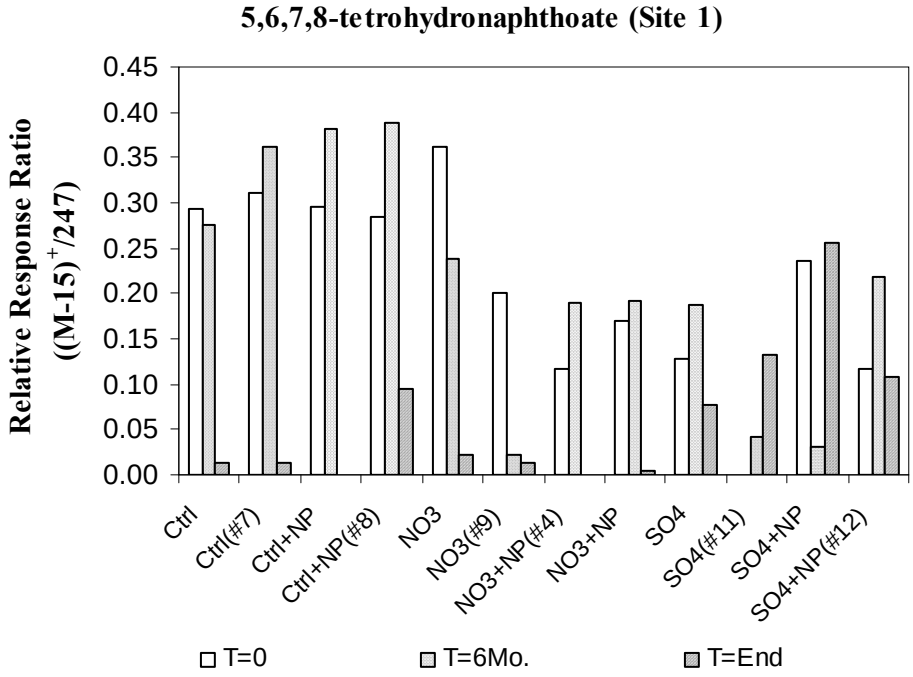

Figure G1-7. Changes in the abundance of 5,6,7,8-tetrohydronaphthoate (as relative response ratio) over time in Site 1 mesocosms.

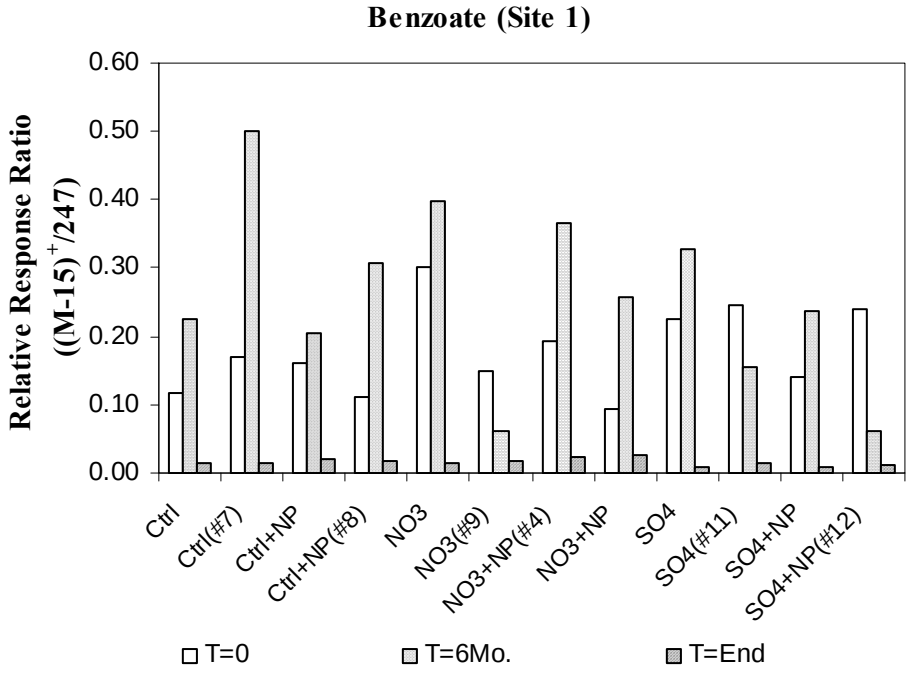

Figure G1-8. Changes in the abundance of benzoate (as relative response ratio) over time in Site 1 mesocosms.

