

## ABSTRACT

Title of Thesis: EFFECT OF TERMINAL ELECTRON  
ACCEPTING PROCESSES ON ACETATE  
THRESHOLDS IN CONTAMINATED  
SEDIMENTS

Gayle E. Davis, Master of Science, 2006

Directed By: Associate Professor, Eric A. Seagren  
Civil and Environmental Engineering  
Department  
Assistant Professor, Jennifer G. Becker  
Biological Resources Engineering Department

Demonstrating the success of *in situ* bioremediation is challenging, requiring multiple lines of evidence, e.g., “footprints” of microbially-mediated processes that transform contaminants, like metabolic intermediates. This project evaluated the hypothesis that characteristic threshold concentrations of acetate, a key intermediate under anaerobic conditions, occur in contaminant plume regions dominated by different terminal electron accepting processes (TEAPs). The evaluation included characterizing the initially predominant TEAP(s) in sediment-slurry microcosms, and then measuring acetate thresholds in the sediment-slurries when dominated by different TEAPs. Based on the characterization, the sediments were dominated by methanogenesis, consistent with field observations. Interestingly, in the threshold experiments, similar acetate thresholds were observed under methanogenic conditions and in chlorinated-aliphatic degrading microcosms, although thermodynamics predict lower thresholds under the latter conditions. Therefore, this study demonstrated some of the potential, as well as

complications, in using and interpreting acetate thresholds as an indicator of the dominant TEAP.

EFFECT OF TERMINAL ELECTRON ACCEPTING PROCESSES ON ACETATE  
THRESHOLDS IN CONTAMINATED SEDIMENTS

By

Gayle E. Davis

Thesis submitted to the Faculty of the Graduate School of the  
University of Maryland, College Park, in partial fulfillment  
of the requirements for the degree of  
Master of Science  
2006

Advisory Committee:  
Dr. Eric A. Seagren, Chair  
Dr. Jennifer G. Becker  
Dr. Alba Torrents

© Copyright by  
Gayle E. Davis  
2006

## ACKNOWLEDGEMENTS

This research was supported by a grant from the Maryland Water Resources Research Center. In addition to this support, the entire efforts of this project were in part made possible by the United States Army and to this organization I owe much gratitude for this significant and elite opportunity.

“A leader is a person who takes a little more than his share of the blame and a little less than his share of the credit” (author unknown). This research would not have been possible without the support of those around me. First, I express my deepest appreciation to Dr. Jennifer G. Becker and Dr. Eric A. Seagren as my co-advisors. Your patience, guidance and advice have motivated me to limits I never thought were possible in an academic environment. I appreciate your commitment to higher education, but most of all for sharing this passion with me. In addition, Dr. Alba Torrents, thank you for your guidance, and to the entire committee, thank you for meeting my timelines set forth by the U.S. Army.

My fellow lab mates; Emily Devillier, Deyang Huang, Yen-Jung “Sean” Lai, Supida Piwkhaw and Hong Yin, thank you for your assistance in this wonderful experience and for sharing your cultures (not the bug type) with me, it is this I cherish and will surely miss.

To my parents, I am grateful for your support. You have shown me how to be successful in all aspects of my life. I wish to thank my husband, Avery for pushing me. I had my doubts but you never did. I am grateful for your constant encouragement and for making me a better person.

## Table of Contents

ACKNOWLEDGEMENTS .....	ii
CHAPTER 1 Introduction .....	1
1.1 <i>In situ</i> Bioremediation .....	2
1.2 Evaluating <i>In situ</i> Bioremediation .....	2
1.3 Increasing Importance for Evaluating Bioremediation .....	4
CHAPTER 2 Scope of Study and Objectives .....	5
CHAPTER 3 Literature Review .....	8
3.1 Demonstrating <i>In situ</i> bioremediation .....	8
3.2 Microbial Footprints .....	9
3.2.1 Concept .....	9
3.2.2 Key processes used to monitor footprints .....	10
3.2.3 Summary .....	14
3.3 Terminal Electron Acceptors .....	14
3.4 Redox potential .....	17
3.5 Use of acetate as an indicator of shifts in predominant TEAPs .....	21
CHAPTER 4 Materials and Methods .....	25
4.1 Experimental Materials .....	25
4.1.1 Sediment and Groundwater Collection .....	25
4.1.2 Sediment Slurry and Microcosm Preparation .....	26
4.2 Experimental Design and Methods .....	29
4.2.1 Characterization Experiment .....	29
4.2.2 Contaminated Sediment Acetate Threshold Determination .....	32
4.3 Analytical Methods .....	38
4.3.1 Oxidized Electron Acceptors and Reduced Products .....	38
4.3.2 Global TEAP Indicators .....	44
4.3.3 Aggregate Measurements .....	48
CHAPTER 5 Results and Discussion .....	50
5.1 Characterization Experiment .....	50
5.1.1 Nitrate Reduction .....	50
5.1.2 Dehalorespiration .....	51
5.1.3 Iron Reduction .....	51
5.1.4 Sulfate Reduction and Methanogenesis .....	53
5.1.5 Acetate Concentrations .....	57
5.1.6 Hydrogen Concentrations .....	61
5.2 Methanogenic Acetate Threshold Experiment .....	63
5.3 Dehalorespiration Acetate Threshold Experiment .....	68

<b>CHAPTER 6 Summary and Conclusions</b> .....	73
<b>APPENDIX</b> .....	75
<b>Appendix A.1</b> .....	76
<b>Appendix A.2</b> .....	77
<b>Appendix B.1</b> .....	78
<b>Appendix B.2</b> .....	79
<b>REFERENCES</b> .....	80

## LIST OF FIGURES

Figure 2.1 Biodegradation pathways for complex organic compounds under anaerobic conditions (Rittmann and McCarty, 2001). .....	6
Figure 4.1. A cross section of the subsurface showing locations where groundwater and sediment were collected. Sediment samples were collected by hand 12-18 inches below the sediment surface at site WB23 and groundwater was collected using a peristaltic pump at pumping well, WB24. ....	27
Figure 5.1. Fe(II) (diamonds) and Fe(III) (circles) concentrations in the sediment characterization experiment. Data points represent the average of duplicate samples and the error bars represent $\pm$ one standard deviation: (a) T1 = no substrate controls, (b) T2 = acetate added, (c) T3 = molybdate added, (d) T4 = acetate and molybdate added. ....	54
Figure 5.2. Headspace methane levels in four microcosm treatments. T1= no substrate controls, T2 = acetate added, T3 = molybdate added, T4 = acetate and molybdate added. Sediment and groundwater were added to microcosms on day 0. Acetate (2mM) was added to the T2 and T4 microcosms on day 2, and molybdate (2mM) was added to the T3 and T4 microcosms on day 2. Data points represent the average of duplicate values. ....	56
Figure 5.3. Aqueous acetate results in the four characterization study microcosm: T1= no substrate controls, T2 = acetate added, T3 = molybdate added , T4 = acetate and molybdate added. (a) average concentrations in duplicate microcosms (days 1 through 23), and (b) average acetate concentrations measured on day 23. Acetate (2 mM) was added to the T2 and T4 microcosms on day 2, and molybdate (2 mM) was added to the T3 and T4 microcosms on day 2. Data points represents the average of duplicate values, except for the 2 mM values in T2 and T4 on day 2, which were assumed. Error bars represent $\pm$ one standard deviation.....	58
Figure 5.4. Concentrations in the T1 microcosms of methane and (a) acetate and (b) hydrogen. All data points represent the average of duplicate samples. ....	60
Figure 5.5. Acetate and methane concentrations in the T2 microcosms. Acetate (2mM) was added on day 2. Acetate and methane data points represent the average of duplicate samples. ....	61
Figure 5.6 Headspace hydrogen levels in the four characterization study microcosm treatments: T1 = controls, T2 = acetate, T3 = molybdate, T4 = acetate + molybdate. Acetate (2mM) was added to T2 and T4 on day 2 and molybdate (2mM) was added to T3 and T4 on day 2. Data represents the average of duplicate samples. ....	62



Figure 5.7. Acetate and methane data from the methanogenic acetate threshold experiments (a) Average acetate and methane levels for the duplicate reactors (R1 and R2). The acetate concentration on day 2 is an assumed value equal to the acetate spike of 2 mM. (b) Average acetate threshold concentrations for each threshold determined. Error bars represent  $\pm$  one standard deviation. .... 66

Figure 5.8. Methane and hydrogen levels in the methanogenic acetate threshold experiment. Data plotted is the average in duplicate reactors (R1 and R2) and error bars represent one standard deviation. .... 67

Figure 5.9. PCE removal and TCE production data for the Mix1 microcosms, in the presence of excess amounts of acetate (~10 fold). Data plotted is the average of duplicate samples and error bars represent one standard deviation. .... 70

## LIST OF TABLES

Table 3.1. Redox pairs in aerobic and anaerobic environments. (Baker and Herson, 1994) .....	15
Table 3.2. Hydrogen concentrations measured in sediments dominated by different TEAPs. ....	21
Table 3.3. Acetate concentrations under various TEAPs (Lovley and Phillips, 1987). ...	23
Table 4.1. Sediment/groundwater slurry treatments used in the characterization study for identifying dominant TEAPs. ....	30
Table 4.2. Treatments used in the first dehalorespiring condition experiment. Microcosms were prepared in duplicate. ....	36
Table 4.3. Key characteristics of the sediment slurry cultures prepared and maintained by Ms. Emily Devillier and used to monitor acetate thresholds under presumptive dehalorespiring conditions. ....	37
Table 4.4 GC operating conditions for analysis of methane and chlorinated VOCs. ....	41
Table 4.5. Gradient Elution used in HPLC method used to quantify AMP of duplicate values. ....	47
Table 5.1. Background levels measured on day 1 of the methanogenic threshold experiment. ....	64
Table 5.2. Average acetate, CH <sub>4</sub> , H <sub>2</sub> , PCE and TCE for the duplicate microcosms in samples taken on day 3 of the experiment. C1 are the controls, with the other microcosms amended on day 2 as follows; Ac-1 with 80 μM acetate, PCE1 with 40 μM PCE and Mix1 with both 80 μM acetate and 40 μM PCE. ....	70
Table 5.3. Acetate, H <sub>2</sub> and CH <sub>4</sub> levels measured in wetland sediments collected from site WB35 following the dechlorination of PCE and CT in the absence of any added electron donors. ....	71

## CHAPTER 1

### Introduction

Contaminated groundwater systems have become a nationwide problem. For example, a national assessment of 55 volatile organic compounds (VOCs) in groundwater by the United States Geological Survey found that 42 of the 55 VOCs were detected in one or more aquifer samples (Zogorski et al., 2006). Although the concentrations were generally low ( $<1 \mu\text{g/L}$ ), VOCs were detected in 90 of 98 aquifer studies, indicating their ubiquitous nature and the vulnerability of aquifers to low level contamination. Among the factors most commonly associated with the presence of VOCs in aquifers were gasoline storage release sites. A variety of technologies are available for remediation of ground water systems impacted by gasoline and other petroleum hydrocarbons. However, some conventional technologies such as pump-and-treat, activated carbon systems and soil venting can be expensive, consume large amounts of energy and do not destroy contaminants. Furthermore, untenable clean up timeframes and the geological site characteristics also limit remediation of contaminated sites by these technologies. These limitations demonstrate the need for further investigation into alternatives to current, conventional clean-up technologies (NRC, 1993). In particular, these limitations have continued to spur investigations into *in situ* bioremediation, i.e., the use of microbiological activity to destroy or immobilize contaminants in place. Both engineered *in situ* bioremediation and monitored natural attenuation or intrinsic bioremediation are becoming increasingly popular (NRC, 1993).

## **1.1 *In situ* Bioremediation**

*In situ* bioremediation, both engineered and intrinsic, offers great potential for environmental cleanup due to the vast array of contaminants that microbes can potentially destroy or transform (Rittmann et al., 1994). In many instances, the native bacteria can degrade the contaminants of concern via existing metabolic pathways, either as electron donors (e.g., petroleum hydrocarbons) or electron acceptors (e.g., highly halogenated solvents). As a result, *in situ* bioremediation technologies can often be less costly, and safer for the surrounding environment, than other, conventional clean up methods (NRC, 1993). Furthermore, and most importantly, *in situ* bioremediation typically uses the naturally occurring microorganisms to destroy or transform hazardous contaminants to less harmful forms, rather than transfer the contaminant to another phase.

Nevertheless, despite the great potential for microorganisms to destroy or transform contaminants, there are several factors that make engineered and intrinsic *in situ* bioremediation technically challenging. In particular, one key challenge in applying *in situ* bioremediation is how to evaluate the success of the process, i.e., are the contaminant concentrations declining and are the microbes responsible (NRC, 1993). Further, it must be determined whether the microorganisms are capable of reducing the contaminant concentrations at a rate that is faster than the migration of the contaminant plume, which depends on the biodegradability of the contaminant as well as the site's geological and chemical characteristics.

## **1.2 Evaluating *In situ* Bioremediation**

Evaluating the success of *in situ* bioremediation is made challenging by the heterogeneous, dynamic and inaccessible nature of the subsurface. Furthermore, there is

unfortunately no one piece of evidence that can unambiguously show that bioremediation has been successful, i.e., demonstrating that the microorganisms have or can clean up a contaminated site (NRC, 1993). In response to this challenge, the National Research Council's (NRC) Committee on *In situ* Bioremediation recommended an evaluation strategy based on multiple, independent converging lines of evidence be used to measure the success of *in situ* bioremediation. Specifically, it was recommended that such a general strategy include three types of data:

1. documented loss of contamination from the site,
2. laboratory assays showing that microorganisms at the site have the potential to transform the contaminants and,
3. one or multiple pieces of evidence showing that biodegradation potential is actually occurring at the field site.

These three, independent types of evidence, when analyzed together, increase the capability for evaluating whether or not an *in situ* bioremediation application at a given location is successful. Based on this guidance, several field protocols for evaluating the success of *in situ* bioremediation have been developed (e.g., (Wiedemeier et al., 1999)).

