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Anaerobic Degradation of Polycyclic Aromatic Hydrocarbons at a Creosote-
Contaminated Superfund Site and the Significance of Increased Methane Production in
an Organophilic Clay Sediment Cap

by

Kiara L. Smith

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Environmental Sciences and Resources

Dissertation Committee:

William Fish, Chair
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Portland State University
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ABSTRACT

The overall goal of this work was to investigate microbial activity leading to the anaerobic degradation of polycyclic aromatic hydrocarbons and an organophilic clay sediment cap used at a creosote-contaminated Superfund site. To determine whether or not PAHs were being degraded under anaerobic conditions *in situ*, groundwater and sediment porewater samples were analyzed for metabolic biomarkers, or metabolites, formed in the anaerobic degradation of naphthalene (a low-molecular weight PAH). In addition, a groundwater push-pull method was developed to evaluate whether the transformation of deuterated naphthalene to a deuterated metabolite could be monitored *in situ* and if conservative rates of transformation can be defined using this method.

Metabolites of anaerobic naphthalene degradation were detected in all samples that also contained significant levels of naphthalene. Anaerobic degradation of naphthalene appears to be widespread in the upland contaminated aquifer, as well as within the adjacent river sediments. A zero-order rate of transformation of naphthalene-D₈ to naphthoic acid-D₇ was calculated as 31 nM·d⁻¹. This study is the first reported use of deuterated naphthalene to provide both conclusive evidence of the *in situ* production of breakdown metabolites and an *in situ* rate of transformation.

Methane ebullition was observed in areas of the sediment cap footprint associated with organophilic clay that was used as a reactive capping material to sequester mobile non-aqueous phase liquid (NAPL) at the site. Anaerobic slurry incubations were constructed using sediment core samples to quantify the contribution of the native sediment and the different layers of capping material (sand and organophilic clay) to the overall methane

production. Substrate addition experiments using fresh, unused organophilic clay, as well as measured changes in total carbon in organophilic clay over time supported the hypothesis that microbes can use organophilic clay as a carbon source. Quantitative PCR (qPCR) directed at the *mcrA* gene enumerated methanogens in field samples and incubations of native sediment and capping materials. Denaturing gradient gel electrophoresis (DGGE) was also performed on DNA extracted from these samples to identify some of the predominant microorganisms within the sediment cap footprint.

The organophilic clay incubations produced up to 1500 times more methane than the native sediment and sand cap incubations. The organophilic clay field sample contained the greatest number of methanogens and the native sediment contained the least. However, the native sediment incubations had greater numbers of methanogens compared to their respective field sample and comparable numbers to the organophilic clay incubation. An increase in methane production was observed with the addition of fresh, unused organophilic clay to the already active organophilic clay incubations indicating that organophilic clay stimulates methanogenesis. In addition, organophilic clay retrieved from the field lost about 10% of its total carbon over a 300-day incubation period suggesting that some component of organophilic clay may be converted to methane. DGGE results revealed that some of the predominant groups within the native sediment and sediment cap were *Bacterioidetes*, *Firmicutes*, *Chloroflexi*, and *Deltaproteobacteria*. An organism 98% similar to *Syntrophus* sp. was identified in the organophilic clay suggesting this organism may be working in concert with methanogens to convert the organic component of organophilic clay ultimately to methane. The capacity of organophilic clay to sequester organic contaminants will likely change over

time as the organic component is removed from the clay. This, in turn, affects the use of this material as a long-term remedial strategy in reduced, contaminated environments.

DEDICATION

To my father who showed me the path
and to my mother who held my hand along the way

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CHAPTER 1

Introduction

1.1 Background

Creosote is a dark, oily fluid formed from the high temperature fractional distillation of coal tar. It is a mixture composed of hundreds of chemicals that are toxic to microorganisms, insects, and invertebrates, making it an excellent wood preservative regularly used to treat utility poles and railroad ties [1]. Polycyclic aromatic hydrocarbons (PAHs) make up approximately 85% of the mixture; the remainder consists primarily of various phenolic and heterocyclic compounds [2]. Historically, wood treating facilities have been located along rivers and as a result of inappropriate disposal practices, river sediments have become extensively contaminated with creosote and other wood treating chemicals, including pentachlorophenol and heavy metals [3]. Many types of sediment-dwelling fish and invertebrates are consequently exposed to these contaminated sediments, creating significant ecological damage to the biota and their respective food webs [4]. Furthermore, the release of PAHs into surface water and groundwater can greatly increase human exposure to these compounds, many of which are known carcinogens and mutagens [5, 6].

Due to their toxicity, PAHs are often the chemicals of interest when characterizing and monitoring creosote-contaminated sites. Of the PAHs commonly found in creosote, naphthalene, phenanthrene, and anthracene typically make up the largest weight percents. Currently the EPA has over 600 PAH-contaminated sites listed on the National Priority List indicating that PAHs are a significant environmental and human health concern [7]. Low solubility tends to make PAHs very persistent at contaminated sites [8]. Removing residual contamination is extremely difficult especially in aqueous environments, such as aquifers, where excavation is not always an option. However, PAHs are also potentially biodegradable, thus generating interest in natural attenuation processes as a method for removing PAH contamination *in situ*.

The rates and mechanisms of aerobic PAH degradation are well documented [9, 10]. However, in sediments and groundwater impacted by industrial and wood-treatment wastes, oxygen either is not present or is quickly consumed and anoxic conditions predominate. In fact, all but the top 1-10 mm of polluted sediments are typically anoxic and the majority of contaminants in groundwater are degraded under anaerobic conditions, suggesting that anaerobic degradation is the most significant biological process that determines the fate of PAHs within subsurface environments [9, 11]. Anaerobic PAH degradation has been observed under various redox conditions: acenaphthene via nitrate reduction [12]; methylnaphthalene, fluorine, and fluoranthene via sulfate reduction [13, 14]; phenanthrene via nitrate and sulfate reduction [11, 13]; and naphthalene via nitrate, sulfate, and iron reduction [11-13, 15, 16]. The initial step in anaerobic naphthalene degradation is believed to be carboxylation leading to the

formation of naphthoic acid, a compound that can be used as an *in situ* indicator, or biomarker, of anaerobic biotic processes [17, 18].

A classic example of a former wood-treating facility with extensive contamination is the McCormick and Baxter Superfund Site. The McCormick and Baxter Creosoting Company, adjacent to the Willamette River in Portland, OR, preserved wood products from 1944 to 1991 using creosote, pentachlorophenol (PCP) and metal-based solutions containing arsenic, chromium, copper, and zinc. Wastewater from the plant was originally discharged directly into the Willamette River and wood preservative sludge was dumped in several unlined trenches at the site. Thus, soil, groundwater, and river sediments became significantly contaminated [19].

The United States Environmental Protection Agency (EPA) placed the McCormick and Baxter site on the National Priorities List in 1994. The Oregon Department of Environmental Quality (DEQ) was designated to direct site remediation, which has included demolition of the original plant, creosote removal from the aquifer via groundwater extraction, and large-scale excavation of contaminated soil. To date, approximately 6,300 gallons of creosote and 33,000 tons of contaminated soil and debris have been removed. To reduce the overall movement of non-aqueous phase liquid (NAPL) towards the Willamette River and surrounding areas, a barrier wall was installed in 2003 composed of a combination of steel sheet pile and a soil-bentonite slurry surrounding an 18-acre area (Figure 1.1). In 2005, an impermeable cap and an earthen soil cap covering a 14.4-acre area and a 3.6-acre area, respectively, were installed within the confines of the upland barrier wall [19, 20].

To meet federal and state ambient water quality standards and prevent exposure of benthic organisms to site contaminants, a 23-acre sediment cap was installed overlying the contaminated sediment in 2005 (Figure 1.1). The majority of the cap consists of a 2-foot sand layer. In areas with higher levels of contamination, a 5-foot layer of sand was used. Where NAPL seeps had been observed, a 1-foot thick layer of organophilic clay (Aqua Technologies of Wyoming, Inc. ET-1), used as reactive capping material, was placed between the contaminated sediments and the sand cap. In addition, CETCO Organoclay Reactive Core Mats (geotextiles impregnated with organophilic clay) were placed above the sand cap in areas where NAPL sheens had been observed following cap construction (Figure 1.1).

Organophilic clay is a modified clay that has been treated with long-chain quaternary ammonium cationic surfactants (Figure 1.2a). These surfactants are exchanged with the cations that exist naturally on clay surfaces (primarily Ca^{2+} and Na^{+}), thus creating a hydrophobic material with high affinity for organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), pentachlorophenol (PCP), benzene, and toluene (Figure 1.2b) [21-24]. Based on the predicted NAPL flow rate of about $2.3 \times 10^{-4} \text{ m}^3/\text{day}$, the organophilic clay cap was estimated to provide protection for at least 500 years assuming the material can hold 50 percent of its weight in NAPL (Figure 1.3) [25].

Prior to the installation of the sediment cap moderate gas ebullition was observed in areas with contaminated sediments. Occasionally, NAPL sheen bursts at the water surface coincided with ebullition suggesting that bubbles may provide a transport pathway for NAPL to reach the overlying Willamette River. Once capped, gas ebullition increased dramatically in areas associated with the organophilic clay cap. However, with

Figure 1.2. (a) A typical quaternary ammonium cationic surfactant used to manufacture organophilic clays. (b) Organophilic clay schematic [26].

the increase in ebullition there did not appear to be an increase in NAPL sheen bursts, and the source of the gas production was unclear [25]. This phenomenon suggests that there may be a direct relationship between the organophilic clay and increased biogas formation (predominately as methane), possibly affecting the long-term performance of the organophilic clay cap. It is possible that with the construction of the sediment cap an upward shift of redox zones encouraged methanogenic activity in the highly reduced sediments below. However, if the biogas originated from the organophilic clay, itself,

Figure 1.3. Conceptual model of the sediment cap at the McCormick and Baxter Superfund Site [25].

questions arise regarding the mechanism that has enhanced anaerobic decomposition processes within the reactive capping material.

1.2 Purpose and Relevance of this Research

The overall intent of this research was to determine whether or not anaerobic degradation of PAHs was occurring at the McCormick and Baxter Superfund Site and to elucidate the source and mechanism behind increased methane production within the remedial cap used at the site. Until fairly recently (within last 22 years), anaerobic microorganisms

were deemed incapable of degrading complex organic compounds such as long-chain hydrocarbons and PAHs [10]. Currently, a great deal remains to be understood regarding factors controlling the extent and rates of anaerobic degradation in the laboratory and *in situ*.

Anaerobic degradation, depending on conditions, may be a slow process, however, it also may be the only potential option available to remove residual or inaccessible contaminants. Using geochemical signatures, biomarkers, and an *in situ* rate study, this research provides a broader understanding of microbial processes and natural attenuation, and it will aid site managers in making decisions regarding predictions of contaminant containment, bioavailability, and remedial options.

Likewise, as reactive capping materials provide a useful remedial option for contaminated sediments, it is of upmost importance to assess the feasibility of these caps *in situ*. The research presented here provides knowledge regarding the effect of anaerobic microbial processes on the effectiveness and lifetime of a reactive sediment cap.

1.3 Reader's Guide to the Thesis

The next three chapters describe the results of my research and my analysis of that research as it applies specifically to the McCormick and Baxter Superfund Site and its implications to environmental science. Chapter 2 is a report submitted to the Oregon Department of Environmental Quality as a preliminary investigation regarding potential anaerobic PAH degradation and its link to geochemistry within the sediment cap footprint at the site. A cohort of students in the OSU/PSU Earth's Subsurface Biosphere IGERT

program developed the report. I was the principle coordinator of the project and I contributed extensively to method development, field study implementation, data analysis, and report preparation. Chapter 3 is a comprehensive study subsequent to that report providing greater resolution to anaerobic PAH degradation within the sediment cap footprint as well as in the upland creosote-contaminated aquifer. A novel method was developed and successfully employed *in situ*, providing both strong evidence of active anaerobic PAH degradation and a conservative rate of biodegradation.

In Chapter 4, the spatial distribution of the apparent methane production was vertically mapped through the profile of the sediment cap at the McCormick and Baxter Superfund Site. Additional studies were conducted with organophilic clays to describe the association of this material and methane production. Furthermore, molecular tools were used to explore the relationship between absolute numbers of methane-producing microorganisms and rates of methanogenesis, as well, to gain insight into microbial diversity within the sediment cap footprint at the site. The findings of the experiments and subsequent analyses provided sufficient information to propose a mechanism by which components within the organophilic clay could be ultimately converted to methane.