APPENDIX G2: PLOTS OF IDENTIFIED METABOLITES IN SITE 3 MESOCOSMS

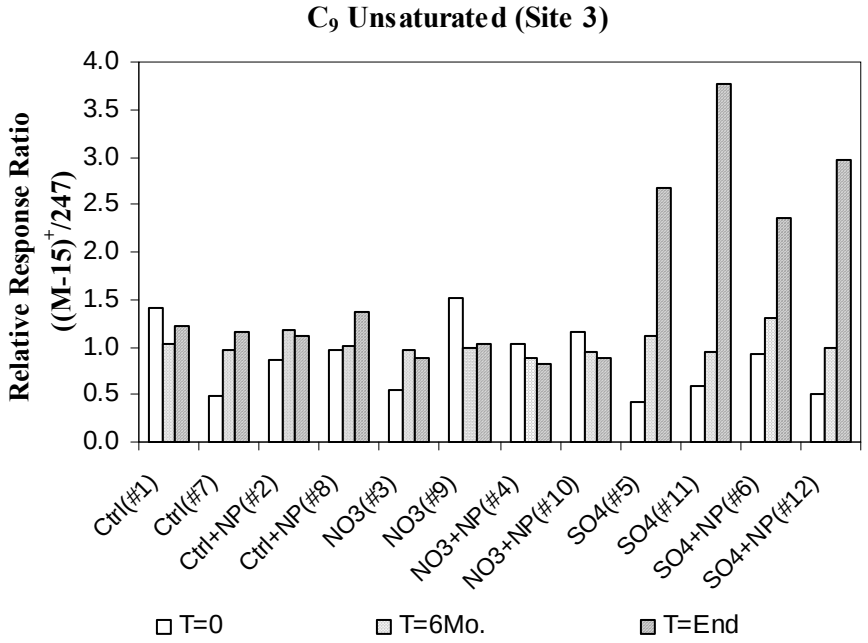

Figure G2-1. Changes in the abundance of C_9 unsaturated metabolite (as relative response ratio) over time in Site 3 mesocosms.

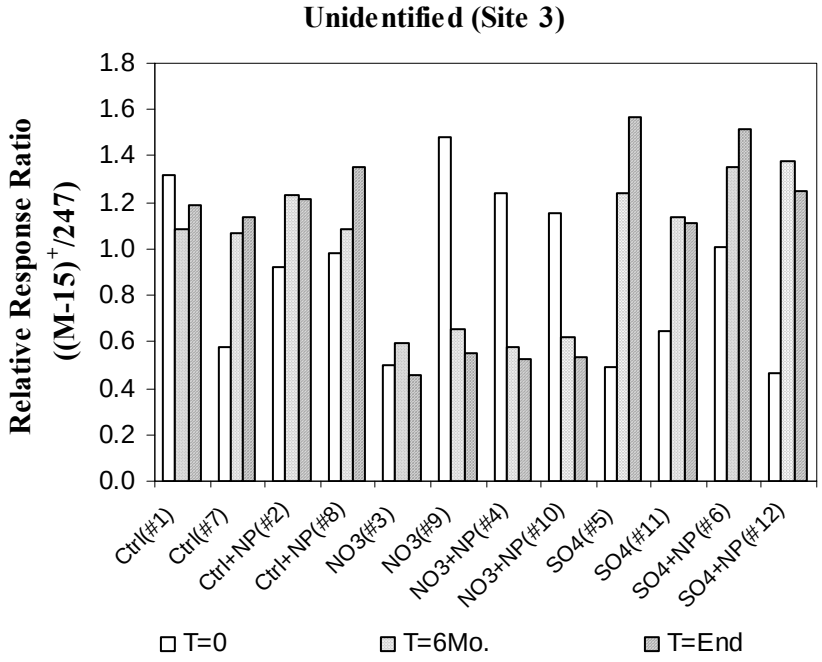

Figure G2-2. Changes in the abundance of one unidentified metabolite (as relative response ratio) over time in Site 3 mesocosms.