Of the three types of evidence suggested by the NRC (1993), the most critical and difficult to obtain is the third type, that linking *in situ* contaminant removal with microbial activity, which establishes a cause and effect relationship. Unfortunately, microbial mechanisms that occur within the sub-surface usually cannot be measured directly. However, bacterial metabolism of contaminants often consume or produce other materials that leave "footprints" of microbial processes that can be measured thereby providing evidence of biodegradation (NRC, 2000). Typical footprints useful for

demonstrating biodegradation at petroleum-contaminated sites include numbers of bacteria, rates of biological activity, carbon stable isotopes, metabolic byproducts, intermediary metabolites, growth-stimulating materials and the ratio of non-degradable to degradable compounds (Seagren and Becker, 2002).

### **1.3 Increasing Importance of Evaluating Bioremediation**

As bioremediation technologies advance, moving from the laboratory to the field, the importance of sound techniques for documenting the success of *in situ* bioremediation will also increase (NRC, 1993). Correspondingly, the NRC Committee on *In situ* Bioremediation recommended that further research be performed in the area of developing innovative site characterization techniques (NRC, 1993). Specifically, they noted that, “rapid, reliable, and inexpensive site characterization techniques would have a significant impact on the ease of evaluating bioremediation.” This remains a rich area for research today, as measurement and interpretation of the footprints of microbial metabolism using available techniques are often complicated and/or ambiguous (Becker et al., 2005). Therefore, this research focused on demonstrating the use of threshold acetate levels as a biodegradation footprint monitoring tool for evaluating the success of *in situ* bioremediation.

## CHAPTER 2

### Scope of Study and Objectives

One of the major challenges associated with the successful implementation of intrinsic or engineered *in situ* bioremediation is how to demonstrate that the contaminant(s) of concern is(are) being destroyed and that the contaminant removal is a result of microbiologically-mediated processes. As discussed further in Chapter 3, it is generally not easy to directly measure these microbial processes. However, these processes often leave “footprints” that can be monitored. For example, key intermediate products of microbial metabolism have been shown to provide useful indicators of the predominant microbially-mediated electron-accepting reactions. Under anaerobic conditions, hydrogen and organic acids, such as acetate, are key intermediates in the terminal electron accepting processes (TEAPs) of interest (Figure 2.1) (Lovley and Phillips, 1987b). Correspondingly, hydrogen concentrations have been proposed as a universal indicator of the predominant TEAPs within a given system. In fact, the use of hydrogen as a footprint for identifying the shift in dominant TEAPs of contaminated sites has been widely studied (Lovley et al., 1994c; Vroblesky et al., 1997; Lu et al., 2001). The results of these studies have demonstrated that characteristic ranges of H<sub>2</sub> concentrations exist for various TEAPs. Nevertheless, there are several difficulties associated with this approach. For example, in order to identify the aqueous threshold values of H<sub>2</sub> (nM) in a system, a highly sensitive reduction gas detector is required. As a result of such challenges, alternative techniques for providing an indicator of the

predominant TEAP continue to be explored. As shown in Figure 2.1, acetate and H<sub>2</sub> play a similar role in anaerobic metabolism.

Therefore, in the larger project of which this research is a part, it is hypothesized that different characteristic threshold acetate concentrations also occur in regions of contaminant plumes that are dominated by different TEAPs and could be a useful component of bioremediation monitoring programs as potential indicators of dominant TEAPs. Specifically, based on thermodynamic considerations outlined below, the characteristic acetate thresholds are expected to increase as the amount of energy released by electron acceptor reduction decreases.

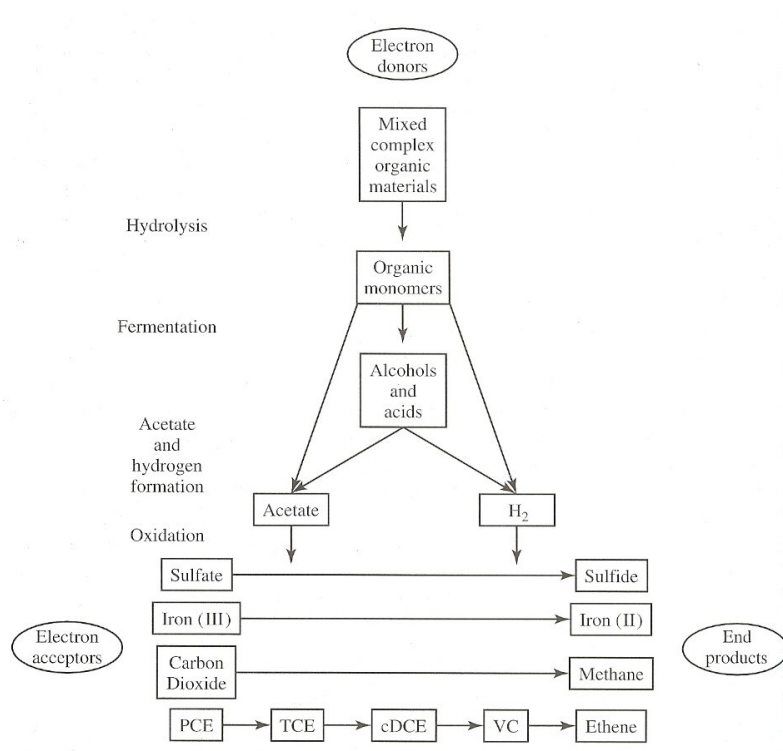


Figure 2.1 Biodegradation pathways for complex organic compounds under anaerobic conditions (Rittmann and McCarty, 2001).



The overall goal of this research was to examine this hypothesis by performing proof-of-concept experiments using environmental samples in order to demonstrate that acetate thresholds do occur and are influenced by the dominant TEAP. To accomplish this goal, this study had two specific objectives and corresponding experimental stages. First, an evaluation was performed to characterize the TEAPs that initially dominated within a selected contaminated sediment. Second, the acetate thresholds and appropriate indicators of TEAPs were measured in the contaminated sediment samples when dominated by different TEAPs.

The following chapters describe in detail this experimental study for evaluating the use of acetate thresholds as indicators of the dominant TEAPs in contaminated sediments. First, Chapter 3 provides a literature review of the key topics relevant to this study. Next, Chapter 4 describes in detail the experimental materials and methods used in conducting this research. Then, the experimental results are presented and discussed in Chapter 5. Finally, Chapter 6 provides a summary of the key findings and conclusions of this research.

## CHAPTER 3

### Literature Review

#### 3.1 Demonstrating *In situ* Bioremediation

Petroleum hydrocarbon contamination is an excellent candidate for cleanup by engineered *in situ* bioremediation, or intrinsic *in situ* bioremediation, given the widespread presence of hydrocarbon degraders in the environment (Rittmann et al., 1994). Nevertheless, one of the ingredients to widespread acceptance of *in situ* bioremediation has been a lack of an agreed upon approach for demonstrating that *in situ* bioremediation is occurring and that the rates of degradation are significant enough to limit the threat to human health and the environment (NRC, 1993).

Obviously, demonstrating loss of contaminants through biodegradation is by far the most critical observation for successful *in situ* bioremediation, but it is made challenging by three main factors. First, the complex nature of groundwater systems can affect the ability to measure concentrations. Second, contaminant concentrations may decrease in specific spatial locations but quite possibly could transform to another equally or more hazardous chemical form. Finally, reactions that initiate contaminant degradation may not be sustainable for the life of the contamination (NRC, 2000).

Because of the challenges associated with monitoring *in situ* bioremediation, most of the protocols developed for providing evidence of successful *in situ* bioremediation use a multiple lines of evidence approach, as described by the National Research Council (1993). Three types of evidence are required in such an approach, and the different protocols all use variation on this theme (Wiedemeier et al., 1999). The first type of

evidence is documenting the loss of contaminants from the site (Wiedemeier et al., 1996). The second type of evidence requires information from the literature or microbial assays from the site that can be used to determine if the site has the potential to transform the contaminants with the given conditions. Finally, the third necessary line of evidence is that which demonstrates that biodegradation is actually occurring in the field site location. The latter type of evidence is the most difficult to obtain and focus of this research.

Techniques for demonstrating that biodegradation is actually occurring in the field requires a thorough evaluation of the characteristics of the contaminated site's microbial population and chemistry. However, microbial mechanisms in contaminated sediments are not easily measured due to the heterogeneous, inaccessible, and dynamic nature of the subsurface. In addition, the simple cause-and-effect measurements of microbial processes that are easily obtained in laboratory experiments are difficult to duplicate at complex field sites.

## **3.2 Microbial Footprints**

### **3.2.1 Concept**

Although the mechanisms used to degrade contamination in the subsurface are not easily measured directly, these processes often leave evidence of degradation in the form of "footprints," such as changes in the concentrations of electron donors and electron acceptors that can be used as indicators of the effectiveness of *in situ* bioremediation (Becker et al., 2005). Thus the monitoring of the loss of contaminants coupled with such footprints helps establish the cause and effect relationship necessary for documenting bioremediation, although there may not always be a basic connection (NRC, 2000).

### **3.2.2 Key processes used to monitor footprints**

In the following paragraphs the key bioremediation footprints are briefly reviewed including: electron acceptors and/or their reduced products processes, inorganic carbon, alkalinity, metabolic intermediates, oxidation-reduction (redox) potential, microbial numbers and activity, carbon stable isotopes and finally the ratio of non-degradable to degradable substrates (NRC, 1993). Then, in the subsequent two sections the monitoring of terminal electron-accepting processes and redox potential are examined in more detail.

#### **3.2.2.1 Electron accepting processes**

The many bacteria present in the subsurface at petroleum hydrocarbon-contaminated sites, degrade organic contaminants by using them as electron donors, and in the process, consuming inorganic compounds like nitrate ( $\text{NO}_3^-$ ), ferric iron (Fe(III)), sulfate ( $\text{SO}_4^{2-}$ ), or carbon dioxide ( $\text{CO}_2$ ) as terminal electron acceptors. Thus, monitoring the loss of electron donor contaminants and correlating it with the consumption of electron acceptors yields evidence that bioremediation is occurring or has occurred. For example, Lovley (1997a) demonstrated that the decrease in Fe(III) concentrations and the increase of Fe(II) concentrations during petroleum hydrocarbon oxidation was evidence of contaminant loss through iron reduction.

#### **3.2.2.2 Inorganic carbon**

In microbially-mediated reduction processes for the mineralization of petroleum hydrocarbons, bacteria produce inorganic carbon, present as either a gas,  $\text{CO}_{2(g)}$ , or as dissolved  $\text{CO}_2$  or  $\text{HCO}_3^-$ . Therefore, increased levels of inorganic carbon can yield evidence of active biodegradation. However, as discussed further below, accurately measuring the dissolved inorganic carbon resulting from biodegradation is challenging

due to the carbonate buffering capacity of groundwater, which is usually measured as alkalinity (Seagren and Becker, 2002).

### **3.2.2.3 Alkalinity**

Alkalinity can also be a useful footprint mechanism, but some caution is required when interpreting alkalinity data, as previously mentioned. Dissolved CO<sub>2</sub> produced during microbially-mediated biodegradation is rapidly transformed to carbonic acid (H<sub>2</sub>CO<sub>3</sub>), which increases the acidity of the groundwater system. The carbonic acid may dissolve carbonate minerals if present, and thereby increase the alkalinity. However, addition of carbonic acid itself does not affect the alkalinity because alkalinity is a measurement of the proton deficiency with respect to the reference proton level, which is CO<sub>2</sub> (Stumm and Morgan, 1996). On the other hand, proton consumption under nitrate-, iron-, and sulfate-reducing conditions will result in an increase in total alkalinity that can be used as a footprint of bioremediation.

### **3.2.2.4 Metabolic intermediates**

Many microbially-mediated processes for the transformation of contaminants result in the formation of metabolites, which can potentially be used as footprints of the biodegradation process of interest. For example, hydrogen and organic acids are key intermediates in the anaerobic metabolism of organic compounds including petroleum hydrocarbons. Therefore, the formation of organic acids could conceivably provide a footprint of petroleum hydrocarbon biodegradation. In fact, at several petroleum hydrocarbon contaminated sites, various aliphatic and aromatic acids were detected in anoxic contaminated plumes (e.g., (Cozzarelli et al., 1990; Kampbell et al., 1996)), as discussed further below. However, using organic acid concentrations as a footprint of

viable degradation poses some challenges. For example, the fate of organic acids in the subsurface is often diverse and complex because many organic acids are both biologically and geochemically reactive, which may make it difficult to directly relate organic acid concentrations with microbial processes.

#### **3.2.2.5 Oxidation-Reduction (Redox) potential**

As reviewed further below, as organic contaminants in the subsurface are biodegraded, there is typically a sequential depletion of the most energetically-favorable electron acceptors. Correspondingly, the redox potential of a particular subsurface environment should theoretically decrease as biodegradation proceeds and provide an indicator of the electron accepting processes that occur within the subsurface. Thus, the fate and transport of contaminants can theoretically be predicted using redox potentials because redox processes affect the rate and extent of biodegradation of the organic compounds (Chapelle et al., 1996). The most common method for monitoring redox processes is the use of platinum electrode probe to measure the redox potential ( $E_h$ ) (Chapelle et al., 1996); however, as discussed below (section 3.4) such measurements are subject to several well known shortcomings. Thus, alternative methods for assessing redox potential in complex systems are needed. The potential usefulness of  $H_2$  and organic acid concentrations as tools for monitoring redox potentials is discussed in more detail in section 3.4.

#### **3.2.2.6 Microbial Activity and Numbers**

Successful bioremediation is highly dependant upon cell growth. Thus, an overall increase in biomass as a result of contaminant degradation can be used as a footprint of biodegradation. For example, petroleum hydrocarbon metabolism is often associated

with microbial growth as demonstrated by Salanitro (1993) who demonstrated that with respect to microbial numbers at a petroleum hydrocarbon contaminated site, found that the highest presence of hydrocarbon-degrading microorganisms were located close to the hydrocarbon source, possibly due to substrate enrichment.