Overall conclusions are presented in Chapter 5, along with suggestions for future studies on the anaerobic degradation of PAHs and the modification of organophilic clays mediated by anaerobic microorganisms.

CHAPTER 2

Geochemical status of a remedial cap on creosote-contaminated sediments at the McCormick and Baxter Superfund Site and its potential link to natural attenuation

2.1 Executive Summary

Polycyclic aromatic hydrocarbons (PAHs), along with their metabolites, are considered possible carcinogens and mutagens, often making them chemicals of interest when characterizing and remediating creosote-contaminated sites. In many PAH-contaminated sites, large concentrations of PAHs with 2-4 fused rings are often present. Because these PAHs are potentially biodegradable, interest in natural attenuation processes as a method for removing PAH contamination *in situ* is frequently considered.

The purpose of this project was to determine whether or not PAHs were degrading under anaerobic conditions within the sediment cap footprint at the McCormick and Baxter Superfund Site and to assess whether this biodegradation corresponds to a specific geochemical signature. Sediment and porewater were collected from the site and subsequently analyzed for a variety of conditions that may indicate anaerobic degradation. The results showed that sampling areas differed chemically and in the

information they yielded relative to biodegradation. Only one site had significant PAH contamination and metabolites indicative of anaerobic naphthalene degradation. Thus, natural attenuation of the PAHs could be occurring in contaminated sediments below the capping material. Evidence of anaerobic degradation suggests that natural attenuation may provide an additional layer of protection to complement the containment provided by the capping material at the site.

2.2 Methods

Sampling procedures and field analyses. Sediment cores and porewater samples were collected from three locations at the McCormick and Baxter Superfund Site on March 23, 2006 (Figure 2.1). At each location, porewater was collected from the capping material using Push Point samplers (1.22m long, 1.91cm I.D., MHE products). Porewater samples were taken at two depths, upper (1.0-1.5 ft) and lower (2.5-3.5 ft). The intention was to sample the sand cap (upper porewater sample) and the native sediment below (lower porewater sample). The porewater samples are henceforth referred to as 1, 2, or 3 according to site number, and -U or L according to the depth at which sample was taken. For example porewater from Site 1 upper is sample 1-U. Temperature, dissolved oxygen (DO), pH, total dissolved solids (TDS), specific conductance, and oxidation/reduction potential (ORP) of the surface and porewater were measured using a water quality probe (Horiba model U-22). After surface water and Site 1 measurements were obtained, DO measurements of subsequent samples indicated a meter malfunction. Thus, DO concentrations were subsequently measured using visual colorimetric Vacuvials (CHEMetrics, Calverton VA).

Water samples collected for the measurement of major cations, anions, nutrients, and dissolved metals were filtered using 0.45- μ m, flow-thru filter cartridges (Millipore, Billerica, MA). Samples for aqueous cation analysis were immediately preserved in concentrated trace metal grade hydrochloric acid (HCl), while samples for metal analysis were acidified to a pH of <2 using trace metal grade nitric acid. Water samples for methane and volatile fatty acid analyses were collected in nitrogen-purged, zero headspace serum vials. Water samples for hydrogen sulfide and Fe (II) analysis were preserved in zinc acetate solution and HCl, respectively. Porewater samples collected for metabolites indicative of anaerobic PAH degradation were acidified in 50% HCl and shipped to the University of Oklahoma for further sample processing and analysis. Remaining porewater samples were preserved according to the analytical methods shown in Table 2.1 and immediately placed on ice.

Table 2.1. Analyses performed by TestAmerica Laboratories, Inc.

Analyte	Matrix	Method
Ferrous Iron	Water	SM3500-Fe D.
Major Cations	Water	EPA 200.7
Metals (total and dissolved)	Water	EPA 6020
PAHs	Water	EPA 8270m
Dissolved Organic Carbon	Water	EPA 415.1
Sulfide	Water	EPA 376.2
Carbon dioxide	Water	SM 4500-C
Nitrate-Nitrogen	Water	EPA 300.1
Nitrite-Nitrogen	Water	EPA 300.2
Sulfate	Water	EPA 300.3
Sulfide	Water	EPA 376.2
Total Alkalinity	Water	SM 2320B
Methane	Water	GC/FID

Sediment cores were also taken at each site at a depth no greater than one meter below the sand cap/surface water interface for measurements of total and extractable iron. All core samples collected in the field were cooled on ice, transported to the laboratory within 12 hours, and subsequently refrigerated.

Laboratory analytical procedures. TestAmerica Laboratories, Inc., Beaverton, Oregon, determined the concentrations of the porewater analytes listed in Table 2.1. Ammonium was determined colorimetrically using the continuous segmented flow procedures described in Gordon et al. [27]. Volatile fatty acids were measured using ion chromatography.

Total reducible Fe (III) in sediment was extracted using the dithionite-citrate-bicarbonate method [28]. “Bioavailable” Fe (II) and Fe (III) were determined by weak acid extraction [29]. Sediment digests were then measured for Fe (II) and total Fe [Fe (II) + Fe (III)] using a modified Ferrozine method [30, 31]. For analysis of total iron, sediment samples were digested with sequential HF, HNO₃, and HCl hot digestions (80-85°C) using a microwave digestion technique (CEM MARS 5000), and analyzed by inductively-coupled plasma optical emission spectroscopy (ICP-OES, Varian Liberty 150).

Porewater samples were analyzed for anaerobic PAH metabolites by gas chromatography-mass spectrometry (GC-MS, Agilent with a HP-5MS column) following the extraction and derivatization as described in Gieg and Suflita [32]. Briefly, 1-L porewater samples were extracted using ethyl acetate and dried with sodium sulfate. Samples were then concentrated in a rotary evaporator followed by further concentration

under a stream of nitrogen to a final volume of 50 μl . The samples were then derivatized using BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetimide; Pierce Chemical Co., Rockford, IL) forming trimethylsilyl esters. For GC analysis, the oven temperature was held at 45°C for 5 min., then ramped at a rate of 4°C min⁻¹ to 270°C and held for 15 min. The metabolites were identified by comparing the GC retention times and MS profiles to those of known standards given in Gieg and Suflita [32].

2.3 Results and Discussion

On-site field parameters. All water quality parameters, other than pH, measured at each location varied significantly from the surface water values suggesting that the subsurface water samples were isolated from the overlying river water (Table 2.2). The pH values were consistent throughout the site with porewater values being approximately equal to that of the surface water with a pH of 6.7. The porewater was essentially devoid of oxygen at every location it was measured. The higher conductivity readings and total dissolved solids (TDS) at Site 3 corresponded with elevated levels of metals, in particular iron and manganese, as well as other cations and contaminants in solution at this location (Tables 2.2, 2.3, and 2.4). The negative oxidation-reduction potential (ORP) values found at Sites 2-L, 3-U, and 3-L indicate reducing conditions in anaerobic sediments, whereas the other sampling locations do not appear to be as reduced (Table 2.2).

Aqueous porewater and sediment geochemistry. Although difficult to determine whether the deep porewater samples were in fact taken within the native sediment, based on the *in situ* parameters and geochemistry, it is likely both the upper and lower porewater samples

Table 2.2. Measured *in situ* parameters.^a

Sampling Site	Depth	pH	DO	Temperature	Conductivity	TDS	ORP
	feet		mg/l	°C	ms/cm	g/l	mV
Surface Water	n/a	6.7	n/a	8.3	0.09	0.06	331
1-U	1	6.7	n/a	9.6	0.12	0.10	309
1-L	3	6.8	n/a	10.6	0.15	0.10	145
2-U	1.5	6.3	0.8	11.3	0.18	0.11	153
2-L	3.5	6.7	0	11.4	0.20	0.13	-65
3-U	1.5	6.7	0	11.1	0.46	0.31	-99
3-L	2.5	6.8	0	10.7	0.57	0.37	-173

^a n/a, not analyzed.

at Site 1 were taken from within the sand cap (Tables 2.2 and 2.3). At every location the DOC was higher in the deep porewater sample relative to the shallow sample taken at that same location. Higher concentrations of DOC in porewater samples from Site 2-L and Site 3-L suggest the partial breakdown of organic compounds within the sediments. Site 3 porewater, with elevated CO₂ levels and increased alkalinity, indicates the mineralization of organic compounds to inorganic species. In the absence of oxygen, the accumulation of reduced species, such as Fe (II), H₂S and NH₄⁺ results from microorganisms readily consuming electron-accepting compounds in the biodegradation of organic compounds [33]. Again, the porewater at Site 3 displays these characteristics, with the exception of the accumulation of H₂S. Sulfide is a reactive compound and thus its concentration can be affected by its precipitation with metals such as iron. Sulfide concentrations were insignificant at all of the site locations; however, due to the high concentrations of Fe (II) at Site 3, the binding of sulfide to Fe (II) may mask sulfide production (Table 2.3). Moreover, sulfate is present at all other sampling locations, except at Site 3, suggesting the reduction of sulfate to sulfide at this location.

Table 2.3. Dissolved organic carbon (DOC), total alkalinity and oxidized and reduced chemical species involved in anaerobic metabolic processes measured in sediment porewater.

Sample ID	Depth	DOC	Total Alkalinity	CO ₂	CH ₄	NO ₃ ⁻	NO ₂ ⁻	NH ₄ ⁺	SO ₄ ²⁻	H ₂ S	Fe (II)
	feet	mg/l	mg/l as CaCO ₃	mg/l	μg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
1-U	1	1.62	63	<10	365	<0.1	<0.1	<0.01	3.65	<0.2	<1
1-L	3	2.35	64	<10	1230	<0.1	<0.1	<0.01	4.65	<0.2	<1
2-U	1.5	1.81	81	<10	7640	<0.1	<0.1	<0.01	5.52	<0.2	<1
2-L	3.5	5.97	83	<10	3080	<0.1	<0.1	<0.01	4.71	<0.2	1.3
3-U	1.5	2.02	179	13.8	8750	<0.1	<0.1	1.009	<1	<0.2	3.8
3-L	2.5	4.41	218	53.4	3890	0.97	<0.1	1.405	<1	<0.2	14.5

Table 2.4. Dissolved major cations and total toxic metals measured in sediment porewater; dissolved toxic metals are in parentheses.^a

Sample ID	Depth	Calcium	Magnesium	Potassium	Sodium	Arsenic	Chromium	Copper	Manganese	Zinc
	feet	mg/l	mg/l	mg/l	mg/l	μg/l	μg/l	μg/l	μg/l	μg/l
1-U	1	12	3.6	1.48	8.81	<1.0 {<1.0}	<1.0 {<1.0}	8.69 {<2.0}	8.07 {4.98}	6.62 {<5.0}
1-L	3	13.4	3.96	1.48	8.79	1.15 {<1.0}	<1.0 {<1.0}	2.34 {<2.0}	54.0 {54.2}	<5.0 {<5.0}
2-U	1.5	17.5	5.29	1.84	10.8	<1.0 {<1.0}	<1.0 {<1.0}	<2.0 {<2.0}	21.8 {6.89}	11.0 {<5.0}
2-L	3.5	17.4	5.9	1.27	9.69	1.34 {1.27}	<1.0 {<1.0}	<2.0 {<2.0}	1020 {n/a}	<5.0 {<5.0}
3-U	1.5	28.3	6.98	3.13	29.7	4.91 {4.62}	<1.0 {<1.0}	<2.0 {<2.0}	3420 {3440}	<5.0 {5.53}
3-L	2.5	45.1	11.5	3.39	27.9	8.8 {7.79}	<1.0 {<1.0}	5.02 {<2.0}	2580 {2780}	<5.0 {<5.0}

^a n/a, not analyzed.

Nitrate was detected in Site 3-L porewater, however, this is likely a laboratory error, as it would be highly unlikely to have measureable nitrate in a highly reduced environment, as demonstrated by the negative ORP and high levels of reduced species, such as Fe (II) (Tables 2.2 and 2.3).