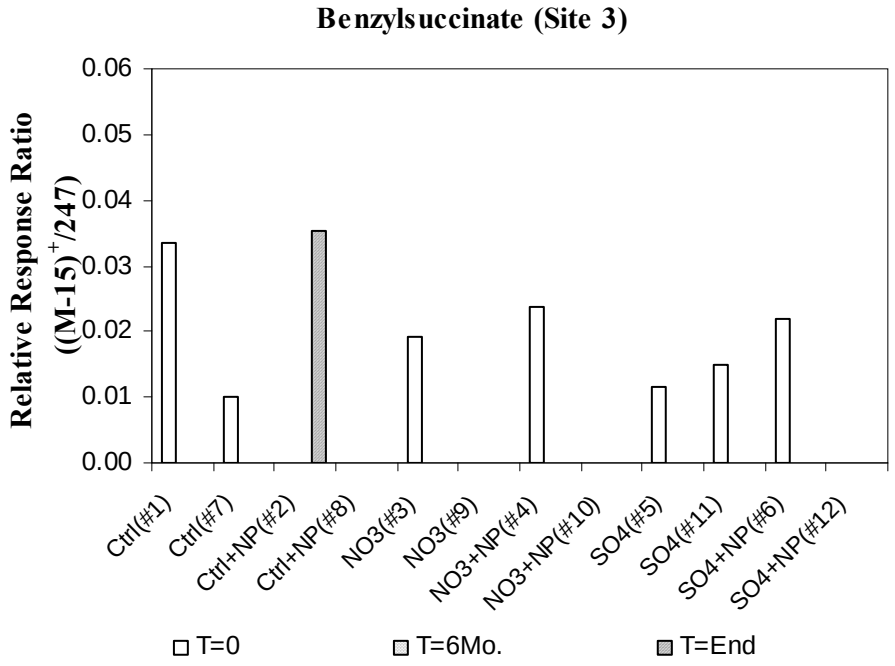

Figure G2-3. Changes in the abundance of benzylsuccinate (as relative response ratio) over time in Site 3 mesocosms.

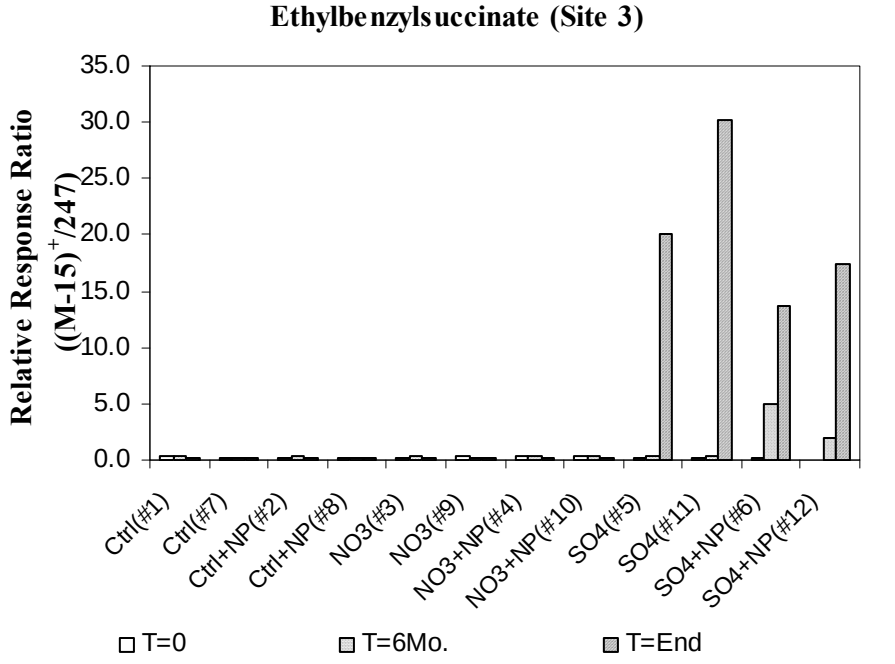

Figure G2-4. Changes in the abundance of ethylbenzylsuccinate (as relative response ratio) over time in Site 3 mesocosms.