### **3.2.2.7 Carbon stable isotopes**

As reviewed above, although changes in dissolved inorganic carbon or soil gas CO<sub>2</sub> can be an indicator of contaminant mineralization, interpretation of such changes can be made challenging due to abiotic processes such as dissolution of minerals or atmospheric CO<sub>2(g)</sub> (Seagren and Becker, 2002). Carbon stable isotopes can be a useful tool for interpreting such challenging dissolved organic carbon measurements.

Specifically, the correlation between contaminant degradation and the formation of inorganic carbon can be examined through the use of the <sup>13</sup>C:<sup>12</sup>C stable isotope ratio, which is expressed as δ<sup>13</sup>C. A positive change in δ<sup>13</sup>C represents an enrichment of <sup>13</sup>C compared to <sup>12</sup>C, and a negative change represents an enrichment of <sup>12</sup>C compared to <sup>13</sup>C. For example, inorganic carbon from carbonate minerals or the dissolution of atmospheric CO<sub>2</sub> contains more <sup>13</sup>C than carbon derived from most organic contaminants. Thus, if the inorganic C from the site has a more negative δ<sup>13</sup>C value than the inorganic carbon from mineral sources, then it is concluded that the C comes from biodegradation (NRC, 1993).

### **3.2.2.8 Ratio of degradable to non-degradable substrates**

Normally, contaminated sites contain a mixture of many contaminants. Some of these compounds may be completely resistant to biodegradation and thus can serve as biomarkers of the biodegradation of other compounds in the mixture. Thus, the ratio of biodegradable to non-biodegradable organic compounds should decrease with time as the

amount of biodegradable substances is transformed or mineralized (NRC, 1993) and could serve as an indicator of bioremediation. Ideal biomarker compounds are not only non-biodegradable, but also have similar physical/chemical properties to the degradable compound of interest.

### **3.2.3 Summary**

Clearly, there are potentially several footprints of microbial processes that could be used for assessing *in situ* bioremediation at a given site. However, as indicated above and reviewed by Seagren and Becker (2002), measuring footprints as quantitative indicators to establish the link between microbial activity and contaminant removal in bioremediation can be challenging, particularly for CO<sub>2</sub> and alkalinity. Thus, it is necessary to measure several footprints to establish a weight-of-evidence that can be used to demonstrate successful bioremediation (NRC, 2000). Of the microbial footprints reviewed above, one of the most important and commonly applied for petroleum contaminated plumes, and the focus of this research, is demonstrating that a shift occurs in the predominant TEAP of a contaminated environment. Therefore, indicators of the dominant TEAPs are reviewed in more detail in the next section.

### **3.3 Terminal Electron Acceptors**

Microbially-mediated electron-accepting processes play a major role in the bioremediation of petroleum hydrocarbon contaminated sites. Specifically, the petroleum hydrocarbons typically serve as an electron donor by bacteria native to a site. Generally, this hydrocarbon oxidation is initially coupled to the reduction of the available oxygen. This is because, if possible, these microbial communities typically utilize the most highly oxidized electron acceptor, which yields the most energy. A variety of terminal electron



acceptors used in respiring processes is listed in reverse order of energy yield in Table 3.1. Typically, the mass of electron donors supplied by the spilled petroleum hydrocarbons greatly exceeds the oxygen available to be used as an electron acceptor, and the aerobic bacteria quickly deplete the available oxygen, creating anaerobic conditions. Theoretically, in environments where oxygen has been depleted, the remaining available electron acceptors are depleted by anaerobic hydrocarbon-oxidizing bacteria in the order of decreasing free energy change for the electron acceptor half reactions. In the process of anaerobic respiration process, inorganic compounds like nitrate ( $\text{NO}_3^-$ ), ferric iron (Fe(III)), sulfate ( $\text{SO}_4^{2-}$ ), and carbon dioxide ( $\text{CO}_2$ ) become the electron acceptors. Important byproducts of these anaerobic respiratory processes are nitrogen gas or ammonium, reduced forms of metals, hydrogen sulfide, and methane, respectively (NRC, 1993).

Table 3.1. Redox pairs in aerobic and anaerobic environments. (Baker and Herson, 1994)

<b>Redox Pair</b>	<b>pE</b>	<b><math>E_h</math> (volts)</b>	<b>Microbial Process</b>	<b>Soil Type</b>
$\text{CO}_2/\text{CH}_4$	-7	-0.42	Methanogenesis	Highly reduced
$\text{CO}_2/\text{acetate}$	-5	-0.28	Homoacetogenesis	Highly reduced
$\text{SO}_4^{2-}/\text{H}_2\text{S}$	-4	-0.22	Sulfate Reduction	Highly reduced Moderately reduced
$\text{NO}_3^-/\text{NO}_2^-$	7.1	0.42	Nitrate Reduction	Anoxic/oxidized
$\text{NO}_3^-/\text{N}_2$	12	0.74	Denitrification	Anoxic/oxidized
$\text{Fe}^{3+}/\text{Fe}^{2+}$	13	0.76	Iron Reduction	Anoxic/oxidized
$\text{O}_2/\text{H}_2\text{O}$	14	0.82	Aerobic Respiration	Oxidized

As a result of these trends, it is generally expected that shifts in the predominant TEAP will occur over time and distance from the source zone, as biodegradation proceeds in petroleum hydrocarbon contaminated plumes and consumes the most preferential TEA. Thus, demonstrating a shift from aerobic to anaerobic TEAPs is key to demonstrating that hydrocarbon contaminant biodegradation is occurring. In addition, knowing the predominant TEAP is also helpful for predicting the potential biotransformations that may occur.

Ground water systems can be monitored to determine the predominant TEAPs within each system by measuring removal of the oxidized form of the electron acceptor or the production of the reduced form of the electron acceptor (Chapelle et al., 1996; McGuire et al., 2000; Smith, 2002). Lovley et al. (1994c) noted that there are several ways to monitor TEA loss and the accumulation of by-products that can aid in properly determining TEAP spatial zones. For example, the loss of Fe(III) and the lack of sulfide accumulation or methanogenesis can reasonably reveal that a site is dominated by iron reducers that out compete sulfate-reducers and methanogens for electron donors.

Nevertheless, obtaining such evidence of electron acceptor consumption can be challenging for subsurface samples, especially when the entire suite of potential oxidized and reduced forms of electron acceptors is considered. For example, difficulties arise because monitoring the presence or absence of end products may or may not identify all microbiological reactions that are occurring. Redox reactions and their products are a function of one-dimensional analysis and do not always clearly define the entire system (McGuire et al., 2000). For instance, sulfate reducing zones may not be easily definable by monitoring the decrease in sulfate concentration and/or production of sulfide. Sulfate

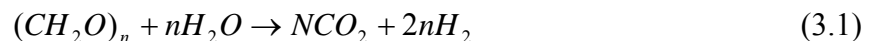
is replenished from existing mineral sources (Plummer et al., 1990) and likewise dissolved sulfides are easily precipitated in the presence of metals; therefore, sulfide is not a quantifiable indicator of sulfate reduction (Chapelle et al., 1995). Another complication is that TEAPs occur on extremely small spatial scales at the microbial level in comparison with the regional scale of contamination. Thus, measurement of these processes is a challenging but necessary task in documenting *in situ* bioremediation.

### **3.4 Redox potential**

An alternative approach for demonstrating a shift in TEAPs, instead of monitoring removal of the oxidized form of the electron acceptor or the reduced product, is to monitor for shifts in the redox potential. The classic technique for monitoring a redox potential is using platinum electrode measurements (Table 3.1). However, such measurements in environmental samples have been widely questioned for their reliability because of the redox disequilibrium (Keating and Bahr, 1998) and also due to the overlapping redox potentials for varying reactions (Lovley and Goodwin, 1988). For example, platinum electrode measurements require the redox pairs to be in equilibrium, but natural aquatic environments are seldom in total equilibrium. One main reason for the lack of equilibrium is how the speciation or complexation of metals affects the redox potential. Most importantly, the complexing of iron is extremely prevalent in natural aquatic environments, and the redox potential for iron decreases in the presence of complex formers or chelates such as citrate and silicate. These chelators are known to form stronger bonds with Fe(III) than Fe(II) (Stumm and Morgan, 1996). Nevertheless, despite the shortcomings of this method, it continues to be widely used, simply because these measurements are easily obtained in a field environment.

As noted above, one potential alternative to using a platinum electrode is to use H<sub>2</sub> and organic acid concentrations as indicators of the redox potential. Competitive interactions between different electron accepting processes have been modeled for both H<sub>2</sub>-consuming and acetotrophic environments as a means for defining site characteristics and degradation potential (Lovley, 1985; Lovley and Goodwin, 1988; Conrad, 1996; He and Sanford, 2004). In particular, aqueous hydrogen concentrations may be a microbially-controlled, nonequilibrium alternative to the master variable pe as an indicator of the redox reactions that take place in contaminated sediments (Lovley et al., 1994c). In fact, measuring H<sub>2</sub> has proven to be a successful approach for monitoring the redox potential (Chapelle et al., 1995; McGuire et al., 2000). H<sub>2</sub> is applicable to a range of redox reactions and is dynamic enough to be applied across an entire system (Lovley and Goodwin, 1988).

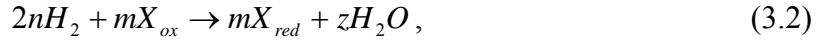
The rationale for this approach can be explained as follows. Organic molecules, through the process of sequential hydrolysis and fermentation produce H<sub>2</sub>, acetate, and CO<sub>2</sub>; therefore, H<sub>2</sub> and acetate are known to be key intermediates in organic mineralization processes (Novelli et al., 1988). During anaerobic metabolism under electron acceptor-depleted conditions, fermentation of organic matter produces hydrogen H<sub>2</sub>:



where (CH<sub>2</sub>O)<sub>n</sub> is a carbohydrate-like organic compound (Hoehler et al., 1998).

In contaminated plumes and other complex environments, as H<sub>2</sub> is produced, it is quickly consumed by and limits, respiratory microorganisms as an electron donor for

various TEAPs, e.g. using compounds like nitrate, sulfate and carbon dioxide as terminal electron acceptors:



where  $X_{ox}$  and  $X_{red}$  are the oxidized and reduced forms of the inorganic electron acceptor, e.g.  $SO_4^{2-}$  and  $H_2S$ , or Fe(III) and Fe(II) (Hoehler et al., 1998). The coupling of anaerobic fermentation and respiration processes via  $H_2$  was first termed interspecies hydrogen transfer by Iannotti and Kafkewit (1973).

Lovley and Goodwin (1988) showed that with pure cultures of hydrogen-consuming organisms, for each electron acceptor, there existed a hydrogen threshold concentration below which hydrogen could no longer be utilized by the cells. Based on these results, they proposed that dissolved  $H_2$  concentrations could be used as a universal indicator of the predominant TEAP in sediment systems. They used the concept of  $S_{min}$  to explain the relationship between  $H_2$  and dominant TEAPs in anaerobic systems.

Based on Monod kinetics,  $S_{min}$  can be defined as the substrate concentration below which steady-state biomass cannot be supported. Thus, for suspended growth microbial cultures, this concentration,  $S_{min}$ , represents steady-state threshold below which biomass washout occurs and for a steady-state biofilm it represents the steady-state threshold concentrations below which net biomass decay occurs.  $S_{min}$  can be calculated as:

$$S_{min} = \frac{K_s b}{Yq_{max} - b} \quad (3.3)$$

where  $K_s$  is the half saturation constant describing the affinity of cells for the substrate (S),  $b$  is the cell decay coefficient,  $q_{max}$  is the maximum specific substrate utilization rate, and  $Y$  is the cell yield coefficient (Rittmann et al., 1994; Becker et al., 2005). Based on

equation 3.3,  $S_{\min}$  is a function of  $Y$ , which is controlled by the thermodynamics and stoichiometry of the electron donor, electron acceptor, and synthesis half reactions (McCarty, 1972), and kinetic factors such as  $K_s$ . Thus, according to equation 3.3, assuming  $H_2$  is the limiting substrate, and  $K_s$  does not vary significantly among the TEAPs, as the predominant TEAP becomes less energetically favorable (i.e.  $\Delta G^\circ$  decreases), the yield is reduced and  $S_{\min}$  threshold will increase.

It has been well documented that hydrogen threshold concentration levels do tend to be inversely related to changes in the Gibbs free energy ( $\Delta G^\circ$ ) of the TEAPs (Table 3.2). That is, in the order of decreasing available free energy, microorganisms uptake decreasing amounts of  $H_2$  (Vroblesky et al., 1997). Thus hydrogen threshold concentrations decrease in order from least to most energetically favorable TEAP: acetogenesis > methanogenesis > sulfate reduction (sulfidogenesis) > Fe(III) reduction > Mn(IV) reduction > denitrification (Löffler et al., 1999). For instance, because nitrate is the most efficient metabolic process listed above, microbes in the denitrification zone will be able to maintain  $H_2$  concentration levels at a threshold well below the rest of the other processes. Similarly, if Fe(III) is available in a system, then hydrogen concentrations will be maintained at a concentration below the thresholds at which sulfate reduction or methanogenesis are not possible (Chapelle et al., 1995).

As a result of such trends, dissolved hydrogen is often described as a potential bioremediation monitoring tool as potential indicators of dominant TEAPs. Nevertheless, there are some shortcomings associated with using dissolved  $H_2$ -levels in this way. One, highly sensitive and specialize reduction gas detectors are required to quantify  $H_2$  at low (nM) concentrations representative of these thresholds. Two,  $H_2$  threshold concentrations

can be affected by a variety of non-redox factors, such as solute concentrations and temperature (Postma et al., 1996).

Table 3.2. Hydrogen concentrations measured in sediments dominated by different TEAPs.