Weak acid extraction targets amorphous iron oxides, which are the most bioavailable portion of iron within sediments. For all three sites, the iron most readily available for microbial respiration is only a minor component of the total mineral iron present in the samples (Table 2.5). Additionally, only a fraction of the total reducible iron [Fe (III)] is bioavailable in samples from Sites 1 and 3, whereas, in samples from Site 2, the total reducible iron is also considered entirely bioavailable. Elevated Fe (II) porewater concentrations and decreased amorphous sediment iron content suggests microbial dissolution of iron oxides within Site 3 sediment (Tables 2.3 and 2.5). However, the Fe (II) could also be actively transported from another location, such as the upland aquifer.

Table 2.5. Total Fe and extractable Fe (III) and Fe (II) in solid core samples (mg/kg).

Sampling Site	ICP-OES ^a	Total Reducible (DCA) Iron ^b	Weak Acid Extraction Ferrozine	
	Total Fe	Fe (III)	Fe (II)	Fe (III)
Site 1	33735	450	67	143
Site 2	36531	273	106	299
Site 3	33688	273	13	82

^a ICP-OES data represents a minimum total sediment Fe values due to incomplete digestion;

^b DCA, Dithionate-Citrate-Acetate extractible.

Methane levels are expected to increase with depth in sediments as electron-acceptors become more limited, leaving methanogenesis as the only possible anaerobic metabolic pathway. This relationship is observed at Site 1, however, at Sites 2 and 3, the upper depth porewater samples had higher concentrations of methane than the lower samples (Table 2.3). The only clear physical difference between the sampling sites is the presence of the organophilic clay cap, located below the sand cap, at Sites 2 and 3 (Figure 2.1). It is possible that the methane observed in the upper samples originates between the upper and lower sample locations and migrates upward. This would be consistent with the observed ebullition at Sites 2 and 3.

Depending on the type of microorganism, different fatty acids are formed during the fermentation of organic compounds. Porewater samples from every location contained pyruvic acid, with Sites 2-U and 2-L having the highest concentrations of 7.5 and 9.4 mg/l, respectively (Table 2.6). Porewater samples from Sites 1-L, 2-U, and 2-L contained acetic acid, whereas Site 3 had the lowest pyruvic acid concentrations and was the only site with detectable butyric acid at 5.6 mg/l. These variances suggest that different anaerobic fermentation processes may be occurring at Sites 2 and 3. The lack of acetic acid at Site 3 suggests the possibility that acetoclastic (acetate-using) methanogens are actively using acetate for the subsequent formation of methane. In addition, *Clostridia*, strict anaerobes known to produce butyric acid, are frequently found in contaminated environments [34, 35]. The use of denaturing gradient gel electrophoresis (DGGE) or clone libraries could differentiate between groups of microorganisms present in these sampling locations to elucidate their connection to PAH transformations or other pertinent microbial activities (see Chapter 4).

Table 2.6. Fatty acid concentrations measured in sediment porewater (mg/l).

Sampling Site	Pyruvic Acid	Lactic Acid	Acetic Acid	Propionic Acid	Butyric Acid
1-U	3.9	<1.0	<1.0	<1.0	<1.0
1-L	4.1	<1.0	1.1	<1.0	<1.0
2-U	7.5	<1.0	1.2	<1.0	<1.0
2-L	9.4	<1.0	1.6	<1.0	<1.0
3-U	1.6	<1.0	<1.0	<1.0	<1.0
3-L	2.3	<1.0	<1.0	<1.0	5.6

Creosote-polluted sites often exhibit metal co-contamination, which can have an effect on the biodegradation of organic pollutants. Metals can both inhibit and enhance biodegradative processes, depending on concentration, valence state, and interactions with soil particles and other metals [36]. Additionally, the flux and concentration of toxic metals into the aqueous phase may increase due to microbial interaction in the biodegradation of PAHs, posing a threat to the environment and human health [37]. Dissolved copper, chromium, and cadmium were below the detection limit at all of the sites (Table 2.4). Dissolved manganese and arsenic concentrations at Sites 2-L, 3-U, and 3-L were considerably higher than at the other sites. The significant reducing conditions found at these locations may create conditions that promote the dissolution of metals into sediment porewater. In addition, manganese and arsenic can be reduced during anaerobic microbial processes [36]. Consequently, the breakdown of organic matter may increase arsenic and manganese concentrations in the sediment cap due to the upward movement of labile species from the contaminated sediment below. Total aqueous metal concentrations were slightly higher than the dissolved concentrations in some locations (Table 2.4). However, the concentrations are below levels that have been reported to inhibit microbial degradation [36].

Site 3 was the only location to display considerable levels of PAH contamination (Table 2.7). Naphthalene, acenaphthene, fluorine, and phenanthrene are the dominant PAHs at 3-L, reflective of their relative concentrations in creosote, as well as their greater solubility, and thus increased potential for movement into porewater [38]. This suggests that Site 3 may be the only sampling location with the potential for natural attenuation of PAHs.

Table 2.7. Concentrations of selected PAHs in sediment porewater samples (µg/l).^a

Sampling Site	ACN	ANT	FLA	FLU	NAP	PHE	PYR
1-U	<0.0251	<0.0238	<0.0238	<0.0238	<0.0238	<0.0238	<0.0238
1-L	<0.0251	<0.0238	<0.0238	<0.0238	0.546	<0.0238	<0.0238
2-U	<0.0251	<0.0238	<0.0238	<0.0238	<0.0238	<0.0238	<0.0238
2-L	<0.0251	<0.0238	<0.0238	<0.0238	<0.0238	<0.0238	<0.0238
3-U	0.0868	0.0687	0.100	0.133	0.117	0.445	0.0543
3-L	57.9	2.60	2.06	30.0	478	23.3	0.948

^a ACN, acenaphthene; ANT, anthracene; FLA, fluoranthene; FLU, fluorine; NAP, naphthalene; PHE, phenanthrene; PYR, pyrene.

Putative anaerobic naphthalene metabolites were not found at any location except Site 3-L. The metabolites identified were naphthoic acid, methylnaphthoic acid, and 1,2,3,4- and 5,6,7,8-tetrahydronaphthoic acid (Figure 2.2). The identification of metabolites at Site 3-L is consistent with the PAH contamination at this location (Table 2.7). Naphthoic acid can also be formed during the aerobic degradation of methylnaphthalene and acenaphthylene [39-41]. However, the production of 1,2,3,4- and 5,6,7,8-tetrahydronaphthoic acid is unique to the anaerobic degradation pathway of naphthalene [32, 42, 43]. Therefore, in addition to the anaerobic conditions observed at Site 3, the detection of tetrahydronaphthoic acids provides further confirmation that naphthalene degradation is occurring under anaerobic conditions.

The observed natural attenuation is potentially reducing the flux of PAHs into the capping material. If PAHs are moving into the overlying sand cap from the contaminated sediment below, degrading microbial communities will likely follow and promote further natural attenuation in the capping material. However, little is known about the effect of organophilic clay on natural attenuation and it is possible that the sequestering of PAHs in the organophilic clay could either inhibit or enhance PAH degradation. A small number of studies have examined the effect of organophilic clay on biodegradation processes, but no research has been conducted under anaerobic conditions. Prior studies have shown that several factors affect the biodegradation potential in an organophilic clay environment including contaminant chemistry, the type of surfactants used to produce the organophilic clay, and the amount of surfactant exchanged onto the clay surface [22, 44, 45]. Sorption of organic and inorganic compounds into organophilic clay was previously thought to slow biodegradation processes, since it was assumed that microorganisms can only attack dissolved material. However, more recent evidence suggests that this assumption is not necessarily valid because the degradation of contaminants sorbed to organophilic clay and other types of organic matter has been convincingly demonstrated [24, 46-48]. In addition, high levels of contaminants that are toxic to microorganisms can often be attenuated by sorption to organophilic clay, the organophilic clay thereby stimulating biodegradation [44].

Figure 2.2. Mass spectral profiles of putative anaerobic naphthalene metabolites detected at Site 3-L (analyzed as trimethylsilyl, TMS, derivatives). (a) Metabolite detected in the sediment porewater having similar GC retention time and MS profile as a 2-naphthoic acid standard. (b) Metabolite detected in the sediment pore water having similar GC retention time and MS profile as a methyl-naphthoic acid standard. (c) Metabolite detected in the sediment pore water having similar GC retention time and MS profile as a 1,2,3,4-tetrahydronaphthoic acid standard. (d) Metabolite detected in the sediment pore water having similar GC retention time and MS profile as 5,6,7,8-tetrahydronaphthoic acid. All metabolites published in Gieg and Suflita [32].

2.4 Conclusions

Site 1—Summary and conclusion. Site 1-U had no detectable concentrations of PAHs and Site 1-L contained a trace level of naphthalene (0.546 $\mu\text{g/l}$). Site 1 had the least variance in geochemical parameters between upper and lower sampling depths, appearing to be fairly homogenous throughout. Porewater samples contained very low Fe (II) and low dissolved methane (Figure 2.3). No evidence of PAH degradation was present.

Site 2—Summary and conclusion. Site 2 had the most variance between sampling depths, but showed no evidence of PAH contamination. The positive ORP and DO concentration of 0.8 mg/l at Site 2-U suggest a less reduced environment, whereas the lower layer had a negative ORP and no detectable dissolved oxygen, indicating anoxic conditions (Table 2.2). A relatively high level of DOC, 5.97 mg/l, where no obvious contamination was present, implies that another carbon source exists at Site 2-L (Figure 2.3b). The source of the DOC may be from the dissolution of organic compounds derived from the organophilic clay capping material. Additionally, Site 2 showed the presence of increased acetic and pyruvic acids, indicating different fermentation pathways at Site 2 than those at Site 3 (Table 2.6).

Site 2 porewater samples contained low amounts of Fe (II) and relatively high methane concentrations, which together, suggest that methanogenesis may be the dominant microbial process at this location (Figure 2.3). Higher concentrations of methane in the upper layer suggests that the methane originated somewhere between the upper and lower sample locations.

Site 3—Summary and conclusion. Site 3 showed substantial PAH contamination and several parameters suggest extensive microbial activities. High concentrations of total dissolved solids and high conductivity values at Site 3 are most likely a result of the increased metals, particularly iron and manganese, and major cations in solution (Tables 2.2, 2.3, and 2.4). The negative ORP and non-detectable DO indicate anaerobic conditions exist at this location and increases with depth.

Figure 2.3. Porewater data collected from each sampling site. (a) Total manganese and dissolved iron (II), magnesium, sodium, and calcium. (b) Methane, sulfate and dissolved organic carbon (DOC).

Site 3 was the only site to show significantly elevated carbon dioxide levels and increased alkalinity, indicating the degradation of organic matter to inorganic species (Table 2.3). Site 3 porewater samples contained significant concentrations of dissolved methane, sulfide, and Fe (II). Site 3-L porewater contained notably higher Fe (II) concentrations and the sediments had the lowest weak acid extractable iron content (Table 2.5 and Figure 2.3a). Since weak acid extraction measures bioavailable iron, the higher porewater concentrations and lower sediment values suggest that microbial iron reduction was occurring in Site 3 sediments. However, the dominant terminal electron accepting process is difficult to define, as these processes can co-occur over very short distances [33, 49].

Chemical analysis of porewater from Site 3-L shows the presence of anaerobic degradation products of naphthalene. These products are unique to anaerobic degradation and thus provide definitive evidence that some degree of natural attenuation was occurring at Site 3. Both methane and carbon dioxide have been found to be byproducts of the anaerobic degradation of organic contaminants [35, 50-53]. The chemistry of Site 3 shows high concentrations of methane and carbon dioxide and the absence of sulfate and several fatty acids. Methanogenesis can be inhibited in the presence of nitrate, sulfate, and various substrates including fatty acids [50]. Thus the depletion of these compounds at Site 3 could indicate the degradation of PAHs by bacteria under methanogenic conditions. The methane produced would then migrate upward and may contribute to the bubbling occurring at the McCormick and Baxter Superfund Site. It is also possible that microorganisms using Fe (III) as an electron acceptor could be degrading PAHs, as significant concentrations of reduced iron [Fe (II)] were present. In

general, microbial processes are responsible for generating Fe (II) in solution, and previous research has shown the involvement of iron-reducing bacteria in PAH degradation [16].