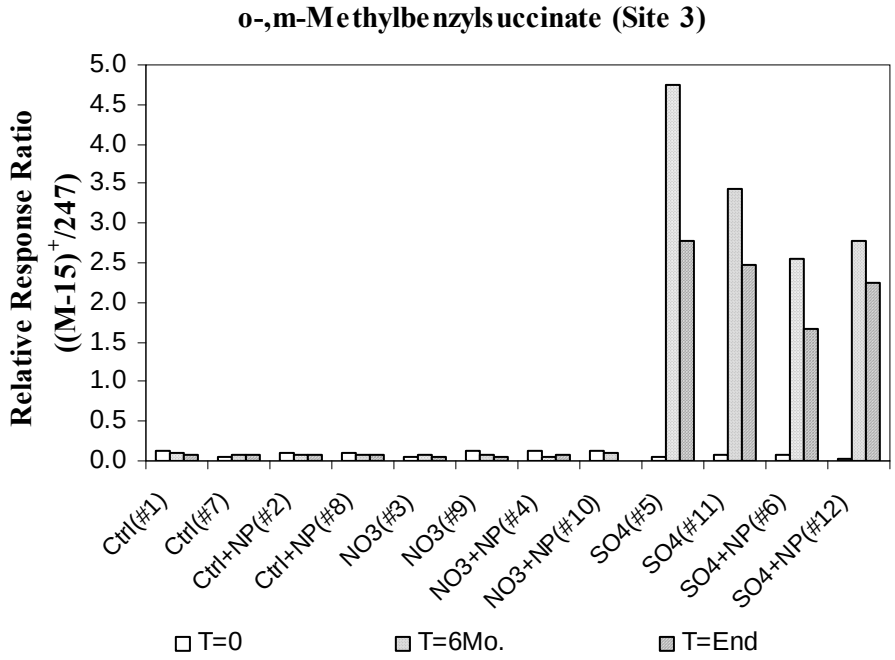

Figure G2-5. Changes in the abundance of o-, and m-methylbenzylsuccinate (as relative response ratio) over time in Site 3 mesocosms.

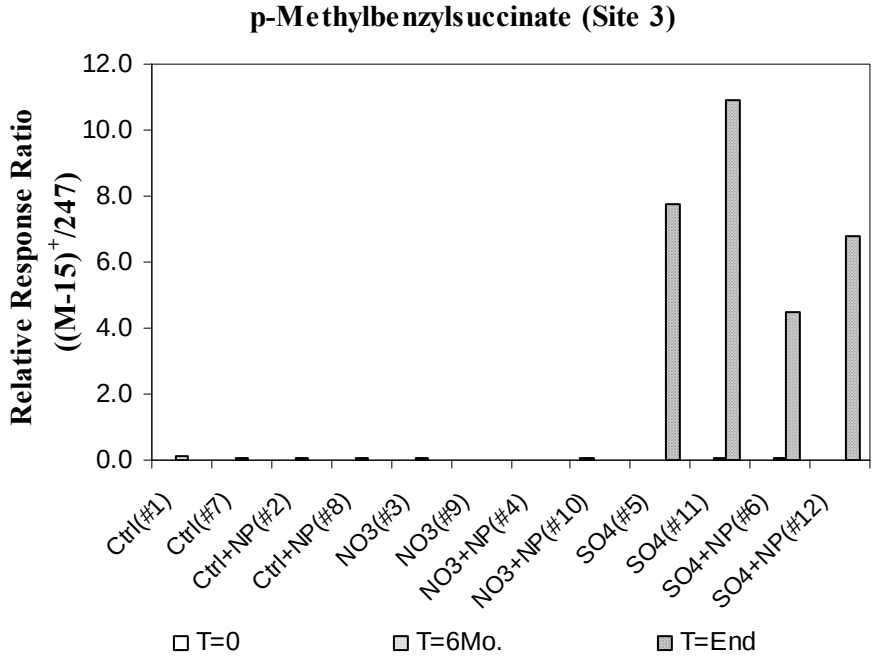

Figure G2-6. Changes in the abundance of p-methylbenzylsuccinate (as relative response ratio) over time in Site 3 mesocosms.