<b>Aqueous Hydrogen Concentration Study</b>			
<b>Sediment TEAP</b>	<b>Lovley and Goodwin, 1988</b>	<b>Vroblesky et al, 1997</b>	<b>Lu, Tao et al., 2001</b>
<b>Methanogenic</b>	Range 7-10 nM	Range 5-25 nM	Range 5-30 nM
<b>Sulfate- Reducing</b>	Range 1-1.5 nM	Range 1-4 nM	Range 1-4 nM
<b>Fe(III)- Reducing</b>	0.2 nM	Range 0.2-0.8 nM	Range 0.2-0.8 nM
<b>Mn(IV)- and/or Nitrate- Reducing</b>	<0.05 nM	not studied	<0.1 nM

### 3.5 Use of acetate as an indicator of shifts in predominant TEAPs

Based on Figure 2.1, it is clear that acetate similar to H<sub>2</sub> is a key intermediate substrate in anaerobic environments. Specifically, in the presence of excess electron donors, organic compound fermentation produces both H<sub>2</sub> and acetate. The products of fermentation serve as electron donors for inorganic TEAP(s). Acetate plays a key role as a primary substrate for terminal electron accepting processes for nitrate reducing, sulfate reducing, iron reducing and methanogenic bacteria (Shaw and McIntosh, 1990). Other low molecular weight carboxylic acids studied have been determined to have a minor role in comparison with acetate (Reeburgh, 1983). Therefore, in a contaminant plume, acetate behavior could be conceptually modeled as follows (Becker et al., 2005): (1) near the contaminant source, the respiratory terminal electron acceptors are depleted, and fermentation of hydrocarbons leads to accumulation of aromatic and aliphatic acids such

as acetate; (2) moving down gradient of the hydrocarbon source zone, the demand for TEAs decreases as does the concentration of acetate is used as an electron donor in a series of sequentially more favorable TEAPs. Using the  $S_{\min}$  concept in equation 3.3, the acetate threshold is expected to increase as the free energy of each TEAP decreases, or more importantly, the dominant TEAP becomes less favorable and the cell yield is reduced (Becker et al., 2005).

Consistent with the conceptual model outlined above, several laboratory studies, as well as field studies of sites with both contaminated and uncontaminated aquifers have shown positive correlations between acetate concentrations and the dominant TEAPs in sediment. The potential for a relationship between the thermodynamics of the electron acceptor half reaction and the acetate concentration in anaerobic sediments was probably first demonstrated in the laboratory by Lovley and Phillips (1987b). By measuring acetate concentrations in sediments in which  $\text{CO}_2^-$ ,  $\text{SO}_4^{2-}$ , or Fe(III)- reduction was the predominant TEAP, they documented that the acetate concentration in the sediment increased as the redox potential of the terminal electron acceptor half reaction decreased (Lovley and Phillips, 1987b) (Table 3.3). More recently, He and Sanford (2004), in studies conducted with the iron- and 2-chlorophenol respiring isolate *Anaeromyxobacter dehalogens* strain 2CP-C, acetate thresholds of 69 ( $\pm 4$ ), 19( $\pm 8$ ) and  $<1$  nM were measured during respiration of 2-chlorophenol, amorphous Fe(III)-reduction and Fe(III)-citrate reduction, respectively. He and Sanford (2004) suggested that 2-chlorophenol cells need to exclude toxic substances from within the cell. Thus, more energy is required to maintain a low concentration gradient of toxic by-products like 2-chlorophenol and this might explain why the acetate threshold is higher under 2-



chlorophenol respiring conditions compared to iron-reducing conditions. In comparison, Jetten et al. (1992) measured acetate threshold concentrations for *Methanothrix* and *Methanosarcina* of 70  $\mu\text{M}$  and 1.2 mM, respectively.

Table 3.3. Acetate concentrations under various TEAPs (Lovley and Phillips, 1987).

<b>Sediment Type</b>	<b>Redox Couple</b>	<b><math>E^{\circ}</math> (V)</b>	<b>Acetate (<math>\mu\text{M}</math>)</b>
Fe(III)- Reducing	Fe(III)/Fe(II)	+0.77	0.5 $\pm$ 0.1
Sulfate- Reducing	SO <sub>4</sub> <sup>2-</sup> /HS <sup>-</sup>	-0.217	2.2 $\pm$ 0.2
CO <sub>2</sub> -Reducing	CO <sub>2</sub> /CH <sub>4</sub>	-0.24	5.2 $\pm$ 0.8

The concentrations of acetate in two uncontaminated locations of the Middendorf aquifer (South Carolina) were studied by Chapelle and Lovley (1992). They found that in a zone of the aquifer with a low concentration of Fe(III) that was dominated by SO<sub>4</sub><sup>2-</sup> - reduction, the acetate concentrations were higher than in a high Fe(III) concentration zone of the aquifer. In another uncontaminated field site study, Barcelona (1980) examined volatile fatty acid concentrations in coastal sediments and found that aliphatic acid concentrations (principally formate, acetate, and butyrate) in anoxic sulfate reducing zones contained up to 5 times higher than in oxic zones within the study site.

Data from petroleum contaminated sites is of particular interest to this research and the available data also support the trend of increasing organic acid concentrations as the terminal electron acceptor becomes more reduced. For example, Cozzarelli et al. (1990) evaluated organic acid concentrations from the Bemidji, MN site contaminated by crude-oil. They found that organic acid concentrations in the methanogenic to iron-

reducing zone (DO = 0 mg/L) near the oil lens were higher than in the down gradient transitional (DO < 1.0 mg/L) and aerobic zones. Near the oil source, acetate was the most prevalent aliphatic acid, although it disappeared rapidly in the anoxic zone. No organic acids were detected in the oxygenated water outside the plume.

Cozzarelli et al. (1995) concluded, based on a study at the gasoline-impacted Galloway, NJ site, that understanding the biogeochemical fate of organic acids is essential to predicting the evolution of degradable organic compounds in contaminated aquifers. Low molecular weight organic acids pools within that study aquifer changed composition and concentration over time as the available electron acceptors changed. For example, in well VW9 at the Galloway site, which was a predominantly Fe(III)- to sulfate-reducing location, the ratio of acetate to benzoate concentrations was significantly lower compared to the ratio of these organic acids observed at well locations that were predominantly methanogenic: well 421 at the Bemidji site (Cozzarelli et al., 1990), and well 3 at a creosote contaminated site in Pensacola, FL (Cozzarelli et al., 1994).

## **CHAPTER 4**

### **MATERIALS AND METHODS**

The following chapter outlines in detail the materials and analytical methods used during the characterization and threshold experiments. First, the source of sediment and groundwater used in this research site and other materials used for the experiments are described. This is followed by a description of the design of the characterization and threshold experiments. Lastly, the analytical methods used during the project are described.

#### **4.1 Experimental Materials**

##### **4.1.1 Sediment and Groundwater Collection**

Sediment samples were collected on January 9, 2006 from the riparian perimeter of the estuarine West Branch Creek, at site WB23, of the Aberdeen Proving Grounds, Edgewood Area, Aberdeen, Maryland (Figure 4.1) with the assistance of Mr. Masten Mount, of the United States Geological Survey. The grab samples were collected by hand, 12-18 inches below the sediment surface, during a period of low tide and subsequently placed into canning jars without headspace. Afterwards, the sediment samples were stored at 19°C, the average site groundwater temperature (Lorah et al., 1997), until used.

Groundwater was collected January 8, 2006 by pumping a well at site WB24 of the aforementioned area using a screened, peristaltic pump. As it was pumped, the groundwater was collected into a clean, 5-gallon bucket, which was then capped and transported back to the laboratory. Within 48 hours of groundwater collection, the 5-

gallon bucket was placed into an anaerobic glove bag under N<sub>2</sub> and transferred to canning jars that were sealed and stored without a headspace at 19°C until used.

#### **4.1.2 Sediment Slurry and Microcosm Preparation**

The experiments in this study were performed using a 10% (by volume) sediment/groundwater slurry batch microcosm reactors. The batch reactors used included 72-ml serum bottles (Wheaton) used in the characterization study, as well as 160 ml bottles (Wheaton) and 2-l reactor bottles used in the acetate threshold experiments. The 2-l bottles were specifically designed with a sampling port for slurry or headspace sampling (Becker, 1998).

In all cases, the same sediment slurry preparation protocol was followed. First, before initiating the slurry preparation, all of the equipment described below was washed with Alconox Detergent, triple rinsed with deionized water, allowed to air-dry, and then placed inside an anaerobic glovebox (Coy Laboratories, Model A). The glassware (72-ml and 160-ml) used for the sediment slurry microcosms were cleaned and baked at 380 °C for three hours. The 2-l reactor bottles were cleaned as mentioned above, openings covered with aluminum foil, and autoclaved (MarketForge Sterilmatic) at 250°C for 40 minutes. The butyl rubber septa (Geo-Microbial Technologies, Inc., No. 1313) were cleaned, triple rinsed with deionized water, wrapped in aluminum foil and autoclaved at 250°C for 20 minutes. Subsequently, a sufficient volume of previously collected groundwater was placed in the anaerobic chamber under H<sub>2</sub> and allowed to equilibrate with the anaerobic atmosphere for 24 hours to aid in deoxygenation of the groundwater. The site sediment was removed from storage and placed in the anaerobic chamber, immediately before preparing the slurries.

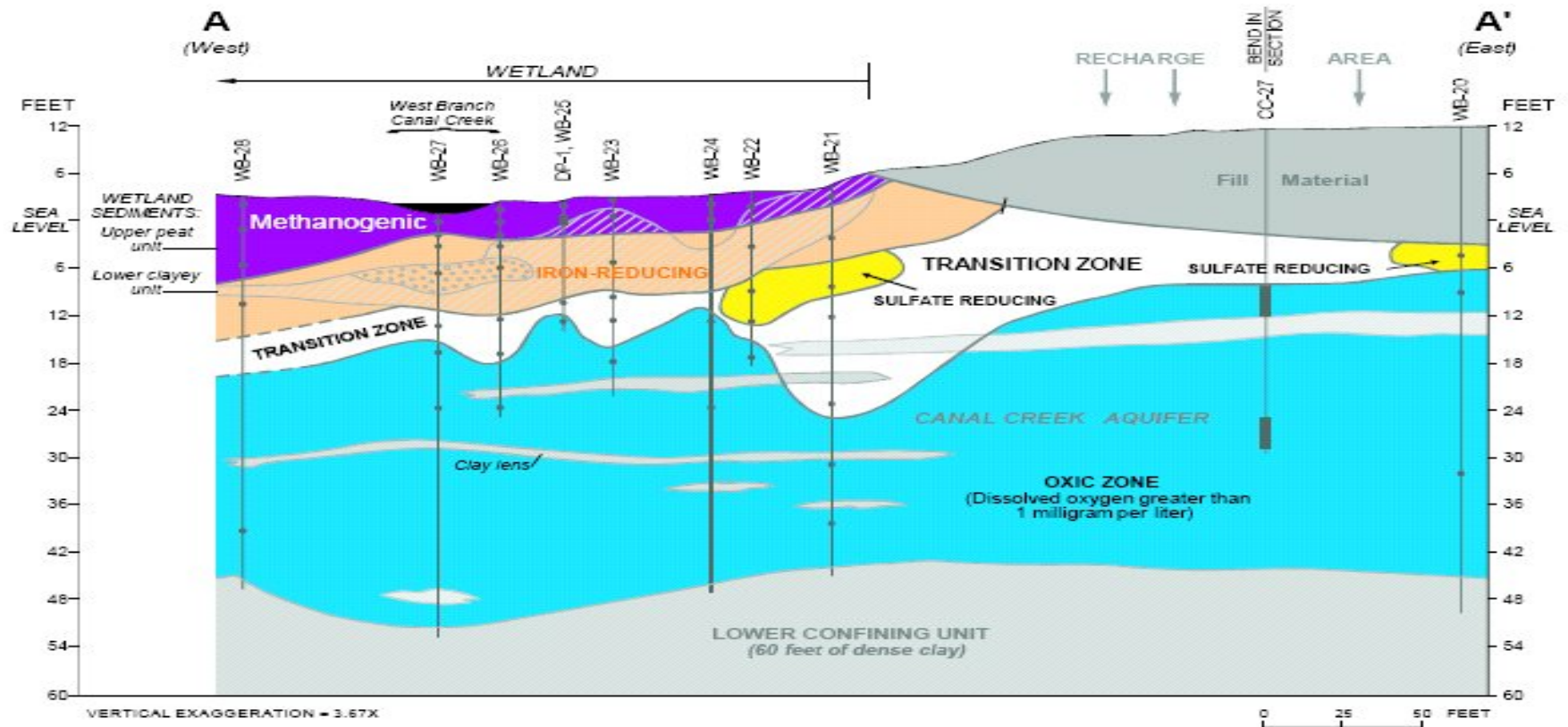


Figure 4.1. A cross section of the subsurface showing locations where groundwater and sediment were collected. Sediment samples were collected by hand 12-18 inches below the sediment surface at site WB23 and groundwater was collected using a peristaltic pump at pumping well, WB24.

All subsequent steps in the slurry preparation were performed in the anaerobic chamber and followed the same basic procedure. For example, in setting up the 72-ml serum bottles for the characterization study, a 500 ml slurry was created by placing approximately 50 ml of sediment in a 50-ml beaker and approximately 450 ml of groundwater in a 500-ml graduated cylinder. Half of the groundwater was then poured into a 1000-ml beaker and the sediment was transferred from the 50-ml beaker into the 1000-ml beaker with the groundwater. The remaining groundwater was used to rinse the 50-ml sediment beaker to ensure that an approximate 10% by volume slurry was obtained in each batch. This mixture was then passed through a sieve (U.S. Standard Series Sieve, No. 3, W.S. Tyler Company) with a 4.76 mm opening into a second 1000-ml beaker to remove large rocks and roots.