CHAPTER 3

Detection of anaerobic metabolites of naphthalene degradation at a creosote-contaminated Superfund site and *in situ* transformation of deuterated naphthalene to a naphthoic acid analogue

3.1 Introduction

Natural attenuation, or intrinsic bioremediation, occurs when indigenous microbial communities are responsible for the breakdown or transformation of contaminants independent of any human manipulations to stimulate or enhance the process [54]. According to the National Research Council, three conditions must be demonstrated to recognize biodegradation as actually occurring: 1) contaminant levels at the site of interest have decreased over time, 2) native microorganisms are responsible, to some degree, for this loss, and 3) there is documented evidence biodegradation is occurring in the field [54]. In general, the first condition is easy to observe, although physical and chemical processes also play a substantial role in contaminant loss. The second and third criteria are often best met by the detection of metabolic biomarkers, specifically,

intermediates in the degradation pathway of a contaminant [55]. Generally, an intermediate is considered a good biomarker if the compound is exclusive to the biochemical process considered. The biomarker should not be a common, commercially produced compound or typical component in industrial wastes, nor should it be a non-specific product shared among several metabolic processes [56]. Ideally, the biomarker is transitory, presumably as a biochemical intermediate to be further degraded, signifying that biodegradation is currently taking place [57].

As discussed in Chapter 1, naphthoic acid is the first intermediate formed in the anaerobic degradation of naphthalene [17]. This compound is not commonly found in nature, and thus it can be used as an *in situ* indicator of naphthalene degradation [56]. However, naphthoic acid can also be formed during the aerobic degradation of methylnaphthalene and acenaphthylene [39-41]. Thus, if the presence of oxygen is unknown, confirming anaerobic degradation requires the detection of additional putative metabolites, unique to the anaerobic degradation of naphthalene, formed subsequently to naphthoic acid, such as 1,2,3,4- and 5,6,7,8-tetrahydronaphthoic acid (Figure 3.1a) [32, 42, 43].

The detection of biomarkers provides a snapshot of biodegradation occurring *in situ* but gives no information on rates of degradation. The rate of biodegradation is an important aspect of natural attenuation and crucial to model prediction. Push-pull tests can be used to quantitatively evaluate the physical, chemical and biological characteristics of an aquifer *in situ*. During a push-pull test, groundwater is extracted from a well and amended with a conservative tracer and reactive compound(s) of interest. The augmented groundwater is subsequently “pushed” back into the well. Over time,

groundwater samples are then “pulled” from the well and measured for changes in tracer and reactive compound concentrations and, if applicable, the formation of metabolic products [58]. Several studies have targeted microbial processes using push-pull tests to define rates of degradation [59-61]. However, a rate of anaerobic degradation of PAHs has not been previously defined using the push-pull method. A similar study conducted by Reusser et al. [59] conducted a push-pull experiment to define rates of transformation of BTEX constituents under denitrifying redox conditions. Deuterated parent products (toluene- d_8 and *o*-xylene- d_{10}), a conservative tracer, and nitrate, serving as a final electron acceptor, were injected into BTEX-contaminated aquifers. Detection of deuterated metabolites provided proof of *in situ* anaerobic transformation of the injected parent products. Additionally, conservative rates of formation were successfully quantified using the concentrations of deuterated transformation products measured over time.

The objective of this study was to determine whether or not anaerobic degradation of PAHs was occurring where contamination was present at the McCormick and Baxter Superfund Site. Groundwater and sediment porewater samples were assessed for putative metabolites of anaerobic naphthalene degradation and general geochemistry. In addition, a push-pull test was conducted using an approach similar to Reusser et al. [59] to evaluate whether the transformation of deuterated naphthalene to deuterated naphthoic acid (naphthoic acid- D_7 ; Figure 3.1b) could be monitored *in situ* and if conservative rates of transformation can be defined using this method.

^a Figure taken from Annweiler et al. [62]

Figure 3.1. (a) Proposed pathway of the anaerobic degradation of naphthalene and methyl-naphthalene. The respective metabolites labeled as I to VIII are 2-naphthoic acid, 5,6,7,8-tetrahydro-2-naphthoic acid (or 1,2,3,4-tetrahydro-2-naphthoic acid, not shown), octahydro-2-naphthoic acid, decahydro-2-naphthoic acid, hydroxydecahydro-2-naphthoic acid, β -oxo-decahydro-2-naphthoic acid, and $C_{11}H_{16}O_4$ -diacid, *cis*-2-carboxycyclohexylacetic acid.^a (b) Naphthalene- D_8 is converted to naphthoic acid- D_7 following the same pathway.

3.2 Methods

Metabolite detection and geochemistry. Porewater was collected from six locations with known contamination within the sediment cap footprint using Push Point samplers (0.91 and 1.82 m long, 1.91 cm I.D.; MHE Products, East Tawas, MI) (Figure 3.2). Most of the samples were taken from the native sediment below the remedial cap, however one sample was also taken from the organophilic clay cap layer and one retrieved from the sand cap. Temperature, dissolved oxygen, pH, turbidity, and specific conductance of the porewater samples were measured using water quality probes (Horiba model U-22 and YSI model 6820). These bulk parameters were measured before and after sampling to reveal any significant changes in water quality data that may have occurred during porewater sampling at each depth. Groundwater samples were taken using a bailer from 11 upland monitoring wells with known PAH contamination (Figure 3.3). Porewater and groundwater samples were preserved according to the analytical methods shown in Table 3.1 and immediately placed in a cooler for transport to Portland State University (PSU). Test America Laboratories, Inc., Beaverton, Oregon, determined the concentrations of the porewater analytes listed in Table 3.1, other than putative anaerobic naphthalene metabolites. Anaerobic naphthalene metabolites were analyzed at PSU using gas chromatography-mass spectrometry (GC-MS, HP-5MS column; Agilent, Santa Clara, CA) following extraction and derivatization as described in Gieg and Suflita [32]. Briefly, 1-L porewater samples were extracted into ethyl acetate and dried with sodium sulfate. The samples were concentrated by rotary evaporation and further concentrated to a final volume of 50 μ l under a stream of N₂. The samples were derivatized using BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetimide; Pierce Chemical Co., Rockford, IL) to

Table 3.1. Analyses performed on porewater samples retrieved from the remedial cap and the native sediments at the McCormick and Baxter Superfund Site.

Analyte	Container/replicates	Preservation	Analytical Method
Naphthalene	1L amber glass/2x	--	EPA 8270m
Naphthalene metabolites	1L amber glass	50% HCl	GC/MS [32]
Methane	40ml VOA/3x	HCl	GC/FID
Carbon dioxide	40ml VOA/2x	--	SM 4500-C
Major anions	500ml poly container	--	EPA 300.0
Major cations	250ml poly container	HNO ₃	EPA 200.7

form trimethylsilyl esters. The GC oven temperature was held at 45°C for 5 min., then ramped at 4°C min⁻¹ to 270°C and held for 15 min. The metabolites were identified by comparing the GC retention times and MS profiles to purchased standards described in Gieg and Suflita [32].

Push-pull test. A single-well push-pull test was performed on Monitoring Well 45s, located outside of the barrier wall and hydraulically connected to the Willamette River (Figure 3.3). Well 45s was selected based on the presence of naphthalene degradation metabolites and hydraulic characteristics suitable for a push-pull test. The *in situ* anaerobic degradation of naphthalene is assumed to be relatively slow based on reported laboratory rate studies [9]. Therefore to allow enough time for biotransformation before the bulk of the labeled parent compound has drifted down gradient, a well was selected that displayed a loss of less than 55% of a conservative tracer over a two-week period (data not shown).

A peristaltic pump was used to transfer approximately 200 L of groundwater from the aquifer into a large plastic carboy. The extracted groundwater was continuously bubbled

with N₂ gas to strip the groundwater of any oxygen introduced while pumping the water out of and back into the well. The test solution was prepared by amending the extracted groundwater with bromide (from KBr, Fisher, Pittsburgh, PA) as a conservative tracer, and deuterated naphthalene-D₈ (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO; Fisher, Pittsburgh, PA). The DMSO acted as a carrier solvent and constituted less than 2% (v/v) of the test solution to avoid significant co-solvent effects [63]. The concentration of each analyte is listed in results section (Table 3.3). The amended groundwater was then injected (“pushed”) back into the well with the peristaltic pump at a rate of about 7 L·min⁻¹. Three samples of the test solution were taken to ensure that the solution was well mixed and to obtain initial (C₀) concentrations for bromide, naphthalene-D₈, naphthoic acid-D₇, and unlabeled naphthalene and naphthoic acid. Groundwater samples were also taken from the well prior to withdrawing the groundwater from the aquifer and analyzed for the same test solution analytes as discussed above, as well as for carbon dioxide, methane, sulfate and nitrate. Total iron was measured using visual colorimetric Vacuvials (CHEMetrics, Calverton VA). Oxidation-reduction potential was measured using a water quality probe (Horiba model U-22).

Groundwater samples were then taken (“pulled”) from the well on a daily to weekly basis for 32 days. On all occasions the well was purged of two well casing volumes before taking samples for analysis. Samples for naphthalene-D₈, naphthoic acid-D₇, and unlabeled naphthalene and naphthoic acid were collected in clean amber bottles (1 L) with Teflon-lined lids and preserved with hydrochloric acid (HCl). Samples for bromide, nitrate, and sulfate analysis were collected in polypropylene vials (60 ml). Samples for

methane and carbon dioxide were collected in VOA vials (40 ml). All samples were immediately placed on ice, refrigerated upon returning to the laboratory and analyzed within one month.

Push-pull analytical procedures. Naphthalene (99+%) and fluoronaphthalene (98%) (Acros Organics, Morris Plains, NJ) were used as an external and internal standard, respectively, to obtain naphthalene-D₈ and naphthalene concentrations. Naphthoic acid (98%; Acros Organics, Morris Plains, NJ) and fluoronaphthoic acid (98%; Sigma-Aldrich, St. Louis, MO) were used as external and internal standards, respectively, to obtain naphthoic acid-D₇ and naphthoic acid concentrations. Fluoronaphthalene and fluoronaphthoic acid, were prepared in ethyl acetate at 40 mM and 0.19 mM, respectively. Qualitative standards of 1,2,3,4- and 5,6,7,8-tetrahydronaphthoic acid were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl acetate and acetone were purchased from Fisher Scientific (Pittsburgh, PA).

Samples analyzed for naphthalene-D₈, naphthoic acid-D₇, and background naphthalene and naphthoic acid were determined using GC-MS as described above. Calibration curves were constructed for naphthalene-D₈, naphthalene, naphthoic acid, fluoronaphthalene, and fluoronaphthoic acid. Quantification of naphthoic acid-D₇ was estimated using the naphthoic acid calibration curve; a response factor of unity was assumed because a commercially available standard was not available. Loss of analyte due to evaporation and sorption to glass, as well as inaccuracy in determining the final sample volume or insufficient derivatization during sample processing was accounted for by calculating the percent loss/gain of internal standards.

Bromide, carbon dioxide, and methane concentrations were measured at a commercial lab, Pace Analytical Services, Inc, Seattle, WA. Bromide was analyzed using EPA method 300.0. Methane and carbon dioxide were analyzed using method AM20GAX. Sulfate and nitrate concentrations were analyzed at PSU using ion chromatography (Dionex ICS-2500 Ion Chromatograph).