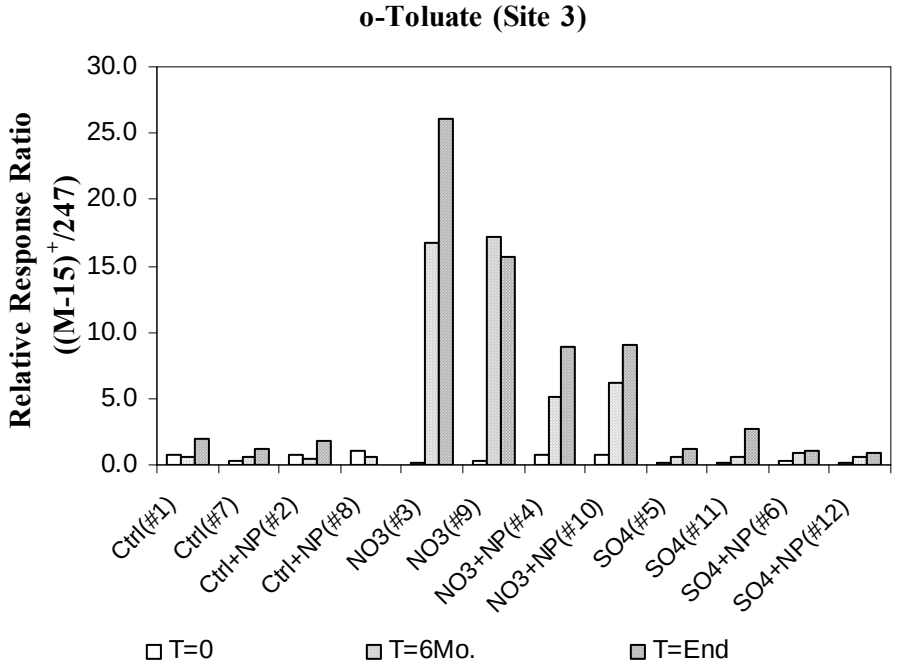

Figure G2-7. Changes in the abundance of o-toluate (as relative response ratio) over time in Site 3 mesocosms.

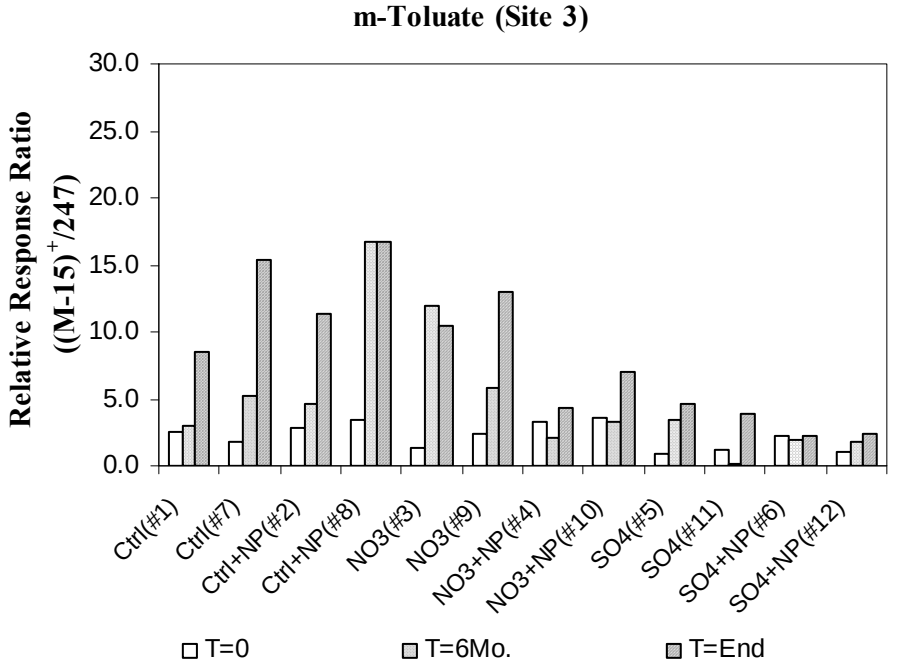

Figure G2-8. Changes in the abundance of m-toluate (as relative response ratio) over time in Site 3 mesocosms.

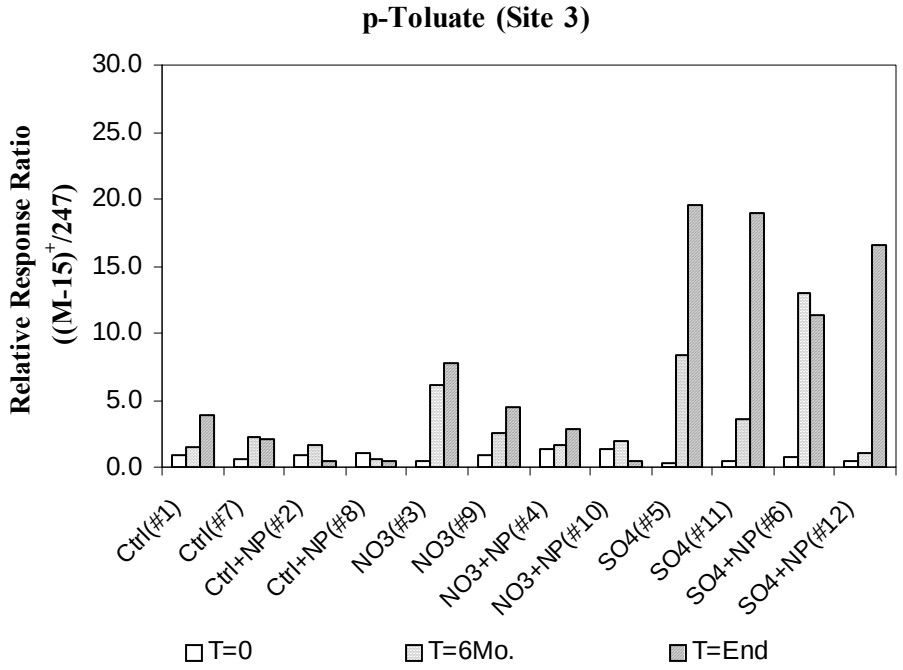

Figure G2-8. Changes in the abundance of p-toluate (as relative response ratio) over time in Site 3 mesocosms.