Sediment slurry microcosms were prepared either without headspace or with headspace. In both cases, before filling each serum bottle, the slurry mixture was stirred to avoid sediment settling and uneven solids distribution in the serum bottles. The no-headspace slurries were used during the characterization study. In that case, the 72-ml serum bottles were filled completely with sediment slurry and sealed while inserting a venting needle through the butyl rubber septa to allow excess liquid to escape when capping the serum bottles. At the same time, 72-ml headspace slurries were set up for use in the characterization study by adding 50 ml of the sediment slurry mixture to each serum bottle which were capped with butyl rubber septa and crimp caps. The same procedure was used for the 160-ml and 2-l headspace bottles used in the threshold experiments, except that 125 ml and 1250 ml, respectively, of sediment slurry were used to maintain the same ratio of headspace slurry in the 72-ml headspace bottles. Once set

up, all sediment slurry bottles were incubated at 19°C without shaking until used in the characterization and threshold experiments described below.

## **4.2 Experimental Design and Methods**

### **4.2.1 Characterization Experiment**

A preliminary, characterization study was performed prior to the acetate threshold experiments to determine the dominant TEAP(s) occurring within the source sediment and groundwater. Specifically, the characterization study was designed to systematically test for aerobic respiration, PCE-reduction, nitrate-reduction, Fe(III)-reduction, sulfate-reduction and methanogenesis. In addition, by monitoring acetate in the characterization study, initial information could be obtained on the acetate levels associated with the dominant TEAPs.

The first planned step in the systematic characterization was to measure the *in situ* DO levels at the site. If groundwater DO is greater than 50 µM, it can be presumed that the groundwater is dominated by aerobic processes (Smith, 2002). A number of complications prevented the DO measurement from being made, but based on historical data at the site (Lorah et al., 1997), which have shown that the wetland groundwater has not contained measurable amounts of DO in recent years, it was assumed that the site sediment and groundwater samples were not aerobic.

The characterization of the remaining TEAPs of interest in the sediment slurry was based on: (1) measurement of oxidized electron acceptors (e.g., Fe(III), NO<sub>3</sub><sup>-</sup>, tetrachloroethene (PCE)) and/or their reduced products (e.g., Fe(II), and dichloroethene (DCE)), (2) measurement of global indicators of TEAPs (i.e., H<sub>2</sub> and acetate), and (3) by evaluating the effects of the addition of acetate or molybdate on methane production and

threshold H<sub>2</sub> and acetate concentrations. These measurements and comparisons were made in sediment slurry serum bottles that were treated in one of four ways (Table 4.1).

Table 4.1. Sediment/groundwater slurry treatments used in the characterization study for identifying dominant TEAPs.

	<b>Treatment</b>	<b>Purpose</b>
<b>T1</b>	Control	To determine natural processes without the addition of electron donors or electron acceptors
<b>T2</b>	Spike with 2 mM Sodium Acetate	Acetate functions as an electron donor
<b>T3</b>	Spike with 2 mM Sodium Molybdate	Molybdate functions as an inhibitor of sulfate reduction
<b>T4</b>	Spike with both 2 mM Sodium Acetate and 2 mM Sodium Molybdate	Acetate functions as an electron donor and molybdate as a sulfate reduction inhibitor

As explained further below, the four treatments were designed following the general strategy of Lovley et al. (1994c). Treatment 1 (T1) microcosms received no additions and represented the background controls for the characterization experiment. Treatment 2 (T2) microcosms received a one-time addition of 2 mM (nominal concentration) of sodium acetate trihydrate (Fisher-Scientific, 99.8%). Treatment 3 (T3) microcosms received a one-time addition of 2 mM of sodium molybdate dihydrate, (Sigma-Aldrich, 99%) to inhibit sulfate reduction (Patidar and Tare, 2004) and treatment 4 (T4) microcosms received a one-time addition of both 2 mM sodium acetate trihydrate and 2 mM (nominal concentration) of sodium molybdate dihydrate. The amount of acetate



added to the T2 and T4 microcosms was (1) within the range of acetate concentrations measured in similar environmental samples (0.5-5 mM) (Lovley et al., 1994c), and (2) if exceeded the background acetate concentration measured on day 1 of the characterization experiment as recommended by Jetten et al. (1992).

Each treatment was set up using 72-ml serum bottles with two headspace serum bottles containing 50 ml of sediment slurry, and twelve non-headspace bottles containing 72-ml of sediment slurry. The headspace bottles were used for repeated monitoring of PCE, DCE, methane, and hydrogen levels (using 0.5 ml headspace samples for each) as well as the total headspace pressure in triplicate bottles for the duration of the experiment. The non-headspace bottles, which were sacrificed in duplicate at each sampling interval, were used to monitor for Fe(II) and Fe(III) in the slurry and aqueous nitrate and acetate levels.

In addition to monitoring the oxidized electron acceptors and/or their reduced products, as well as the global TEAP indicators, as described above, the four treatments were also designed so that they could be used to evaluate the importance of sulfate-reduction, methanogenesis and Fe(III) reduction (Lovley et al., 1994c). First, if methane was produced in the T1 and T2 microcosms, then the sediments were methanogenic. Two, if the methane production was higher in the T4 microcosms than in the T2 microcosms, and higher in the T3 microcosms than in the T1 microcosms, then sulfate-reduction was inhibited in the T4 and T3 microcosms and may have also been important. Thus, if acetate was removed in the T2 and T4 microcosms with no methane production and there was no indicator of sulfate reduction, then Fe(III) reduction was also important.

The characterization experiment was conducted over a 23 day period. All no headspace microcosms were sacrificed in duplicate at regular sampling intervals. On day 1, the first pairs of sacrificed no-headspace bottles were analyzed to obtain the concentrations for acetate,  $\text{NO}_3^-$ , Fe(II), Fe(III), and the headspace bottles were analyzed to obtain PCE, DCE,  $\text{CH}_4$  and  $\text{H}_2$  for all of the treatments. Then, on day 2, T2, T3, and T4 treatments received the chemical additions summarized in Table 4.1. The headspace bottles were next sampled on day 6 and on every 4<sup>th</sup> day thereafter. The no headspace bottles were next sampled on day 7, and then on subsequent 4 day intervals. The non-headspace bottles were sacrificed and sampled until day 23; however, the headspace bottle monitoring continued an additional 26 days with sampling every 7 days, to confirm the final methane and hydrogen threshold levels. The duration of the entire headspace experiment was 49 days.

## **4.2.2 Contaminated Sediment Acetate Threshold Determination**

### **4.2.2.1 TEAP 1: Methanogenic Acetate Threshold Design**

The goal of this experiment was designed to obtain the acetate threshold values for the sediment slurry under methanogenic conditions. This TEAP was selected for threshold analysis first because, as discussed in Chapter 5, the characterization study suggested that methanogenesis was one of the major TEAPs active in the sediments. To ensure that the threshold was measured under methanogenic conditions an excess electron donor in the form of acetate was added. In this way, any other remaining TEAs (e.g., iron, sulfate), would be consumed such that the ultimate threshold would be measured under methanogenic conditions.

To eliminate the variability in data associated with sacrificing numerous serum bottles at each sampling interval, as done in the characterization study, the acetate threshold study was conducted using duplicate 2000-ml reactor bottles.

At each sampling interval of the experiment, the bottles were sampled for total pressure, CH<sub>4</sub>, and H<sub>2</sub> in the headspace, and aqueous acetate levels. First, the total headspace pressure was monitored, followed by removal of headspace samples (0.5 ml each) for analysis of CH<sub>4</sub> and H<sub>2</sub>. Next a sample was taken for acetate analysis by removing 2 ml of sediment slurry using a 3 ml syringe (Becton, Dickinson and Company) while purging each reactor with N<sub>2</sub>/CO<sub>2</sub> (80%/20%) to prevent air from entering the bottles and/or creating a negative pressure during sampling of the slurry phase. This 2 ml sample was then used for acetate analysis as described below. Then, after the slurry sample was removed for the aqueous acetate analysis, a second headspace sample for analysis of CH<sub>4</sub> and H<sub>2</sub> was taken and a second total pressure measurement was made. The latter was necessary because the process of flushing the headspace with N<sub>2</sub>/CO<sub>2</sub> (80%/20%) gas during removal of a slurry sample strips out CH<sub>4</sub> and H<sub>2</sub>. Therefore, cumulative concentrations of methane and hydrogen were calculated by taking into account the total headspace volume and the amount that was stripped by this procedure.

The sediment and groundwater was combined to form the 10% slurry and placed into the reactors on day 0 of this experiment as described in section 4.1. Once the reactors were sealed and removed from the anaerobic chamber, they were purged with a gas mix of N<sub>2</sub>/CO<sub>2</sub> (80%/20%) for 30 minutes to remove excess H<sub>2</sub> that may have been acquired from the inside of the anaerobic chamber. Subsequently, on day 1, initial measurements were made for total pressure, CH<sub>4</sub>, H<sub>2</sub> and acetate.

Next, on day 2, each reactor was spiked with 2 mM sodium acetate (nominal aqueous concentration). Then, on day 3 the bottles were sampled again for headspace total pressure CH<sub>4</sub>, H<sub>2</sub> and aqueous acetate concentration. This sampling procedure was repeated every second day. This sampling interval was selected based on Madsen (1997), which recommends that samples be obtained at intervals equal to or less than 1/3 or 1/4 of the active period of biological activity. In this case, the active period was assumed to be 12 days based on the characterization study in which acetate thresholds were achieved between day 10 and 13. Sampling was continued until 5 acetate concentrations were measured that could be fitted by a line with a slope that was not statistically different from zero. This was done by obtaining five data points at the assumed threshold concentration and drawing a line through these data points and comparing the slope of this line with zero. If the slope of the line from the data points was not statistically different from zero than the y-intercept, or average of the five data points was the calculated acetate threshold concentration. Each reactor was then spiked again with 0.89 mM sodium acetate (nominal aqueous concentration) to obtain a duplicate data set for demonstrating the reproducibility of the acetate threshold.

#### **4.2.2.4 TEAP 2: Dehalorespiration-Acetate Threshold Conditions**

The goal of this experiment was to obtain acetate threshold values for the sediment slurry under conditions that were presumably dehalorespiring. Two approaches were used to assess acetate thresholds under dehalorespiring conditions. The first approach involved conducting an experiment analogous to that used for methanogenic conditions and involved adding acetate and PCE to the wetland sediment collected from site WB23. When it became clear that PCE respiration was not going to occur at a rate

fast enough given the time constraints for the project, a second approach for measuring acetate thresholds under dehalorespiring conditions was tested. This involved measuring acetate concentrations in wetland sediments that were collected from site WB35, and had been amended with PCE and/or carbon tetrachloride (CT) one or more times but did not receive any exogenous electron donors.

First, the experiment using site WB35 will be explained but due to the aforementioned time constraints, will not be fully analyzed in detail. The dehalorespiring threshold experiment was conducted over 11 days in duplicate. It contained four treatments as summarized in Table 4.2. Each treatment was prepared in duplicate 160-ml serum bottles. Each bottle contained 125 ml of the 10% (by volume) sediment/groundwater slurry mixture and approximately 33.8 ml of headspace.

At each sampling interval of the experiment, bottles were sampled for total pressure, CH<sub>4</sub>, H<sub>2</sub> and PCE, daughter products of PCE (i.e. TCE, DCE, vinyl chloride (VC), ethene), and aqueous acetate levels. First, the total headspace was monitored, followed by headspace sampling (0.5 ml each) for H<sub>2</sub>, as well as for CH<sub>4</sub> and PCE, which were monitored with one sample on the GC. Next a sample was taken for acetate analysis by removing 2 ml of sediment slurry using a 3 ml syringe. Prior to removing the slurry, each bottle was injected with 2.5 ml of sterile N<sub>2</sub>/CO<sub>2</sub> gas mix (80%/20%) to maintain positive pressure, thereby making it easier to remove the 2 ml slurry sample and preventing introduction of air. This sample was then used for acetate analysis as described below. Hydrogen, CH<sub>4</sub>, and PCE and total pressure were measured a second time. After day 5, the practice of analyzing H<sub>2</sub>, CH<sub>4</sub>, PCE and total pressure a second time was discontinued because losses were not significant.

The sediment and groundwater was combined to form the 10% slurry and placed into the 160-ml serum bottles on day 0 of this experiment as described in section 4.1. Once the reactors were sealed and removed from the anaerobic chamber, they were purged with a gas mix of N<sub>2</sub>/CO<sub>2</sub> (80%/20%) for 30 minutes to remove excess H<sub>2</sub> that may have been acquired from the inside of the anaerobic chamber. Subsequently, on day 1, initial measurements were made for total pressure, CH<sub>4</sub>, H<sub>2</sub>, PCE and acetate. Next on day 2, each reactor was spiked with the nominal concentrations of substrates as noted in Table 4.2. Then, on day 3 the bottles were sampled again for headspace total pressure, CH<sub>4</sub>, H<sub>2</sub>, PCE, and aqueous acetate concentration and every second day until day 11. Subsequently, the bottles were sampled every second day as on day 3 and continued until day 11.

Table 4.2. Treatments used in the first dehalorespiring condition experiment. Microcosms were prepared in duplicate.

<b>Microcosm</b>	<b>Treatment</b>	<b>Purpose</b>
<b>C1</b>	Control	To determine natural processes without the addition of electron donors or electron acceptors
<b>Ac1</b>	Spike with 80 μM Acetate	Acetate functions as an electron donor
<b>PCE1</b>	Spike with 40 μM PCE	PCE controls to compare natural degrading conditions in the sediment
<b>Mix1</b>	Spike with both 80 μM acetate and 40 μM PCE	Acetate functions as an electron donor and PCE as the electron acceptor

Analysis of acetate thresholds under presumptive dehalorespiring conditions was also performed using samples provided by Ms. Emily Devillier. A summary of key information relevant to this experiment follows. The groundwater came from the same site, WB24, used in the characterization and methanogenic threshold experiments; however, the sediment for this experiment came from a different location, one known to have high levels of chlorinated compounds (WB35).

The sediment slurries used in this evaluation by Emily Devillier were prepared as summarized in Table 4.3. The protocols used in the set up of the sediment slurry cultures is similar to the protocols used in the characterization experiment.

Table 4.3. Key characteristics of the sediment slurry cultures prepared and maintained by Ms. Emily Devillier and used to monitor acetate thresholds under presumptive dehalorespiring conditions.