3.3 Results and Discussion

Metabolite detection and geochemistry. Putative metabolites formed in the anaerobic degradation of naphthalene were found in five of six sediment porewater samples and five of the eleven groundwater samples suggesting that widespread anaerobic biodegradation of naphthalene was occurring within the sediments and upland aquifer at the McCormick and Baxter Superfund Site (Table 3.2). Metabolites detected were 1- or 2-naphthoic acid, 5,6,7,8-tetrahydronaphthoic acid, and 1,2,3,4-tetrahydronaphthoic acid. At only one location, MBPW0813-36, was naphthalene detected without the presence of breakdown metabolites, although, naphthalene was measured between the method detection limit (MDL) and method reporting limit (MRL), and thus is reported as an estimated value. Not surprisingly, at this location and others without detectable naphthalene, metabolites were not detected, presumably due to the absence of substrate available to the anaerobic microorganisms responsible for naphthalene degradation (Table 3.2). Anaerobic conditions in the sampled porewater and groundwater were evidenced by the elevated levels of carbon dioxide and methane, the diminished levels of sulfate (possible periodic sulfate reduction), and moderate to high levels of dissolved iron (probable iron reduction) (Table 3.2). However, these analyses do not necessarily specify

Table 3.2. Geochemistry, naphthalene and naphthalene metabolites detected in groundwater and porewater samples from a creosote-contaminated aquifer and river sediments.^a

Sample name	Well type/ sediment layer	Depth (ft.)	Naphthalene (ug/L)	Nitrate (mg/L)	Sulfate (mg/L)	Carbon dioxide (mg/L)	Methane (ug/L)	Total {dissolved} iron (mg/L)	Metabolites detected
MW-44s	shallow	5 - 30	3150	0.07	5.39	48.4	9370	n/a	+
MW-45s	shallow	5 - 30	1170	nd	2.32	37	7220	n/a	+
MW-41s	shallow	5 - 30	nd	0.50	0.88	15.7	2080	n/a	-
MW-43i	intermed.	30 - 50	nd	nd	0.76	nd	nd	n/a	-
MW-62i	intermed.	30 - 50	1.21	0.03	2.50	n/a	1320	n/a	+
MW-44d	deep	50 - 150	2.5	0.54	3.52	n/a	32.4	n/a	+
MW-45d	deep	50 - 150	0.49	0.47	2.47	n/a	1.29	n/a	+
MW-38d	deep	50 - 150	nd	0.02	6.52	nd	nd	n/a	-
MW-39d	deep	50 - 150	nd	0.09	2.44	nd	nd	n/a	-
MW-36d	deep	50 - 150	nd	nd	3.31	nd	nd	n/a	-
MW-60d	deep	50 - 150	nd	0.91	4.65	n/a	1.72	n/a	-
MBPW0802-06	WC-SC	0.5	265	nd	nd	86.9	10600	47.2 {48.1}	+
MBPW0813-36	TFA-OC	3.0	0.0226	nd	nd	105	7950	36.9 {40.8}	-
PSU5-36	TFA-NS	3.0	3.97	nd	1.29	59.5	10100	23.6 {23.2}	+
MBPW0815-40	TFA-NS	3.3	1.26	nd	nd	222	9460	75.7 {75.2}	+
MBPW0802-50	WC-NS	4.2	0.382	nd	1.42	47.8	191	27.3 {28.0}	+
MBPW0801-60	WC-NS	5.0	0.743	nd	5.56	30.5	629	17.5 {16.5}	+

^a WC, Willamette Cove; TFA, Tank Farm Area; SC, sand cap; OC, organophilic clay; NS, native sediment; n/a, not analyzed; nd, below detection limit.

which terminal electron acceptors are involved in the anaerobic degradation of naphthalene at the site. Naphthalene degradation could be coupled with methanogenesis, which has been previously reported in similar studies [64, 65], with iron reduction, or sulfate reduction [13-15, 64, 66]. Degradation is occurring under anaerobic conditions at the site and appears widespread (where naphthalene is available) both in the native sediments and the upland aquifer. However, the microorganisms involved in the breakdown of naphthalene at the site are not known.

Push-pull test. The background concentrations of naphthalene and naphthoic acid in Well 45s were 31.2 μM and 205 nM, respectively (Table 3.3). The aquifer is anoxic around the well as indicated by the negative oxidation/reduction potential (ORP) and elevated levels of carbon dioxide and methane (Table 3.3). Low levels of sulfate, non-detectable levels of nitrate, and high concentrations of iron ($> 179 \mu\text{M}$) were also observed in the groundwater (Table 3.3). Naphthoic acid-D₇ was detected by comparing the retention time and assumed mass spectral profile with the GC retention time and MS profile of naphthoic acid (Figure 3.4). The results of the push-pull experiment are shown using breakthrough curves for naphthalene-D₈ and bromide. Breakthrough curves plot the relative concentration (C/C_0) versus time, where C is the solute concentration at a given time and C_0 is the concentration in the test “push” solution prior to reinjection into the well (Figure 3.5a). Dilution of naphthalene-D₈ was accounted for by dividing its relative concentration to the relative concentration of the conservative tracer (bromide). Any observed decreases in C/C_0 for the dilution-adjusted naphthalene can be attributed to loss via degradation. However, over the duration of the experiment, dilution adjusted

Table 3.3. Geochemistry of Monitoring Well 45s and test solution composition.^a

Analyte	MW-45s	Test Solution
Bromide (mM)	nd	1.55
Naphthalene (μM)	31.2	23.5
Naphthalene-D ₈ (μM)	nd	111.2
Naphthoic Acid (nM)	205	140
Naphthoic Acid-D ₇ (nM)	nd	nd
Nitrate (μM)	nd	n/a
Sulfate (μM)	12.5	n/a
Carbon Dioxide (μM)	5908	n/a
Methane (μM)	449	n/a
Iron (total) (μM)	>179	n/a
ORP (mV)	-34	n/a

^a n/a, not analyzed; nd, below detection limit.

Figure 3.4. Mass spectral profiles of (a) naphthoic acid and (b) deuterated naphthoic acid-D₇ detected during a push-pull test using Monitoring Well 45s (analyzed as trimethylsilyl, TMS, derivatives).

Figure 3.5. Normalized concentrations of (a) bromide and naphthalene-D₈ and (b) the subsequent formation of naphthoic acid-D₇ during a push-pull test in Monitoring Well 45s.

C/C_0 values of naphthalene- D_8 were around or greater than 1. A ratio greater than 1 indicates retardation of the reactive solute relative to the non-reactive tracer, and therefore, using changes in reactive solute concentrations over time to determine anaerobic degradation or transformation rates would be inaccurate [59]. However, the use of a deuterated parent compound (naphthalene- D_8) followed by the detection and quantification of its unique anaerobic metabolic biomarker (naphthoic acid- D_7) provided unequivocal evidence of anaerobic degradation of naphthalene and a rate of naphthalene transformation was determined (Figure 3.5b).

Dilution-adjusted naphthoic acid- D_7 concentrations were calculated by dividing the measured concentration by C/C_0 for bromide. Naphthoic acid- D_7 appeared by day 3, continued to rise until day 8, began to decline by day 12, and reached non-detect levels by day 24 (Figure 3.5b). A similar approach to that of Reusser et al. [59] was applied to calculate zero-order and first-order rates of transformation. The linear portion of the increasing naphthoic acid- D_7 , specifically, day 1 to day 8, was subjected to linear regression and a zero-order rate of naphthoic acid- D_7 production was calculated as $31 \text{ nM} \cdot \text{d}^{-1}$. The apparent first-order rate, $0.006 \cdot \text{d}^{-1}$, was then calculated by dividing the zero-order rate by the average concentration of naphthalene- D_8 measured in the same time period, day 1 to day 8.

These rates likely underestimate the true degradation rates since an extracellular concentration of an intermediate compound may only represent a small fraction of that which remains within the cell to be further degraded [57]. A review of anaerobic PAH degradation showed that published laboratory rates of anaerobic naphthalene degradation vary widely depending on several factors, including the source of inoculum and the

terminal electron accepting process [9]. However, a field study of naphthalene-C¹⁴ degradation in creosote-contaminated river sediments by Bianchin et al. [67] calculated a rate of mineralization of 39 nM·d⁻¹ which is quite similar to our rate of naphthalene transformation of 31 nM·d⁻¹.

As illustrated in Figure 3.5b, unlabeled (i.e., background) naphthalene and naphthoic acid concentrations are constant (except for the initial disturbance induced by the experiment), indicating that the anaerobic degradation of naphthalene is continuous and may be proceeding as a (quasi-) steady state at this location in the contaminated aquifer.

3.4 Conclusions

Widespread anaerobic biodegradation of naphthalene and possibly other PAHs is occurring within the sediments and upland aquifer at the McCormick and Baxter Superfund Site. The mechanism (i.e., the terminal electron accepting process) by which this degradation is occurring is unknown. Future studies could include laboratory microcosms of McCormick and Baxter sediments and aquifer porous media amended with naphthalene and various electron acceptors. Molecular analyses could provide information on microbial diversity in locations with known biodegradation potential and identify key members responsible for the degradation of PAHs.

This study is the first reported use of deuterated naphthalene to provide conclusive evidence of the *in situ* production of breakdown metabolites of anaerobic naphthalene degradation. Using this method, a conservative rate of transformation was successfully measured in the presence of high background concentrations of naphthalene and naphthoic acid. This study sets the groundwork regarding future site characterization.

Rates could vary depending on the location within the contaminated aquifer based on availability of substrates and other environmental factors. Future studies could include push-pull experiments using other wells throughout the site, as well as using additional amendments such as terminal electron acceptors or higher molecular weight PAHs to give a broader perspective regarding biodegradation at the McCormick and Baxter Superfund Site.

CHAPTER 4

Assessment of methanogenesis and its subsequent impact on the lifetime of an organophilic clay sediment cap

4.1 Introduction

The cleanup of contaminated sediments is of considerable concern, but is hindered by complex problems, such as sediment accessibility and contaminant release, that have limited the options for remediation. Dredging and capping are common clean-up techniques, but these can have substantial adverse primary and secondary consequences. Dredging causes considerable environmental disruption and the potential dispersion of contaminants, often re-exposing aquatic biota to toxic chemicals and leaving residual contaminated sediments that may create future environmental problems [68, 69]. Capping, although often less disruptive, requires monitoring and maintenance to ensure that the cap remains intact and functional in the event of floods, navigational activities, and bioturbation.

A major problem associated with sediment caps is that compaction of contaminated sediment by the overlying cap may extrude contaminants in porewater, at least temporarily, increasing the advective flux of contaminants through the sediments and remedial cap to the surface water [70, 71]. Another possible complication is ebullition of

biogenic methane and carbon dioxide from the contaminated sediments through the remedial cap. Gas bubbles frequently form in organic-rich sediments because the concentrations of methane easily exceed saturation [72]. Bubbles can entrain or sparge contaminants, moving these compounds vertically to the surface water [73-77].

New capping technologies have been developed to ameliorate the risks associated with traditional capping practices. Enhancing the sorptive properties of the cap reduces the diffusive and advective flux of contaminants by increasing the capacity of the capping material to bind and retard contaminants as they move through the cap. Laboratory and field-scale studies using activated carbon, coke, organic-rich soil, and organophilic clay (also called organoclay) have demonstrated the ability of these sorbents to reduce organic contaminant migration compared to conventional sand caps [78-81].

Recent studies have shown that the construction of a sediment cap often causes an upward shift in reducing zones as the native sediment becomes isolated from the overlying (oxic) surface water. That is, the oxic/anoxic interface as well as the cascade of terminal electron accepting processes (TEAPs) migrate upward from the native sediment to within the capping material [82]. Where aerobic processes once existed, such as at the sediment/water interface, with the addition of a sediment cap, sulfate-reducing or methanogenic conditions may now prevail. This redox shift may improve the efficacy of the cap by retarding contaminants that are less labile in reduced environments or enhancing the removal of contaminants that are amenable to anaerobic degradation. Or the shift may diminish cap function by increasing biogas production and its subsequent contaminant transport [83]. In addition, ebullition can substantially compromise the physical integrity of the cap by creating fractures or preferential

pathways for contaminants [74, 75, 84, 85].

Studies discussed herein assess the ability and degree to which anaerobic microorganisms use the organic component of organophilic clay as a carbon source. Anaerobic slurry incubations were constructed using sediment core samples to quantify the contribution of the native sediment and the different layers of capping material (sand and organophilic clay) to the overall methane production observed at the McCormick and Baxter Superfund Site. Substrate addition experiments and changes in total carbon over time supported the hypothesis that organophilic clay can be used as a carbon source. Quantitative PCR (qPCR) and denaturing gradient gel electrophoresis (DGGE) were used to measure total numbers of methanogens and to identify some of the predominant microorganisms in each of the capping layers, respectively. Here, we found that a syntrophic consortium of microorganisms may be responsible for the conversion of the organic component of organophilic clay ultimately to methane.