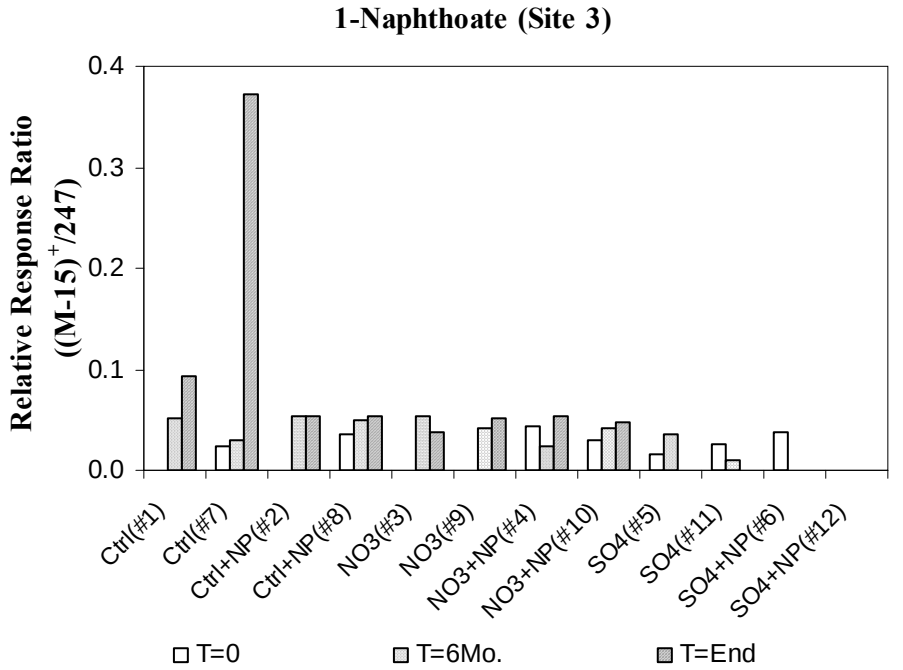

Figure G2-10. Changes in the abundance of 1-naphthoate (as relative response ratio) over time in Site 3 mesocosms.

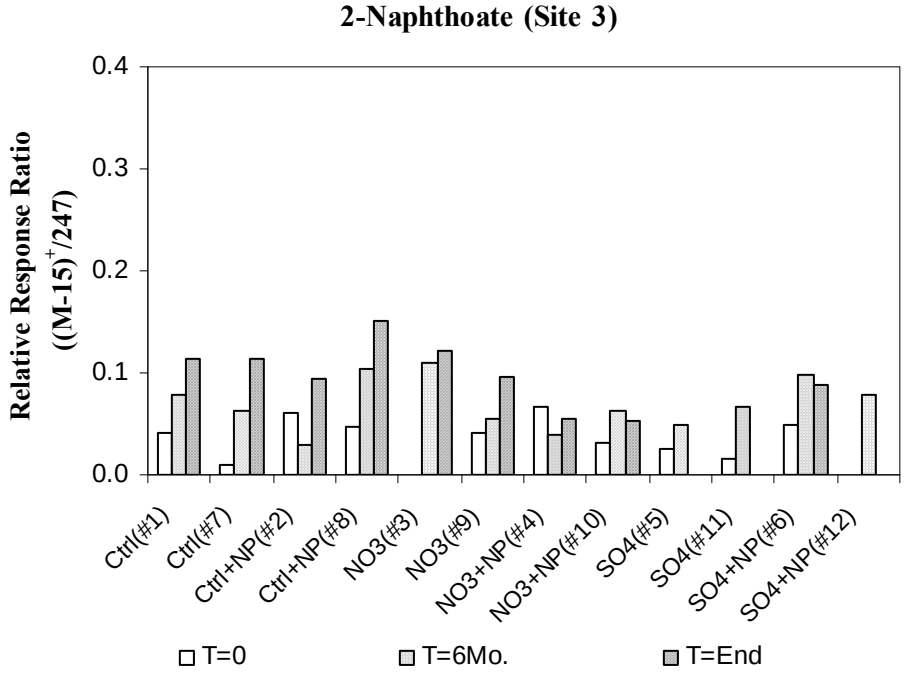

Figure G2-11. Changes in the abundance of 2-naphthoate (as relative response ratio) over time in Site 3 mesocosms.