	<b>PCE</b>	<b>CT</b>
<b>sediment:groundwater</b>	1.5:1	1.5:1
<b>volume of slurry</b>	45 ml	45 ml
<b>storage time on day of sampling (days)</b>	62	59
<b>number of spikes of chlorinated solvent</b>	3	4
<b>concentration of spike (mg VOC/L)</b>	range 4.3-11	range 7.0-9.2

A 2 ml slurry sample was removed from the 72-ml serum bottle once the characteristic VOC had been depleted within the microcosm. This 2 ml sample was then used for acetate analysis as described below.

## 4.3 Analytical Methods

### 4.3.1 Oxidized Electron Acceptors and Reduced Products

#### 4.3.1.1 Iron Analysis

Analysis of iron reduction in the sediment slurry cultures was performed using an HCl-extractable Fe(II) and microbially-reducible Fe(III) method described by Lovley and Phillips (1987a). Application of this method required the preparation of three reagents: (1) the ferrozine reagent, (2) the hydroxylamine reagent, and (3) 0.5 M hydrochloric acid (HCl). The ferrozine reagent (Acros, 98%) solution was made at a concentration of 1 g/L in a 50 mM HEPES buffer (J.T. Baker, 100%) at pH 7. The HEPES buffer is necessary because the ferrozine reaction with the  $\text{Fe}(\text{ligand})_3^{2+}$  can only occur between pH values of 4 and 9. During this study, ferrozine reagent was found to react with the magenta  $\text{Fe}(\text{ligand})_3^{2+}$  if the mixture was made in deionized water and stored at room temperature. Therefore, the ferrozine reagent in 50 mM HEPES buffer was always made immediately before each sampling interval. In addition, a 0.25 M hydroxylamine hydrochloride reagent (Fisher Scientific, 98.5%) mixture was made in 0.25 M HCl (Fisher-Scientific) along with a 0.5 M HCl (Fisher-Scientific) solution. Sufficient volumes of both of these solutions were made to last the entire sampling period for each experiment.

Measuring Fe(II) production and microbially-reducible Fe(III) using this method requires a two step process. First, to measure the Fe(II) in each sacrificed, 72-ml serum bottle culture, the sediment slurry was initially mixed well by vigorously shaking the serum bottle by hand and then opening the bottle to remove a 0.1 ml sediment slurry sample with a 100- $\mu\text{L}$  micro-pipette (Rainin). This sample was added to a previously weighed 7-ml scintillation vial containing 5 ml of 0.5 M HCl. Next, the vial was mixed



by hand for 15 s , after which it was allowed to stand at ambient room temperature for 1 hour. The weight of the added slurry sample was then determined gravimetrically. Subsequently, a 0.1 ml sample of this mixture was added to another 7-ml scintillation vial containing the 5 ml ferrozine reagent and swirled by hand for 30 s. The ferrozine mixture was then immediately passed through a 0.2  $\mu\text{m}$  membrane filter (Nucleopore) into a 1 cm diameter sample cuvette, which was then transferred to the Hach spectrophotometer (Hach DR/2400) and the  $A_{562}$  of the filtrate determined.

In the second step, the microbially-reducible Fe(III) was reduced to Fe(II) and the total Fe(II) determined. To do this, another 0.1 mL sample was withdrawn from the sacrificed serum bottle after mixing and was added to a previously weighed 7 ml scintillation vial containing 5 ml of the 0.25 M hydroxylamine hydrochloride in 0.25 N HCl reagent. Subsequently, the sample was treated and analyzed as described for the Fe(II) measurement. The microbially reducible Fe(III) was calculated by subtracting the initial Fe(II) from the total Fe(II).

The calibration curve was prepared with eight standards of Fe(II) at concentrations of 0.1, 0.5, 1, 5, 10, 30, 50, and 100 mg of Fe(II)/L using a ferrous ethylene diammonium sulfate tetrahydrate (Sigma-Aldrich, 98%) solution in prepared with deionized water. A linear plot of  $A_{562}$  absorbance vs. Fe(II) yielded an  $R^2$  value of 0.9884. This plot was used to determine the actual concentrations measured in mg of Fe(II)/L and subsequently converted to and reported as  $\mu\text{mol}$  of Fe(II), for all sediment slurry samples.

The lower detection limit for the ferrozine reaction has previously been reported to be 4 mg Fe(II)/L (Stookey, 1970). During this study, using Standard Methods 1030E, (APHA, 1989) the method detection limit of this method was determined to be 10 mg Fe(II) /L.

#### **4.3.1.2 Methane**

Analysis of methane in the headspace of sediment slurry cultures was performed by gas chromatography using a Hewlett Packard 5890 Series II Plus gas chromatograph (GC) with a flame ionization detector (FID) and a 1% SP-1000 on a 60/80 Carbowax-B (Supelco) packed column (2.44 m x 3.2 mm with a film thickness of 3  $\mu$ m). A summary of the GC operating conditions is provided in Table 4.4. With these conditions, the run time for each injection was 15.7 minutes, with retention time of 0.45 minutes for methane. The software, HP GC Chemstation (Rev.A.10.02, Agilent Technologies) was used for analysis and integration of the output signal from the GC.

To initiate the analysis, 0.5 ml headspace samples were withdrawn from each headspace bottle using a 1000  $\mu$ l, gas tight syringe (Hamilton, Series A-2) and manually injected into the GC. After each injection, the syringe was purged five times with ambient air and opened to equilibrate. Injections were made in duplicate for each sample to ensure reproducibility.

An external methane calibration curve was obtained using a methane standard (Supelco, 99%). The methane bottle was connected to a 0.5 cm diameter rubber tubing approximately 24 inches in length. The open end of the tubing was first submerged into a 500-ml beaker of tap water, and the methane tank was opened sufficiently to produce methane gas bubbles in the tap water beaker.

Table 4.4 GC operating conditions for analysis of methane and chlorinated VOCs.

Mechanism	Parameter	Condition
Gas Flow Rates	Carrier Gas (Helium)	Helium (Ultra Pure Carrier Grade) 60 ml/min
	Septum Purge	60 ml/min
	Column Flowrate	6.7 ml/min
	Flame Oxidant	Air (Ultra Pure Carrier Grade) 400 ml/min
	Flame Combustible	Hydrogen (Ultra Pure Carrier Grade) 40 ml/min
Oven	Initial Temperature	60°C
	Initial Hold Time	2 min
	Temperature Gradient	10°C/min to 200°C
	Hold Time	4.2 min
	Final Temperature	200°C
Injector	Temperature	200°C
Detector	Temperature	250°C

The gas tight syringe was then inserted through the tubing and methane was withdrawn in varying volumes. This procedure ensured that the gas pressure remained at 1 atm to facilitate calculation of the number of moles of H<sub>2</sub> gas injected into the GC, using the ideal gas law:

$$n = \frac{PV}{RT} \quad (4.1)$$

where  $P = 1 \text{ atm}$ ,  $V = \text{volume of injection (L)}$ ,  $R = 0.0821 \text{ L} \cdot \text{atm} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ ,  $T = 293 \text{ K}$ . To achieve different molar amounts of methane, the following volumes of methane were injected: 0.5 ml, 0.1 ml, 600  $\mu\text{l}$ , 250  $\mu\text{l}$ , 100 $\mu\text{l}$ , and 25 $\mu\text{l}$ . A linear regression of mass ( $\mu\text{mol}$ ) versus peak area was then obtained. Each standard was injected one time with standard curves were produced on two occasions yielding an  $R^2$  value = 0.9976 and 0.9988. Results are reported as  $\mu\text{mol}$  in the gas phase.

#### **4.3.1.3 PCE, TCE and DCE Analysis**

PCE, TCE, and DCE concentrations in the headspace of sediment slurry microcosms were analyzed by gas chromatography using the method described above for methane. The retention times for PCE, TCE, and DCE are 14.7, 9.9 and 7.5 minutes, respectively. The headspace PCE, TCE and DCE concentrations were determined by injecting headspace samples obtained from ten aqueous PCE, TCE, and DCE solutions of known concentrations from a methanol stock solution of PCE, TCE, and DCE (Gossett, 1987). Specifically, the calibration standard concentrations for PCE were 4, 7, 23, 36, 49, 76, 142, 290, 434 and 588  $\mu\text{M}$ , and the concentrations for TCE were 2, 3, 9, 15, 20, 30, 55, 112, 167 and 227  $\mu\text{M}$ . The DCE standard concentrations were 0.9, 3, 6, 9, 13, 28, 53, 107, 160, and 216  $\mu\text{M}$ . The linear calibration curves of peak area as a function of standard concentration yielded  $R^2$  values of 0.99 for all calibration curves. The results are reported as  $\mu\text{M}$  aqueous concentrations of the respective chlorinated compounds.

#### **4.3.1.4 Nitrate**

Analysis of nitrate concentrations in the sediment slurry cultures was performed by spectrophotometry and the Hach, cadmium reduction Method Number 8192. This

method requires a 15 ml sample, NitraVer 6, and NitriVer 3 Nitrite Reagent Powder Pillows and a 10 ml sample cell fitted for the Hach spectrophotometer.

To obtain an aqueous sample for analysis, 20 ml of sediment slurry sample was vacuum filtered through a 0.22  $\mu\text{m}$ -pore size Acrodiscs filter (Gelman Scientific, 0.47 mm) using a polypropylene membrane filter apparatus (Life Sciences). Then 15 ml of the filtrate was poured into a clean 25 ml graduated cylinder. The contents of one NitraVer 6 Reagent Powder Pillow was added to the graduated cylinder, which was then stoppered and shaken vigorously for 3 minutes, after which the graduated cylinder was placed on the bench for a two minute reaction period. Ten ml of this reactant was then carefully poured into a clean, 10 ml Hach sample cell. One NitriVer 3 Nitrite Reagent Powder Pillow was added to the sample cell, and the cell contents shaken gently for 30 seconds. The sample cell was then placed on the bench for a 15 minute reaction period. Subsequently, the sample cell was placed into the Hach DR/2400 spectrophotometer, and measured at a wavelength of 507 nm.

This method was calibrated using four standard solutions with 0.01, 0.05, 0.1, and 0.5 mg/L of  $\text{NO}_3^-$  made using potassium nitrate (Fisher, 98%) and deionized water. A reagent blank for NitraVer 6 Reagent and NitriVer 3 Reagent Powder Pillows was determined for each new lot of reagents and this blank value was subtracted from the absorbance final results. A standard curve was obtained by plotting absorbance versus  $\text{NO}_3^-$ -N mg/L with an  $R^2$  value of 0.9958. Results are reported as mg/L of  $\text{NO}_3^-$ -N in the aqueous phase.

## 4.3.2 Global TEAP Indicators

### 4.3.2.1 Hydrogen Analysis

Concentrations of H<sub>2</sub> partial pressures in the headspace of microcosm serum bottles were measured using a Peak Laboratories, Peak Performer 1 (PP1) gas analyzer with a reducing compound photometer (RCP) and 1/8 column. The detector and column temperatures were set at 265°C and 105°C, respectively. Nitrogen (Ultra Pure Carrier Grade, Air Gas) was used as the carrier gas at a flow rate of 75 ml/min. With the given conditions, the retention time for H<sub>2</sub> was 48 seconds. The total run time for each injection was 3.5 minutes.

To initiate the analysis, 0.5 ml headspace samples were withdrawn from each headspace serum bottle using a 1000 µL gas tight syringe (Hamilton), after which the entire sample was manually injected into the PP1. Following each injection, the syringe was purged five times with ambient air. Injections were made in duplicate for each sample to ensure reproducibility.

A H<sub>2</sub> calibration curve was generated using an 18.7 ppm H<sub>2</sub> (balance N<sub>2</sub>) standard (Ultra Pure Carrier Grade, Air Gas). This standard gas was used to flush 72-ml serum bottles (Wheaton) sealed with a butyl rubber septa (Geo-Microbial Technologies, Inc.) before withdrawing calibration standards. A 1000 µL gas tight syringe was inserted through the septum, and the desired volumes of H<sub>2</sub> were withdrawn. To create the standard curve, four different volumes (50 µL, 0.1 ml, 0.3 ml, and 0.5 ml) of the 18.7 ppm standard gas were injected on the PP1 and used to create a linear plot of H<sub>2</sub> mass (µmol) versus peak area. Each standard volume was injected one time and standard curves were produced twice yielding an R<sup>2</sup> value = 0.9823. Based on the information

provided by the manufacturer, the PP1 gas analyzer is capable of detecting parts per trillion concentration of reducing gases.

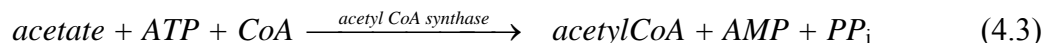
The H<sub>2</sub> μmol values obtained using the standard curve were converted using the sample injection volume to concentrations expressed in terms of parts per million by volume (ppmv) in the serum bottle headspace. The ppmv values were subsequently converted to aqueous concentrations in nM using the following relationship from Löffler et al., (1999) following the convention of Conrad (1996):

$$H_2(\text{dissolved}) = \frac{LP}{RT} \quad (4.2)$$

where L is the Ostwald coefficient for H<sub>2</sub> solubility (0.01941 at 20°C), R is the universal gas constant (0.0821 L · atm · K<sup>-1</sup> · mol<sup>-1</sup>), T is the temperature in Kelvin (293 K), and P is the H<sub>2</sub> partial pressure in atmospheres (Wilhelm et al., 1977; Löffler et al., 1999).

#### 4.3.2.2 Acetate Analysis

Aqueous acetate concentrations in the sediment slurries were analyzed using the protocol in King (1991). This method uses an enzymatic assay to determine acetate concentration through a reaction mediated by acetyl coenzyme A (CoA) that forms AMP:



The AMP produced was assayed using high pressure liquid chromatography (HPLC) as discussed further below.