4.2 Methods

Sediment and porewater sampling. Between August 25 and September 3, 2008, sediment cores were collected from the McCormick and Baxter Superfund Site. Two areas of the sediment cap were sampled, the Willamette Cove (WC) and the Tank Farm Area (TFA). Within each area, two sets of samples were collected based on cap composition—sediment cap containing a sand layer underlain by a layer of Aqua Technologies ET-1 organophilic clay, WC-2 and TFA-13, and sediment cap containing only sand, WC-1 and TFA-14 (Figure 4.1). The cores included material from each characteristic layer of the cap: the sand layer (SC), the organophilic clay (OC; if present), and the underlying native

sediment (NS). The types of samples are referred to as WC-1, WC-2, TFA-13, or TFA-14 according to site location, and -SC, -OC, or -NS according to the type of material. For example sand cap from WC-1 is sample WC-1-SC. A core of sand capping material was also collected from a reference location (REF) within the sediment cap footprint but away from the highly contaminated areas and the organophilic clay capping material. The sediment cores were collected with a track-mounted push probe using a combination of hydraulic pressure and mechanical hammering to drive the sampler into the remedial cap and sediment. The sampler consisted of a hollow 3-inch diameter rod with an acetate liner. The cores were immediately placed on ice, and transported back to the laboratory where the cores were cut into sections based on composition. Subsamples of the native sediment and core materials were removed from the center of each characteristic layer and at the interface between layers (i.e., sand cap/organophilic clay interface, etc.) using sterile technique to avoid cross-contamination. The subsamples were stored at -80°C until further processing for the molecular characterization described below. Environmental core samples of the organophilic clay were also taken from five different cores obtained from WC-2 and TFA-13. These samples were dried at room temperature, ground using a clean mortar and pestle, and sent to Oregon State University (OSU) for the determination of total carbon content. The sectioned cores were placed in airtight ammo boxes that were flushed with N₂ to create an anaerobic atmosphere and refrigerated until further sample processing. Several liters of porewater were collected from the Willamette Cove native sediment using Push Point samplers (0.91 and 1.82 m long, 1.91 cm I.D.; MHE Products, East Tawas, MI). The porewater was used in the construction of laboratory incubations discussed below.

Native sediment and sediment cap incubations. Slurries of native sediment and remedial cap materials (sand and organophilic clay) were prepared in an anaerobic glove box using 50 g ($\pm$ 0.5 g) of sub-sectioned, homogenized core material mixed with 75 mL ($\pm$ 0.5 mL) of porewater retrieved from the site. Prior to mixing, the porewater was bubbled with N₂ for 30 minutes and chemically reduced inside an anaerobic glove box by adding Na₂S to a concentration of 0.012%. Resazurin (0.001%) was added as a redox indicator and the solution was then filtered (0.45 μ m membrane filters; Whatman, Piscataway, NJ). The reduced porewater was combined with the sample solids in 160 ml serum bottles, capped with butyl rubber stoppers, and crimp-sealed. The headspaces of the bottles were exchanged with N₂ to remove any H₂ introduced from the glove box. Incubations were performed in triplicate or more. Sterile controls were prepared using duplicate incubations of each treatment and autoclaving the incubations at 121°C for 20 min on three successive days.

After 215 days, 5 to 15 ml of slurry was removed from an active incubation of each capping layer from all coring locations. The samples were centrifuged (30 min at 3000 rpm) to retrieve at least 500 mg of material. DNA was immediately extracted from these samples as described below. After 300 days, a WC-2-OC incubation and a TFA-13-OC incubation along with one respective sterile control of each treatment were deconstructed. The entire contents were centrifuged (10 min at 3000 rpm) and the supernatant was discarded. The samples were dried at room temperature, ground with a clean mortar and pestle, and sent to OSU to be analyzed for total carbon content. The sterile controls, which had no microbial activity over the 300-day period, are assumed to have the same initial total carbon content as the treatment incubations.

Incubations were also constructed using 10 g ($\pm$ 0.5 g) additions of fresh, unused organophilic clays, CETCO Organoclay (Hoffman Estates, IL) and Aqua Technologies ET-1 (Casper, WY), to TFA-13-OC (15 g $\pm$ 0.5 g) in filter-sterilized, Na₂S-reduced porewater (60 mL $\pm$ 0.5 mL), constructed in quadruplicate. Additions using “washed” fresh organophilic clays were also constructed same as above, only in triplicate. To wash the organophilic clays, CETCO Organoclay and Aqua Technologies ET-1 were suspended in deionized water and shaken for 10 minutes. The wash was then discarded and the process was repeated 24 times to ensure removal of soluble organic compounds. The washed organophilic clays were subsequently dried at 45°C in an oven overnight. Duplicate sterile controls were prepared by autoclaving the incubations for 20 min on each of 3 successive days (121°C). Unamended controls (i.e., without fresh, unused organophilic clay) contained TFA-13-OC (15 g $\pm$ 0.5 g) and reduced porewater (60 mL $\pm$ 0.5 mL), in quadruplicate, and served as controls to account for background methane production. Incubations of both washed and unwashed CETCO Organoclay and Aqua Technologies ET-1 without TFA-13-OC also served as controls. A sample of fresh, unused Aqua Technologies ET-1 was ground using a clean mortar and pestle and sent to OSU for total carbon content analysis.

Methane production in all incubations was measured over time by injecting headspace samples (0.2 mL) into a Shimadzu 2010 gas chromatograph with a flame ionization detector and a fused-silica capillary column (RT-QPLOT; Restek, Bellefonte, PA). Temperatures were held constant at 200°C for the injector and detector column and the oven was held constant at 100°C.

DNA extraction. With the exception of sand cap and native sediment from WC-1, the environmental core samples were extracted using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. The core samples from WC-1 and the sediment and capping materials removed from the incubations were extracted using the MP Biomedicals FastDNA SPIN Kit for Soil (MP Biomedicals LLC, Solon, OH) following the manufacturer's instructions. To confirm the presence of genomic DNA, the extracts were run in a 0.7% agarose gel using electrophoresis. The extracted DNA was used for molecular analyses described below.

Quantitative PCR. Quantitative PCR (qPCR) was performed to detect and quantify the *mcrA* gene in DNA extracts of the environmental core samples and native sediment and sediment cap incubations. A set of standards was produced from a single standard of the *mcrA* gene kindly provided by Anna-Louise Reysenbach, Portland State University and Mary Voytek, U.S. Geological Survey. The standards were diluted in PCR-grade water over 8 orders of magnitude down to 1×10^0 gene copies per μL . The standards and the extracted DNA samples were amplified using the forward primer, 5'-GGTGGTGTGTMGGATTCACACARTAYGCWACAGC-3', and the reverse primer, 5'-TTCATTGCRTAGTTWGGRTAGTT-3' using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA). Each PCR reaction contained the following: 1 μL each of the forward and reverse primers, 1 μL template DNA, 9.5 μL PCR-grade water, and 12.5 μL Quantitect SYBR Green master mix. All qPCR standards and samples were run in duplicate on a Stratagene mx3005P (La Jolla, CA). A "no-template" control was employed using sterile water as well as a negative control (*Desulfovibrio* sp. strain lac 3)

to detect non-specific amplification. The PCR amplification conditions were as follows: 10 minutes hold at 95°C, followed by 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute, followed by 82°C for 10s. Fluorescence readings were taken at both 72°C and 82°C, during each of the 40 cycles on the SYBR channel (excitation at 492 nm). The melting curve analysis was performed with continuous fluorescence readings between 55° C and 95° C to check for amplification artifacts and contamination, as well as to ensure reaction specificity and accurate quantification. The standard curve was constructed and cell numbers were determined using the MxPro QPCR software.

PCR-DGGE. The 16S rRNA gene was amplified from the extracted DNA and run in denaturing gradient gel electrophoresis (DGGE). For DNA amplification, 2 µl of the extracted DNA was combined with 25 µl PCR MasterMix (2X) (Fermentas, Glen Burnie, MD), 20 pmol each of the forward and reverse primers, 27f-GC and 534r for bacteria or U515f-GC and A1041r for archaea (Table 4.1), and PCR-grade water to obtain a final volume of 50 µl. The forward primers used to amplify bacterial and archaeal 16S rRNA genes were modified with a GC clamp to aid in band separation in DGGE analysis. The PCR amplification conditions were as follows: 5 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, 1.5 minutes at 60°C and 1 minute at 72°C, followed by a final 7 minutes at 72°C.

About 250 ng of amplified DNA was loaded into each well of a 6.5% acrylamide gel with a denaturant gradient (formamide and urea) of 40-60%. The DGGE analysis was conducted on a Bio-rad DCode system at 60°C with an electrophoresis time of 16 h at 75 volts. The gels were stained with 1x SYBR Green I (Invitrogen, Carlsbad, CA) and

subsequently visualized under UV light.

Bands were excised from the gels using a clean razor blade and placed in sterile microcentrifuge tubes containing 50 μ L Tris-EDTA overnight at room temperature to elute the DNA from the gel fragment. The tubes were then centrifuged and the band DNA was amplified following the same amplification conditions as previously described for DGGE, however, the forward primers, 27f and U515f were used rather than 27f-GC and U515f-GC. The PCR products were purified using Qiaquick PCR Purification kit (Qiagen, Valencia, CA).

The purified PCR products (25-50 ng) were then added to sterile water and 3 pmol purified forward primer, 27f and U515f (Table 4.1), respective of whether the PCR product is bacterial or archaeal DNA, to achieve a final volume of 12 μ L. The samples were then sent to the University of Calgary Genetic Analysis laboratory to be sequenced using a 48 capillary ABI 3730S automated DNA sequencer. The chromatograms of the DNA sequences were visualized and edited using FinchTV software. The edited sequences were submitted to GenBank using BLASTN to get an indication of their phylogenetic affiliation by comparison to closely related 16S rRNA sequences present in the database.

Table 4.1. PCR primers used in DGGE.

Name	Primer Sequence (5' to 3')	Reference
27f	CAGAGTTTGATCCTGGCTCAG	[86]
27f-GC	ccgcgccgccccggcggcggggcggggcgaggagCAGAGTTTGATCCTGGCTCAG	[86]*
534r	ATTACCGCGGCTGCTGG	[87]
U515f	GTGCCAGCAGCCGCGG	[88]
U515f-GC	ccgcgccgccccggcggcggggcggggcgaggagGTGCCAGCAGCCGCGG	[88]*
A1041r	GGCCATGCACC(W)CCTCTC	[89]

* indicates the primer was modified for this study.

4.3 Results and Discussion

Methanogenesis in native sediment and sediment cap incubations. Slurry incubations of the organophilic clay layer of the sediment cap produced up to 1500 times more methane over an 80-day period than the other (non-organophilic clay) layers incubated under the same conditions (Figure 4.2). Initial rates of methanogenesis for the incubations containing only the sand cap ranged from 0.001 to 0.042 $\text{CH}_4 \cdot \text{day}^{-1} \cdot \text{g}^{-1}$. Initial rates of methanogenesis in the incubations containing native sediment ranged from 0.002 to 0.390 $\mu\text{mol CH}_4 \cdot \text{day}^{-1} \cdot \text{g}^{-1}$. The incubations containing native sediment originating from areas with active NAPL seeps, WC-2-NS and TFA-13-NS, had rates of methane production of 0.284 and 0.390 $\mu\text{mol CH}_4 \cdot \text{day}^{-1} \cdot \text{g}^{-1}$, respectively. These rates are similar to those of methane production in sandstone coring materials containing residual oil, as described in Gieg et al. [35]. In contrast, the rates of methanogenesis in the organophilic clay incubations, TFA-13-OC and WC-2-OC, were significantly higher, at 1.021 and 1.367 $\mu\text{mol CH}_4 \cdot \text{day}^{-1} \cdot \text{g}^{-1}$, respectively (Figure 4.2). Initial lag in methane production was only observed in one treatment, TFA-13-OC, during the first 11 days of incubation, thus the rate was quantified subsequent to this period of time. No methane production was observed in any of the sterile controls.

The greatly elevated rates of methanogenesis in the organophilic clay incubations clearly indicate that the organophilic clay stimulated sustained methane production over the 80-day period. Flux chamber field studies run concurrently with the research presented here estimated a rate of gas production within the organophilic clay footprint of 2.9 $\text{L/m}^2/\text{day}$ (measured at TFA-13 between September 4 to September 14, 2008), a vast

Figure 4.2. Methane production observed in laboratory incubations constructed of native sediment and sediment cap materials retrieved from various locations at the McCormick and Baxter Superfund Site. Incubations contained 50 g of core material and 75 ml native sediment porewater. Error bars represent 1 standard deviation of the mean of triplicate and quadruplicate incubations.