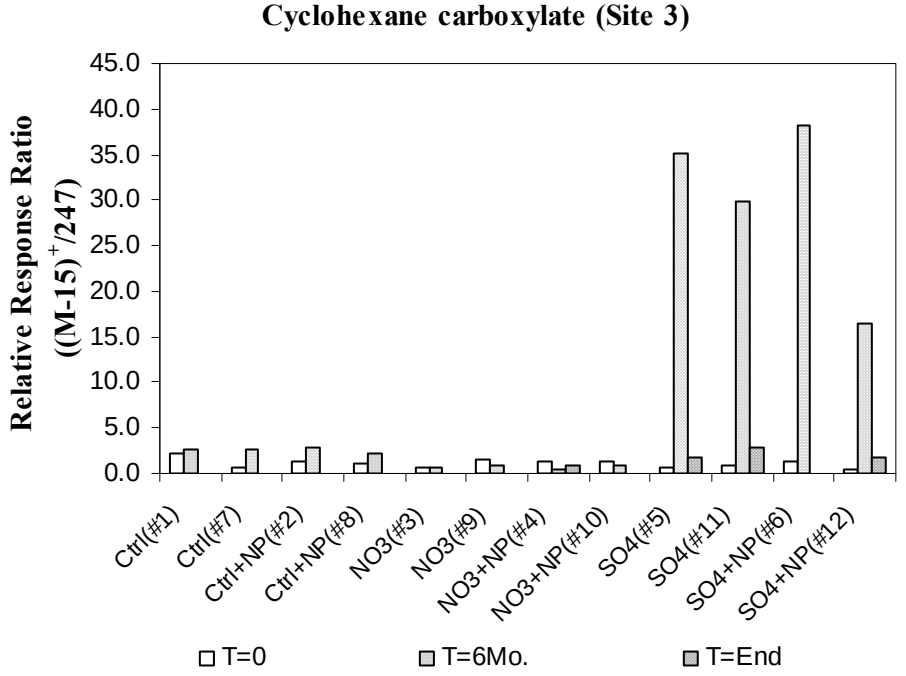

Figure G2-12. Changes in the abundance of cyclohexane carboxylate (as relative response ratio) over time in Site 3 mesocosms.

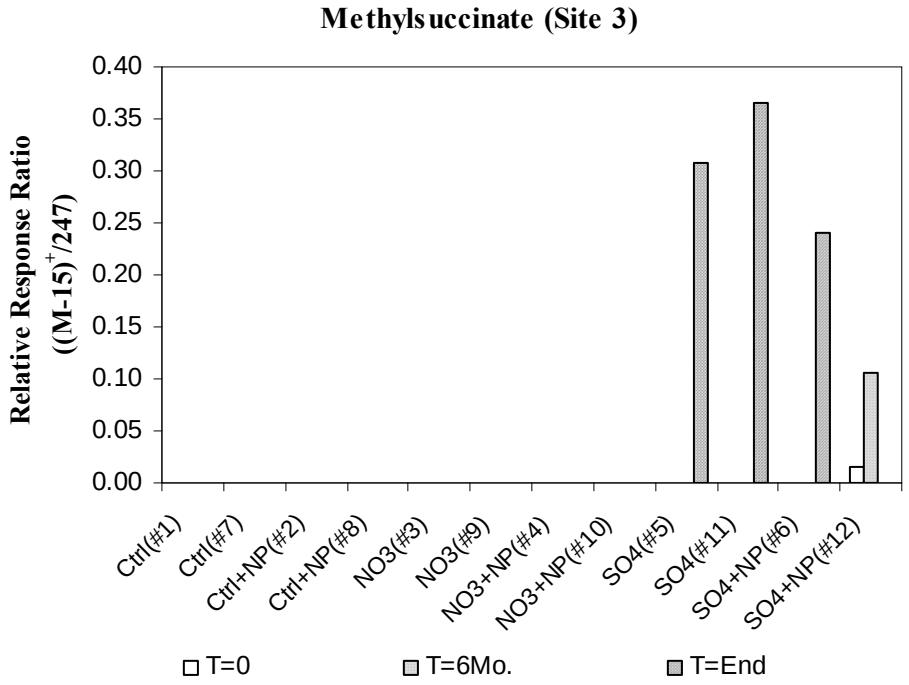

Figure G2-13. Changes in the abundance of methylsuccinate (as relative response ratio) over time in Site 3 mesocosms.