The acetate analysis required the following stock solutions, which were prepared using deionized water: 10 mM adenosine triphosphate (ATP) (Sigma-Aldrich), 200 μg/ml bovine serum albumin (Sigma-Aldrich), 10 mM Coenzyme A disodium salt (Fluka, 27593), and 20 U/ml Acetyl CoA synthase (Sigma, A1765). ATP and BSA were stored in 15 ml centrifuge tubes (Becton, Dickinson and Company), acetyl CoA synthase was

stored in the original container obtained from the manufacturer, and CoA was stored in a 1.7 ml centrifuge tube. All stock solutions were frozen for several months without any apparent loss of activity.

Sample preparation required several steps. First, 2 ml slurry samples were vacuum filtered through a 0.22  $\mu\text{m}$ -pore size acrodiscs filter (Gelman Scientific, 0.47 mm) using a polypropylene membrane filter apparatus (Life Sciences) and 1 ml of the filtrate was placed into a 1.7 ml polypropylene centrifuge tube (Para-Scientific Company). Subsequently, 10  $\mu\text{L}$  of each stock solution was added to the centrifuge tube using a 100  $\mu\text{L}$  Rainin pipette. This resulted in final concentrations of 100  $\mu\text{M}$  of CoA and 50  $\mu\text{M}$  of ATP in the centrifuge tube. Prior to day 15 of the characterization experiment, 100  $\mu\text{M}$  of ATP was used.

Each sample vial was then vigorously shaken for approximately two minutes followed by incubation in a 35°C water bath (Brookfield TC 500) for 12 hours. At the conclusion of the incubation period, the reaction was stopped by placing the sample centrifuge tubes into a boiling water bath for 2 minutes. The samples were then cooled for one hour and subsequently centrifuged on a table top centrifuge (Eppendorf, 5415C) for 10 minutes at 10,000 rpm. Afterwards, a 1000  $\mu\text{L}$  pipette (Rainin) was used to carefully remove 800  $\mu\text{L}$  of the supernatant and transferred into a 1 ml HPLC vial (Fisher-Scientific), which was capped and placed into the HPLC sample tray for analysis.

Analysis of the AMP produced by the reaction in equation 4.3 was performed by HPLC using a Waters system (Waters 600E Multisolute Controller, Waters 717 Autosampler, and Waters 996 Photodiode Array Detector) equipped with a silica C18 silica reverse phase column (Supelcosil LC-18, 25cm x 4.6mm with 5 $\mu\text{m}$  silica particles).



Prior to day 15 of the characterization experiment, a different column (YMC-Pack ODS-AQ, 150 x 4.6 mm with 5  $\mu$ M silica particles) was used. For all injections, a sample volume of 200  $\mu$ L was used. Initially, the mobile phase (1.3 ml/min) consisted exclusively of mobile phase A (0.1M  $\text{KH}_2\text{PO}_4$ ). Mobile phase B (90% Mobile A:10% methanol v/v) was provided according to the gradient outlined in Table 4.5. Adenosine monophosphate (AMP) eluted at approximately 13.7 minutes. This gradient run was continued for a total of 25 minutes to elute any additional proteins produced by the enzymatic reaction or present in the sample. Prior to day 15 of the characterization experiment, an isocratic elution method using 0.05 M potassium phosphate (pH=4.5) buffer with 10% methanol was used. At the conclusion of each injection, an 18 minute stabilization period was maintained with filtered deionized water prior to subsequent injections to avoid ghost peaks in subsequent injections. AMP was detected by monitoring UV absorbance at 254 nm using the Waters 996 Photodiode Array Detector. Empower Software (Waters Corporation, 2000) was used for analysis and integration of the output signal from the HPLC.

Table 4.5. Gradient Elution used in HPLC method used to quantify AMP.

Time (min)	Mobile Phase B
0	0
9	0
15	25
17.5	90
19	100
23	100
24	0

The detector response was calibrated by the addition of known concentrations of acetate to the enzyme reaction mixture. Specifically, the calibration curve was prepared

with up to eight acetate standards (concentrations ranging from 0.1 to 100  $\mu\text{M}$ ) using sodium acetate trihydrate (Fisher, 99.8%) in HEPES buffer solution at  $\text{pH}=7.8$ . Prior to day 15 of the characterization experiment, the acetate standards were made at  $\text{pH}=4.8$ , that of the laboratory deionized water. Duplicate injections of each standard and sample were performed due to variations that occurred with the HPLC. King (1991) reports a detection limit for acetate using this method of approximately 0.1  $\mu\text{M}$  acetate, however, in this study, detection limits were in the  $\mu\text{M}$  range. Results are reported as  $\mu\text{M}$  of acetate in the aqueous phase.

### **4.3.3 Aggregate Measurements**

#### **4.3.3.1 Total Pressure**

Analysis of total pressure in sediment slurry cultures containing headspace was performed using a pressure gauge (Ashcroft, 316). The pressure gauge was fitted with a 22 gage needle (Fisher Scientific, 1") and inserted through the butyl rubber septa of each headspace bottle. Pressure measurements were taken before and after all headspace sampling events to obtain accurate readings of overall pressure and pressure changes within the slurry cultures. Results are reported in psi.

#### **4.3.3.2 Total Suspended Solids**

Analysis of total suspended solids of the sediment slurry mixtures was adapted from Standard Methods, 2540D (APHA, 1989). Prior to sample analysis, a glass fiber filter (Whatman, 4.7 cm) was placed into the polypropylene membrane filter apparatus (Life Sciences), connected to the vacuum system, and washed with three-5 ml volumes of deionized water. The filter was then removed from the filter assembly, and placed onto a marked aluminum weigh dish of known weight. The glass filter and dish

along with two additional, empty aluminum weigh dishes were dried in the 105°C oven for one hour, after which all three items were cooled for at least 10 minutes in a desiccator. Before use in the next step, the initial weight of the dry baked filter and dish was measured to the nearest 0.1 mg.

The dry glass filter was placed on a clean, polypropylene membrane filter apparatus (Life Sciences) and transferred into a 10 ml graduated cylinder, covered with parafilm, shaken, and then poured onto the filter and vacuum filtered through the device. The graduated cylinder was rinsed with a small amount of deionized water and poured onto the filter. The filter apparatus and the sides of the filter were also rinsed with a small amount of deionized water under vacuum until the filter appeared to be dry. The filter was then removed from the filter apparatus, and placed into a second, baked aluminum dish of known weight. This filter and dish were then placed into the 105°C oven and dried for one hour, after which they were placed into a desiccator for 10 minutes before weighing to the nearest 0.1 mg. This process of drying, desiccating and weighing was repeated until a constant weight was obtained or until the weight change was less than 4% of the previous weight. In this experiment, only two cycles were needed to achieve a constant weight. The test was completed in duplicate and results are reported in g/L.

## **CHAPTER 5**

### **RESULTS AND DISCUSSION**

In this chapter, the results of the sediment characterization experiment are first presented and discussed in terms of their implications with respect to the dominant TEAPs in the wetland sediment. Next, the rationale for selecting the TEAP used in the acetate threshold experiment is discussed. Finally, the results of the acetate threshold experiments are reported and discussed.

#### **5.1 Characterization Experiment**

As previously mentioned, wetland sediment and groundwater were collected from two locations at Aberdeen Proving Ground that receive discharge from groundwater that is contaminated with chlorinated solvents (Lorah et al., 1997). Dissolved oxygen has not been detected in previous studies involving this groundwater and sediment. Therefore, aerobic respiration was assumed to be unimportant at the sites, and the analyses and microcosm treatments focused instead on evaluating the importance of nitrate reduction, dehalorespiration, iron reduction, sulfate reduction and methanogenesis. The results of these analyses and treatments are presented and discussed below.

##### **5.1.1 Nitrate Reduction**

Dissolved nitrate concentrations were measured in filtered sediment slurries on day 1 of the sediment characterization experiment. No measurable nitrate was detected in any of the four treatments tested using the cadmium reduction method, which has a lower detection limit of 0.01 mg NO<sub>3</sub><sup>-</sup>-N/L. Therefore, nitrate reduction was ruled out as an important TEAP at these sites. The results of the nitrate analyses were expected because

nitrate reduction in groundwater is feasible only when high concentrations of nitrate arise due to agricultural impacts or contamination from septic systems, neither of which is thought to be important at Aberdeen Proving Ground.

### **5.1.2 Dehalorespiration**

On day 1 of the characterization experiment, the gas phases of the four headspace microcosms were analyzed for the presence of several chlorinated VOCs, including CT, chloroform, dichloromethane, PCE, TCE, DCE. Previous studies have shown that biodegradation of chlorinated VOCs occurs in the sediment at the source site and, along with other natural attenuation processes, reduces the concentrations of chlorinated VOCs below detectable levels in surface waters (Lorah et al., 1997). In this study, no chlorinated VOCs were detected in any of the treatments. Ethene and ethane were detected in the microcosms in low concentrations, along with high levels of methane, as discussed in section 5.1.4. Thus, it seems likely that reductive dehalogenation of chlorinated ethenes and/or ethanes in the groundwater or deeper sediment layers led to the formation of ethene and ethane, but dehalorespiration was not an ongoing process in the sediment microcosms used in this study.

### **5.1.3 Iron Reduction**

After ruling out the importance of aerobic respiration, nitrate reduction, and dehalorespiration in the collected groundwater and sediment based on historical evidence and experimental evaluations, the remaining sediment/groundwater microcosms were subjected to four treatments (Table 4.1) to evaluate the importance of iron and sulfate reduction and methanogenesis. Concentrations of HCl-extractable Fe(III) and Fe(II)

were analyzed to evaluate whether iron reduction was occurring in the various microcosms. The results are presented in Figure 5.1.

It was expected that if active iron reduction were occurring in the sediment microcosms, then Fe(II) would accumulate as the Fe(III) levels decreased with time. However, as demonstrated in Figure 5.1 this trend was not obvious in any of the four treatments. Nevertheless, Fe(II) was present in all four treatments, which may be an indicator that iron reduction has occurred, or possibly that iron reduction is ongoing. In the T2 and T3 microcosms there is a slight trend indicating a reduction in the Fe(III) concentration that may be an indicator of active iron reduction as well. It is also noticeable in Figure 5.1 that Fe(II) increased in all microcosms on day 11 of the experiment and is more likely the result of experimental error and/or ferrozine interference as explained below.

Several factors undoubtedly contributed to the ambiguous iron data. First, the laboratory deionized water apparently interfered with the ferrozine colorimetric reagent when left as a solution at room temperature and may have made the Fe(II) data unreliable. Second, the iron concentrations were measured in samples obtained from different microcosms sacrificed and sampled at various intervals and this sampling technique may have contributed to the variability observed in the Fe(III) and Fe(II) measurements. Specifically, it is possible that solid phase Fe species were not equally distributed among the microcosms during their preparation due to the heterogeneous nature of the sediment. These heterogeneities would not be expected to have as much of an effect on the measurement of dissolved chemical species such as acetate. Finally, it is

possible that the conversion of dissolved or HCl-extractable forms of iron to precipitates that are not extractable with HCl obscured trends in the Fe(III) and Fe(II) data.

Nevertheless, as discussed above, the detection of Fe(II) in the sediment microcosms could suggest that reduction of bioavailable Fe(III) occurred previously in the surface sediment used to prepare the microcosms. Or alternatively, it could indicate that Fe(III) reduction may have been an important TEAP in lower sediment layers and Fe(II) was either transported to the surface sediment or present in the groundwater. A previous study of the wetland site by Lorah (1997) supports the latter explanation. Redox conditions were increasingly reduced along the upward flowpath of groundwater (from the aquifer) and iron-reducing conditions were observed in a lower, clayey sediment layer (Figure 4.1).

#### **5.1.4 Sulfate Reduction and Methanogenesis**

As previously described, the importance of methanogenesis as a TEAP in the wetland sediment was evaluated by monitoring CH<sub>4</sub> production in sediment slurries to which no substrates were added and acetate-amended slurries. Comparison of CH<sub>4</sub> production in these two microcosm treatments with methanogenesis in microcosms that were amended with molybdate, a specific inhibitor of sulfate-reducing bacteria, with or without acetate was used to test for sulfate reduction.