Figure 4.3. Initial rates of methanogenesis of incubated native sediment and sediment capping materials. Note that organophilic clay is present only at WC-2 and TFA-13; only sand cap was incubated from the reference location (REF).

majority of which was methane (76-110%). Whereas, in areas only capped with sand, the rate of gas production was two orders of magnitude lower with a rate of 0.02 L/m²/day (36-61% methane) [90]. The field study alone does not necessarily identify the source of the methane gas, however, in conjunction with the laboratory incubations, the results strongly suggest that the greatest contribution of methane to the ebullition observed at the site originates from the organophilic clay layer of the sediment cap (Figure 4.3).

qPCR to quantify methanogens in the native sediment and sediment cap. Quantitative PCR (qPCR) was used to investigate the relationship between rates of methanogenesis and the number of methanogens measured in each of the respective capping layers. qPCR is a rapid and specific molecular technique frequently used to determine the number of cells present in environmental samples [91, 92]. The abundance of the *mcrA* gene, which is the molecular signature for methanogenesis, was enumerated from DNA extracted directly from field samples and after 215 days of incubation under anaerobic conditions. The *mcrA* gene encodes for the α subunit of methyl coenzyme-M reductase enzyme that catalyzes the reduction of the methyl group attached to coenzyme-M leading to the subsequent release of methane by the organism. Since the α subunit of this enzyme complex is present in all methanogens, targeting this amino acid sequence with qPCR provides a method to quantify methanogens in each sample of interest [93]. Assuming that each cell has one copy of the *mcrA* gene, the number of cells present in a given sample can be measured by the number of copies of *mcrA* gene detected by qPCR [94].

Methanogens were present in all locations, although DNA could not be amplified

Figure 4.4. Numbers of methanogens in the native sediment and sediment cap field samples (grey) and after 215 days of incubation (black). N/A indicates that the DNA was not amplifiable.

from WC-1-NS field sample and TFA-13-OC incubation (Figure 4.4). Of the field samples, WC-2-OC had the greatest number of copies of the *mcrA* gene (8.7×10^5). Conversely, TFA-13-OC field sample contained significantly fewer *mcrA* gene copies compared to the adjacent sand cap and native sediment located above and below this capping layer, and more than two orders of magnitude less gene copies than WC-2-OC (Figure 4.4). Considering the significantly higher gas production rates measured in the field at TFA-13 (compared to the rate measured outside of the organophilic clay cap footprint) and the comparable methane production rates in the TFA-13-OC and WC-2-OC laboratory incubations, it would be expected that the number *mcrA* gene copies in the TFA-13-OC field sample would be similar to that of WC-2-OC. However, this difference may indicate some spatial heterogeneity in the microbial community within

the organophilic clay cap *in situ*. Copies of the *mcrA* gene in sand cap and native sediment field samples ranged from 1.5×10^3 to 2.8×10^4 . At all locations the *mcrA* gene copies were lower in the native sediment field samples than in the sand cap (excluding WC-1-NS; DNA could not be amplified from this field sample). However, this may be due to the ease by which DNA can be extracted from sand compared to contaminated river sediment or sediments with high organic content. Thus, the methanogens in the sediments may be underrepresented, in both the field and incubation samples.

The number of *mcrA* gene copies in the WC-2-NS incubation was comparable to that of WC-2-OC, although the rates of methane production were almost five times greater in the organophilic clay incubations. Because qPCR provides only the absolute number of copies of the *mcrA* gene in the extracted samples, these results do not necessarily imply that all cells are actively expressing the gene and therefore performing methanogenesis. The number of methanogens among the different capping materials is only an indication of the approximate number of methanogens, but may not be directly related to the observed rates of methane production in laboratory incubations or *in situ*. Activity is based not only on the number of cells but also on the availability of substrate and the efficiency of the individual species of organisms and this is clearly demonstrated in the qPCR results.

In every instance, incubation under methanogenic conditions resulted in increased numbers of methanogens. Surprisingly, methanogens from the native sediment from WC-2 and TFA-14 increased over 1000 fold (Figure 4.4). This suggests that, *in situ*, methanogens may be inhibited by the presence of other terminal electron acceptors besides CO₂ [50], whereas, in a closed system (i.e., laboratory incubations without these

electron acceptors), methanogens were able to grow in the native sediments. The organophilic clay exhibited the smallest change in the number of methanogens between the field sample and incubation, only a 3-fold difference, suggesting that cell growth is limited both in the field and in the laboratory. Sand cap from TFA-13 and TFA-14 also displayed relatively small increases in methanogens. This is likely due to substrate availability considering the low organic content typically found in sand. It is clear in this study and those of others, that measuring gene abundance alone, without lab metabolic studies, such as slurry incubations, or without looking specifically at the expression of functional genes (i.e., quantifying mRNA transcripts) does not provide a realistic estimation of the true metabolic capacity of a microbial community [95-98].

Organophilic clay as a carbon source. Laboratory incubations were constructed to determine whether the addition of organophilic clays to already microbially-active organophilic clay could stimulate greater levels of methane production. Two organophilic clay products (Aqua Technologies ET-1 and CETCO Organoclay) were compared to assess whether the methane production is just a function of Aqua Technologies ET-1 or if organophilic clays, in general, have the potential to produce high levels of methane. The addition of both Aqua Technologies ET-1 and CETCO Organoclay produced significantly higher levels of methane compared to the unamended controls over a 485-day period suggesting that anaerobic microorganisms may use these organophilic clays, or some component therein, as a carbon source for respiration and growth (Figure 4.5). If organophilic clay can be used as a carbon source, at least a 66% increase in methane production could be expected with the addition of 10 g fresh

organophilic clay to 15 g of organophilic clay capping material (ET-1) retrieved from the field. The washed and the unwashed CETCO Organoclay additions produced nearly the same amount of methane over the duration of the experiment, 70 and 62% more methane than the unamended controls, respectively. These results indicate that washing did little to modify the CETCO Organoclay and the carbon available to the microbial community could not be removed solely by washing the material. The unwashed Aqua Technologies ET-1 addition produced 135% more methane than the unamended controls compared to an 81% increase in the washed Aqua Technologies ET-1 treatment. Clearly, washing did affect the fresh Aqua Technologies ET-1. These results suggest that some biodegradable material, perhaps an excess of the quaternary amine used to manufacture the ET-1 organophilic clay remains unbound or loosely bound, and can be washed away to some extent. Over the first 208 days, the unamended controls (15 g TFA-13-OC) have a rate of methane production of $1.017 \mu\text{mol CH}_4 \cdot \text{day}^{-1} \cdot \text{g}^{-1}$. This rate is similar to that of the TFA-13-OC incubations described earlier (50 g; $1.021 \mu\text{mol CH}_4 \cdot \text{day}^{-1} \cdot \text{g}^{-1}$ over 80-day period). Methane production slowed after day 208 to a rate of $0.478 \mu\text{mol CH}_4 \cdot \text{day}^{-1} \cdot \text{g}^{-1}$. However, even at day 485, the unamended controls continued to steadily produce methane. No methane was produced in the incubations that contained only fresh, unused CETCO Organoclay and Aqua Technologies ET-1 without the organophilic clay retrieved from the field serving as the inoculum. This suggests that the microorganisms inhabiting the organophilic clay *in situ* likely originated from the contaminated sediments below rather than from the organophilic clay.

The unamended control treatment continued to produce methane at a moderately high rate for at least 485 days suggesting that a large pool of carbon must be available to the

microbial community (Line 1, Figure 4.5). If the carbon is coming from the organophilic clay then the carbon content of the organophilic clay should decrease over time. If we assume the Aqua Technologies ET-1 initially deployed in the field had a total carbon content similar to the fresh ET-1 we analyzed, the ET-1 organophilic clay retrieved 4 years after cap construction did, in fact, show significant reductions in carbon content, especially in the Willamette Cove (Table 4.2). Of the environmental core samples taken

Figure 4.5. Methane production of ET-1 organophilic clay (TFA-13-OC) incubated with fresh, unused organophilic clays under different conditions as follows: (line 1) unamended control, (line 2) inoculum with 10 g unwashed CETCO Organoclay, (line 3) inoculum with 10 g washed CETCO Organoclay, (line 4) inoculum with 10 g washed Aqua Technologies ET-1, and (line 5) inoculum with 10 g unwashed Aqua Technologies ET-1. These incubations contained 15 g organophilic clay (Aqua Technologies ET-1) retrieved from TFA-13 at the McCormick and Baxter Superfund Site (serving as the inoculum) and 60 ml native porewater. Error bars represent 1 standard deviation of the mean of replicate incubations (lines 1, 2 and 5 in quadruplicate; lines 3 and 4 in triplicate).

Table 4.2. Total carbon ($\mu\text{mol C/g}$) and apparent carbon loss in various organophilic clay samples. Apparent carbon loss is an estimated value based on the ratio of total carbon in a sample to that of fresh, unused Aqua Technologies ET-1 organophilic clay.

Sample name	Total carbon	Apparent carbon loss (%)
Fresh Aqua Technologies ET-1 OC	11,697	0
WC-2-OC (core 1)	5,378	54
WC-2-OC (core 2)	6,155	47
WC-2-OC incubation 0 days	5,696	51
WC-2-OC incubation 300 days	4,564	61
TFA-13-OC (core 1)	8,621	26
TFA-13-OC (core 2)	10,555	10
TFA-13-OC (core 3)	10,203	13
TFA-13-OC incubation 0 days	10,739	8
TFA-13-OC incubation 300 days	9,739	17

from WC-2-OC, the total carbon was about half that of the fresh, unused ET-1. The samples from TFA-13-OC were more variable, with the apparent loss in total carbon ranging from 10 to 26% (Table 4.2). This variability indicates that carbon loss may be occurring in discrete pockets in the Tank Farm Area, providing support that heterogeneity exists within the capping material *in situ*, as suggested earlier, regarding *mcrA* gene copies in the environmental sample from TFA-13-OC. More definitively, about a 10% decrease in total carbon was observed in the organophilic clay incubated under anaerobic conditions in the laboratory. The organophilic clay in the WC-2-OC and TFA-13-OC incubations lost about 1100 and 1000 $\mu\text{mol C}\cdot\text{g}^{-1}$ over a 300-day period, respectively (Table 4.2). It appears that the loss of carbon relates to the observed methane production, but it is not feasible to accurately quantify this correlation due to uncertainties regarding the exact chemical make-up the organophilic clay and the production of other carbon-containing compounds. Nonetheless, the amount of methane produced in TFA-13-OC

(unamended control; Line 1, Figure 4.5), over the first 300 days of incubation was about 259 $\mu\text{mol CH}_4\cdot\text{g}^{-1}$, which is about a quarter of the total carbon removed from the organophilic clay in the TFA-13-OC incubation.

Microbial community analysis of the native sediment and sediment cap. DGGE is one of the most frequently used methods for molecular fingerprinting [99]. In addition to providing a rapid fingerprinting comparison for many samples, specific phylogenetic information can be derived from the DGGE bands. All samples extracted for archaeal and bacterial DNA were analyzed using DGGE. However, due to sequencing difficulties, only 9 of the 81 archaeal bands and 39 of the 65 bacterial bands produced readable sequences. Thus our DGGE results are not comprehensive enough to understand the true microbial diversity of these sampling locations. However, the results do show that most of the bands analyzed contained sequences not closely affiliated to any cultured species (Tables 4.3 and 4.4). The microbial diversity in contaminated sediments and associated remedial caps therefore remains relatively unknown. The two archaeal groups identified were affiliated with *Crenarchaeota* and *Euryarchaeota*. The sequences within the *Crenarchaeota* group were most closely related (>97%) to organisms involved in anaerobic digestion of sludge. The sequences within the *Euryarchaeota* were not closely related to any specific group. Several bacterial phylogenetic groups were recovered by DGGE in the native sediments, including fermentative bacteria associated with *Bacteroidetes*, *Firmicutes* (specifically *Clostridia*), and *Chloroflexi*, organisms frequently observed in highly reduced and contaminated environments (Table 4.4) [35]. The sand cap contained groups similar the native sediments, however, sequences related to bacteria

Table 4.3. Phylogenetic affiliation of archaeal DGGE bands to their closest match in GenBank. Identical matches within the same sample denote multiple bands with similar sequences.