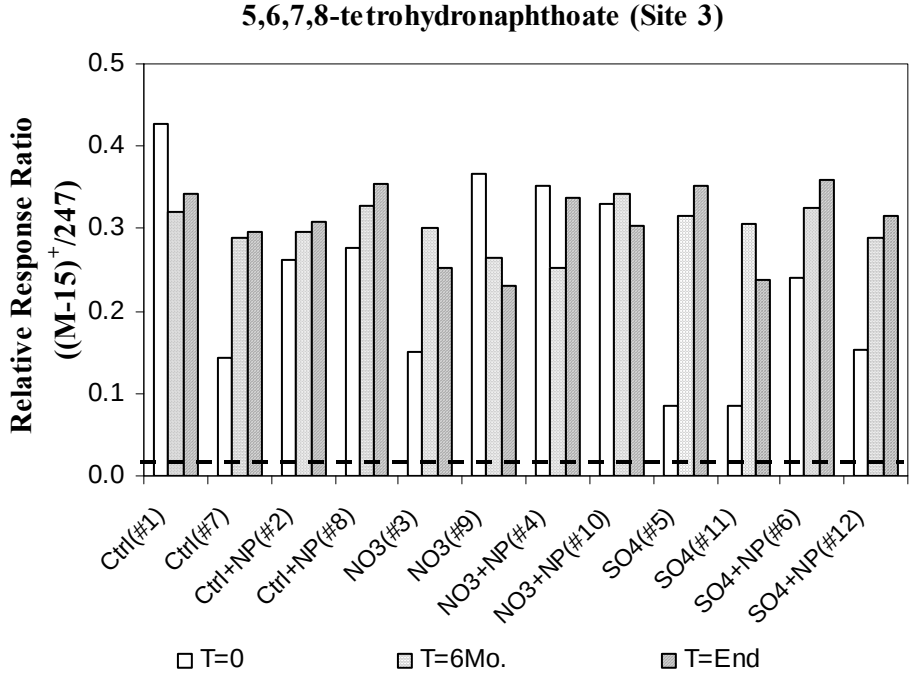

Figure G2-14. Changes in the abundance of 5,6,7,8-tetrohydronaphthoate (as relative response ratio) over time in Site 3 mesocosms.

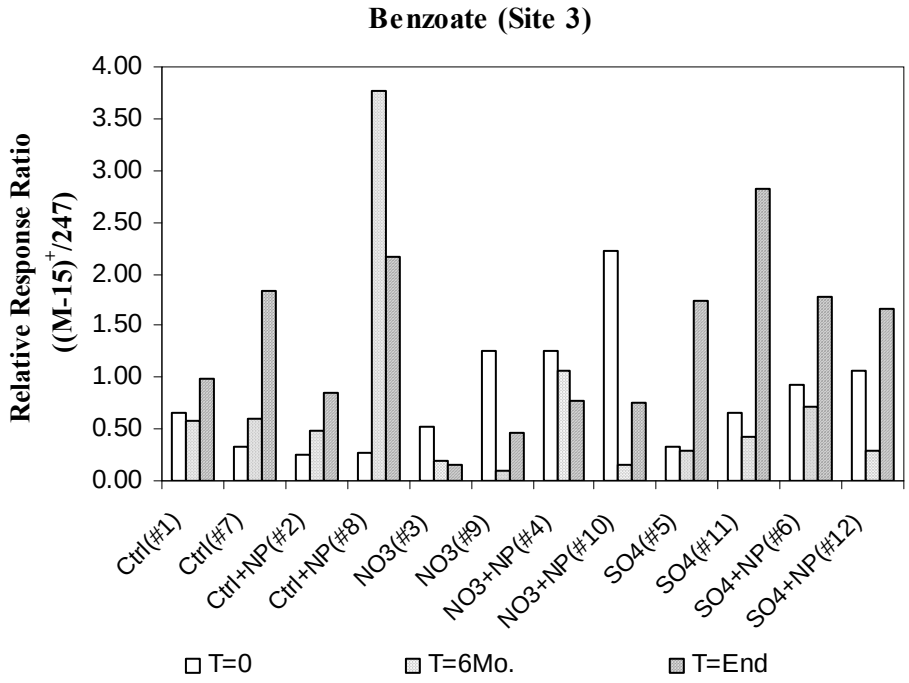

Figure G2-15. Changes in the abundance of benzoate (as relative response ratio) over time in Site 3 mesocosms.