Sample name	Type	Group	Closest Match	Accession No.	Similarity (%)
TFA-13-SC	ENV	Crenarchaeota	Uncultured Crenarchaeotes	CU916960	99
TFA-13-SC	ENV	Crenarchaeota	Uncultured Crenarchaeotes	CU916960	99
WC-2-SC/OC	ENV	Euryarchaeota	Uncultured Euryarchaeote	AB189394	90
WC-2-SC/OC	ENV	Euryarchaeota	Uncultured Euryarchaeote	AB189394	90
WC-2-SC/OC	ENV	Euryarchaeota	Uncultured Euryarchaeote	AB189394	90
WC-2-SC/OC	ENV	Euryarchaeota	Uncultured Euryarchaeote	AB189394	90
WC-2-SC/OC	ENV	Euryarchaeota	Uncultured Euryarchaeote	AB189388	89
TFA-13-OC/NS	ENV	Crenarchaeota	Uncultured Crenarchaeotes	CU916960	98
TFA-13-OC/NS	ENV	Crenarchaeota	Uncultured Crenarchaeotes	CU916960	98

Table 4.4. Phylogenetic affiliation of bacterial DGGE bands to their closest match in GenBank. Identical matches within the same sample denote multiple bands with similar sequences.

Sample name	Type	Group	Closest Match	Accession No.	Similarity (%)
WC-1-SC	INC	Chloroflexi	Uncultured <i>Bellilinea</i> sp.	GU556335	96
WC-1-SC	INC	Bacteroidetes	Uncultured Bacteroidales	FJ888412	93
WC-1-SC	INC	Firmicutes	Uncultured Clostridia	GQ243111	97
WC-1-SC	INC	Betaproteobacteria	<i>Gallionella</i> sp.	FJ391503	97
WC-2-SC	ENV	Bacteroidetes	Uncultured Bacteroidetes	CU924080	96
WC-2-SC	ENV	Deltaproteobacteria	Uncultured <i>Geobacter</i> sp.	AB293264	95
WC-2-SC	INC	Betaproteobacteria	<i>Gallionella</i> sp	FJ391492	96
TFA-14-SC	ENV	Nitrospirae	Uncultured Nitrospirae	GU236094	98
TFA-14-SC	INC	Betaproteobacteria	Uncultured Gallionellaceae	EU266836	96
WC-2-SC/OC	ENV	Deltaproteobacteria	<i>Syntrophus</i> sp.	AJ133795	98
WC-2-SC/OC	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	CU924922	96
WC-2-SC/OC	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	FJ902368	94
WC-2-SC/OC	ENV	Firmicutes	Uncultured Clostridiales	DQ173815	88

Table 4.4. (Continued)

Sample name	Type	Group	Closest Match	Accession No.	Similarity (%)
WC-2-OC	ENV	Firmicutes	<i>Eubacterium</i> sp.	L04711	84
WC-2-OC	ENV	Deltaproteobacteria	<i>Syntrophus</i> sp.	AJ133795	89
WC-2-OC	ENV	Deltaproteobacteria	<i>Syntrophus</i> sp.	AJ133795	91
WC-2-OC	ENV	Deltaproteobacteria	<i>Syntrophus</i> sp.	AJ133795	97
WC-2-OC	ENV	Deltaproteobacteria	<i>Syntrophus</i> sp.	AJ133795	97
WC-2-OC	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	CU924922	95
WC-2-OC	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	FJ902368	94
WC-2-OC	INC	Deltaproteobacteria	Uncultured Deltaproteobacteria	FJ902368	94
WC-2-OC	INC	Acidobacteria	Uncultured <i>Acidobacterium</i> sp.	GQ302574	95
WC-2-OC	INC	Deltaproteobacteria	<i>Syntrophus</i> sp.	AJ133795	97
WC-2-OC	INC	Synergistetes	Uncultured Aminanaerobia	CU926236	93
WC-2-OC/NS	ENV	Deltaproteobacteria	Uncultured <i>Syntrophus</i> sp.	GU112190	93
WC-2-OC/NS	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	CU924922	95
WC-2-OC/NS	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	FJ902368	94
WC-2-OC/NS	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	CU924922	96
WC-2-OC/NS	ENV	Deltaproteobacteria	Uncultured Deltaproteobacteria	FJ902368	94
TFA-13-OC/NS	ENV	Spirochaetes	Uncultured Spirochaetaceae	EU073764	93
TFA-13-OC/NS	ENV	Deltaproteobacteria	<i>Syntrophus</i> sp.	AJ133795	86
WC-2-NS	INC	Bacteroidetes	Uncultured Bacteroidales	FJ888412	98
WC-2-NS	INC	Chloroflexi	Uncultured Chloroflexi	AB293389	92
WC-2-NS	INC	Chloroflexi	Uncultured Chloroflexi	AB293389	92
WC-2-NS	INC	Firmicutes	Uncultured <i>Pelospora</i> sp.	DQ173909	92
TFA-13-NS	ENV	Bacteroidetes	Bacteroidales	GU129114	98
TFA-13-NS	ENV	Spirochaetes	Uncultured Spirochaetaceae	EU073764	93
TFA-13-NS	ENV	Firmicutes	Uncultured Thermoanaerobacteriaceae	AY684086	91
TFA-14-NS	INC	Deltaproteobacteria	Uncultured <i>Syntrophus</i> sp.	GU112190	95

associated with iron cycling (*Geobacter* sp. and *Gallionella* sp.) were also present.

The largest number of the bacterial sequences (49%) recovered by DGGE were most closely affiliated with the group *Deltaproteobacteria*, which were present in every sampling location (Table 4.4). Of particular interest are the sequences similar (>97%) to *Syntrophus* sp. obtained from environmental core samples and laboratory incubations containing organophilic clay. *Syntrophus* sp. is associated with the degradation of long-chain fatty acids (stearate, C18:0; heptanoate, C17:0), long-chain hydrocarbon degradation, and benzoate degradation [100-102]. Based on the 16S rRNA gene sequence information acquired from DGGE, the sequences recovered from the organophilic clay are most closely related to a species that breaks down hexadecane under methanogenic conditions [101]. *Syntrophus* sp. is a type of syntrophic bacteria, a group that cooperates with methanogens to make the breakdown of complex organic molecules thermodynamically favorable under methanogenic conditions. Syntrophic bacteria rely heavily on methanogens to remove inhibitory levels of H₂ and acetate. In turn, methanogens depend on syntrophic organisms to break these compounds down to simpler molecules they can use (e.g., acetate, CO₂ and H₂) [102, 103]. This cooperation of organisms can be highly productive; in fact, in the presence of methanogens, the degradation of 1 g of lipids by syntrophic bacteria can produce up to a liter of methane [103].

The exact chemical formulation of the proprietary Aqua Technologies ET-1 is not publically available, but the company website indicates that it is synthesized from bentonite clay treated with a long-chain (12-18 C) quaternary amine [104]. Similarly, CETCO produces their organophilic clay, PM-199 Organoclay, using a quaternary amine

made from tallow—a compound extracted from animal fat and tissue consisting of long chain triglycerides [105]. Because syntrophic organisms can oxidize complex organic molecules, such as long-chain fatty acids and hydrocarbons, with high yields of methane, it is plausible that the observed anaerobic, highly methanogenic breakdown of the organic component of organophilic clay may proceed by this mechanism.

The purpose of this study was to take an integrated approach to understand ebullition observed at the McCormick and Baxter Superfund Site. The results demonstrate that the majority of the methane originates in the organophilic clay used as a reactive sediment cap, and suggest that a syntrophic consortium of microorganisms may be using organophilic clay as carbon source. The capacity of organophilic clay to sequester organic contaminants will change over time as the organic component is removed from the clay. This, in turn, affects the use of this material as a long-term remedial strategy in reduced, contaminated environments. If the extent of carbon loss observed in the laboratory incubations reported here are reflective of losses *in situ*, the effective lifespan of the sorptive capacity of organophilic clay may be on the order of years or tens of years, rather than the expected hundreds of years. Assessing the heterogeneity of carbon content that may exist within the organophilic clay cap in the field could provide a better picture as to whether organophilic clay degradation (i.e., carbon loss) is widespread throughout the capping material or is only occurring in localized areas. Also, the mechanism by which microorganisms access the organic molecules ionically bonded to the clay surface in organophilic clay is not known. The bacteria may directly attack the bound molecules or, perhaps the surfactants must first move into solution. It is possible that a point may be reached where the organic material can no longer be degraded and a

residual level of the quaternary ammonium cationic surfactant remains, extending the lifetime of the material. Another situation to investigate is that organophilic clay may initially sorb the contaminants but the subsequent breakdown of the organic component of the clay could result in a slow release of contaminants that, in turn, may or may not be degraded by resident microorganisms. In any case, future studies need to assess how microorganisms affect the capacity of organophilic clay to act as a long-term remedial cap, and appropriate modifications be made to improve this commercially available product.

CHAPTER 5

Conclusions and implications for further research

The work presented herein expands what is currently known about anaerobic microbial activity in PAH-contaminated groundwater and sediments, and in sediment capping materials. This research also provides environmental managers with site-specific information, allowing for better understanding and management of the McCormick and Baxter Superfund Site.

Naphthalene was degraded under anaerobic conditions in the upland contaminated aquifer, as well as within the adjacent river sediments. In fact, wherever naphthalene was present, the metabolites of anaerobic naphthalene degradation were detected. It is possible that PAHs other than naphthalene may also be degraded at the site, however, higher molecular weight PAHs have lower solubilities, possibly limiting biodegradation. Microcosm experiments using sediment or aquifer porous media would help define the parameters that are controlling natural attenuation at the McCormick and Baxter Superfund Site, as well as reveal the terminal electron accepting process that may control the extent of biodegradation, both spatially and quantitatively. The capacity of the native

microbial community to break down compounds containing more than two-fused benzene rings could be determined with the addition of other PAHs, such as phenanthrene or pyrene, to the microcosms.

An *in situ* rate of anaerobic naphthalene transformation was successfully quantified using the push-pull method. However, this push-pull experiment almost certainly underestimated the actual *in situ* rate of degradation. Labeling the parent compound with deuterium precluded our ability to measure naphthalene mineralization. Rather, we were confined to describing the *in situ* breakdown of naphthalene as the rate of naphthalene transformation to naphthoic acid. One alternative would be to use carbon isotopes as a method to measure naphthalene degradation. In this way, the mineralization of labeled naphthalene could be quantified by measuring the production of labeled carbon dioxide and possibly even labeled methane. However, this method also has its limitations, as not all degraded naphthalene would be converted solely to these compounds, and this rate, too, would be considered conservative. Moreover, the use of radioactive C¹⁴ and C¹³ in the field embodies additional respective setbacks, namely environmental red tape and cost.

Multiple lines of evidence indicate that the organophilic clay used as a reactive sediment cap at the McCormick and Baxter Superfund Site stimulates methanogenesis. This stimulus is likely caused by a syntrophic relationship of anaerobic microorganisms converting the organic component of the organophilic clay ultimately to methane. This process is not limited to a single brand of organophilic clay; a significant increase in methane production was observed in both of two brands tested. Ideally, the next phase of research would be to isolate the active microbial consortium from the organophilic clay

retrieved from the field and conduct similar experiments to those described here, but having only fresh, unused organophilic clay available to the microorganisms (i.e., no residual organophilic clay from the field). As discussed earlier, the ability of bacteria to access sorbed organic compounds is unclear. Future studies should include looking at the physical characteristics of organophilic clay, specifically equilibrium partitioning and sorption/desorption kinetics of the surfactant used to manufacture organophilic clay, thereby specifying the ratio of surfactant in solution compared to the sorbed fraction at some moment in time. Thus, the availability of the organic component to the microorganisms would be better understood. Another aspect to investigate is whether different types of surfactants could be used to make a less “biodegradable” organophilic clay, possibly extending the lifetime of this material.

Because the organophilic clay was used to cap areas with NAPL seepage in order to impede contamination of the overlying river water, studies concerning the ability of microbial communities to actively degrade PAHs and other organic contaminants sorbed to organophilic clay would be particularly interesting. Furthermore, considering the capacity of these syntrophic microorganisms to degrade long-chain hydrocarbons, organophilic clay could provide a habitat perfect for the selection and success of microorganisms capable of degrading organic contaminants under methanogenic conditions.

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