Methane concentrations in the four microcosm treatments through day 50 are presented in Figure 5.2. Little CH<sub>4</sub> production was observed in the microcosms that were not amended with acetate and reached maximum values of approximately 12 μmol. Headspace methane levels gradually decreased after reaching a maximum in all four sets of microcosms, presumably due to the depletion of endogenous electron donors and/or

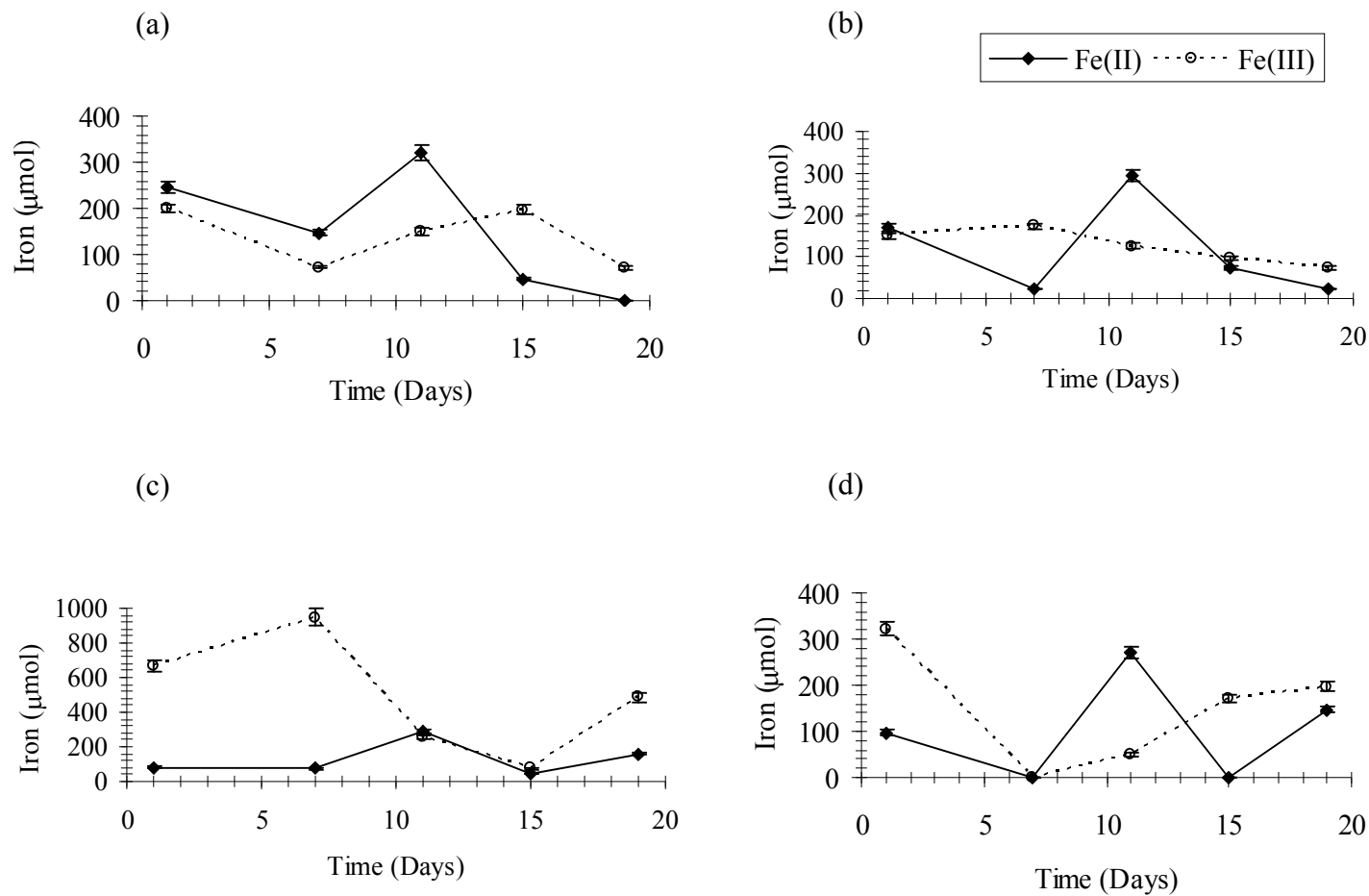


Figure 5.1. Fe(II) (diamonds) and Fe(III) (circles) concentrations in the sediment characterization experiment. Data points represent the average of duplicate samples and the error bars represent  $\pm$ one standard deviation: (a) T1 = no substrate controls, (b) T2 = acetate added, (c) T3 = molybdate added, (d) T4 = acetate and molybdate added.



acetate, which caused CH<sub>4</sub> production to cease while the repeated sampling of the headspace gradually depleted the methane in the bottles.

It is conceivable that sulfate reduction acted as a sink for acetate and other electron donors in the T1 microcosms and diverted reducing equivalents away from methanogens. In that case, higher methane production would be expected in the molybdate-treated T3 microcosms. However, comparison of the mean methane concentrations in the T1 and T3 microcosms between days 6 and 49 using Student's t-test ( $\alpha/2=0.025$ ) showed no statistically significant difference, which suggests sulfate-reduction was not important.

The low methane concentrations in the T1 and T3 treatments also suggest that the site from which the sediment was collected may be electron donor-limited. It is possible that endogenous electron donors were depleted during the reduction of preferable electron acceptors such as Fe(III) or sulfate. An alternative explanation is that reduction of preferable electron acceptors in the T1 and T2 microcosms was not observed because of a lack of bioavailable electron donor.

Not surprisingly, substantially more methane was produced in the T2 and T4 microcosms, which were amended with acetate, as shown in Figure 5.2. Comparison of the mean methane levels in the two treatments between days 22 and 42 using Student's t-test ( $\alpha/2=0.025$ ) indicates that the T4 microcosms produced slightly more methane (average = 63  $\mu\text{mol}$ ) compared with the T2 microcosms (average = 52  $\mu\text{mol}$ ), suggesting that sulfate reduction was occurring in the acetate-amended T2 microcosms. This supports the idea that electron donor availability may have limited the reduction of at least some preferable electron acceptors in the T1 and T3 microcosms. In fact, it seems

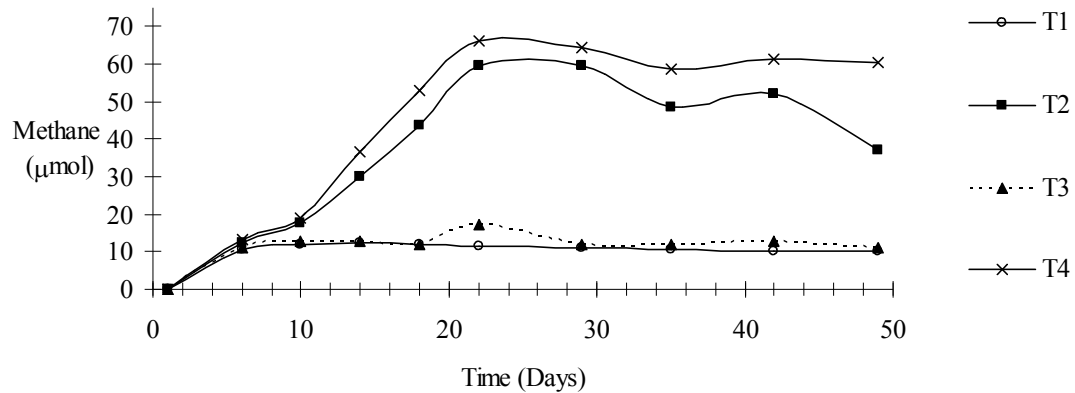


Figure 5.2. Headspace methane levels in four microcosm treatments. T1= no substrate controls, T2 = acetate added, T3 = molybdate added, T4 = acetate and molybdate added. Sediment and groundwater were added to microcosms on day 0. Acetate (2mM) was added to the T2 and T4 microcosms on day 2, and molybdate (2mM) was added to the T3 and T4 microcosms on day 2. Data points represent the average of duplicate values.

likely that the addition of acetate stimulated TEAPs other than methanogenesis, because only a small percentage of the 2 mM of acetate added to each of the T2 and T4 microcosms was apparently converted to methane.

It is interesting to note that, judging by the slope of methane production between days 1 and 6, methane production began with little or no lag period in the T2 and T4 microcosms. This could be interpreted, as evidence that other TEAPs like sulfate-reduction was not occurring in these treatments. However, under some conditions, multiple TEAPs may occur simultaneously, for example, within micro-niches in the sediment. The immediate production of methane in the T2 and T4 microcosms is also significant because it supports the idea that acetate and other substrates had largely been depleted in the surface sediments, which led to low methanogenic activity in the T1 and T3 microcosms.

### 5.1.5 Acetate Concentrations

Acetate results are displayed in Figure 5.3. Acetate concentrations were measured on day 1 of the experiment to determine the initial amounts of acetate present in the sediment slurries. Initial acetate levels in the four treatments ranged from 70 to 276  $\mu\text{M}$ . Additional acetate (2mM) was added to the T2 and T4 microcosms on day 2 (this value is shown in Figure 5.2 but it is an assumed value, as no acetate measurements were made on day 2). It is also possible that the acetate levels in these microcosms on day 7 reflect the effect of some endogenous acetate production during this period. This is demonstrated by the observation that acetate concentrations also increased between days 2 and 7 in the T1 microcosm, which was not amended with acetate or molybdate. Thus, there must have been some endogenous acetate production in the T1 microcosms during this period. This endogenous acetate production could have been the result of fermentation and/or homoacetogenic processes. Acetate concentrations in the T1 and T3 microcosms leveled off between days 11 and 23 and appeared to reach threshold concentrations of 5.7 and 10.2  $\mu\text{M}$ , respectively, based on the day 23 values.

Methane production is plotted along with acetate and hydrogen levels in T1 (Figure 5.4) to show that the most of the methane in the T1 microcosm was probably derived from hydrogen rather than acetate because once hydrogen was depleted, methane production stabilized. Compared with methanogenesis, sulfate-reduction generates a little more free energy (Table 3.1). Thus, according to the central hypothesis of this research, if sulfate reduction was contributing to acetate oxidation in the T1 microcosms, rather than methanogenesis, then a higher acetate threshold should have been detected in the T3 microcosms in which sulfate reduction was inhibited. In fact, the threshold acetate

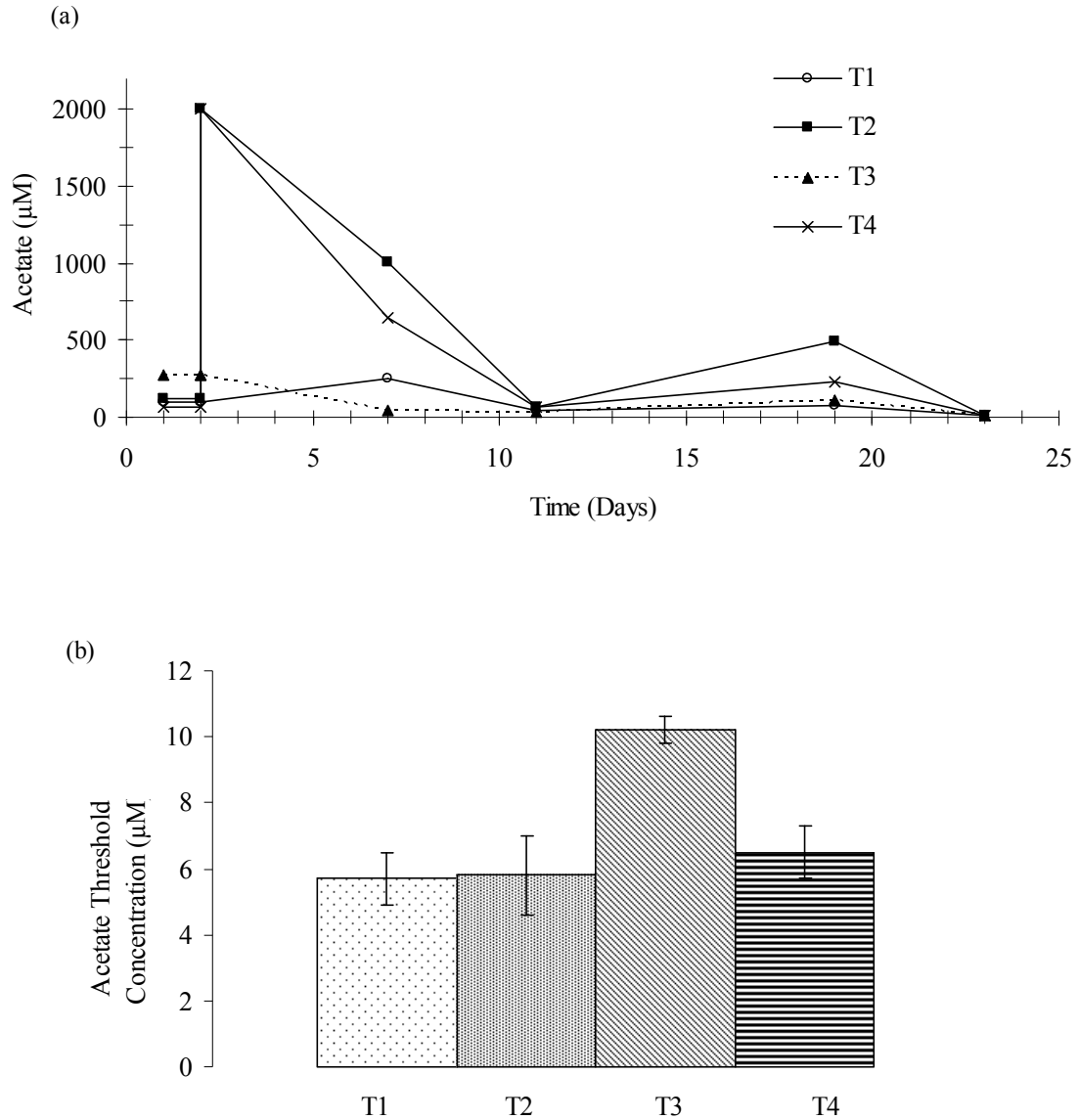


Figure 5.3. Aqueous acetate results in the four characterization study microcosm: T1= no substrate controls, T2 = acetate added, T3 = molybdate added , T4 = acetate and molybdate added. (a) average concentrations in duplicate microcosms (days 1 through 23), and (b) average acetate concentrations measured on day 23. Acetate (2 mM) was added to the T2 and T4 microcosms on day 2, and molybdate (2 mM) was added to the T3 and T4 microcosms on day 2. Data points represents the average of duplicate values, except for the 2 mM values in T2 and T4 on day 2, which were assumed. Error bars represent  $\pm$  one standard deviation.

concentration measured in the T3 microcosms (10.2  $\mu\text{M}$ ) was nearly double the concentration measured in the T1 microcosms (5.7  $\mu\text{M}$ ). Based on the Student's t-test ( $\alpha/2=0.025$ ), this difference was statistically significant. Therefore, acetotrophic sulfate-reduction could explain why methane production in the T1 microcosms appeared to be primarily the result of hydrogen, rather than acetate oxidation (Figure 5.4).

As previously discussed, substantially more methane was produced in the T2 and T4 microcosms compared with T1 and T3 microcosms, presumably because the addition of acetate to the T2 and T4 microcosms stimulated acetoclastic methanogenesis. However, it is somewhat surprising that the greatest amount of methane production in the T2 microcosms occurred after the large decrease in acetate concentration was observed (Figure 5.5).

In the microcosms that were not amended with acetate, the addition of molybdate may have increased acetate levels by inhibiting sulfate reduction, as noted above. This trend continued in the acetate amended bottles. The apparent acetate threshold in the molybdate-treated T4 microcosms was slightly higher (6.5  $\mu\text{M}$ ) than that detected in the T2 microcosms (5.8  $\mu\text{M}$ ), which were not amended with molybdate. However, this slight difference in acetate threshold was not statistically significant ( $\alpha/2 = 0.025$ ).

All four treatments yielded acetate threshold concentrations of approximately 6  $\mu\text{M}$  or higher. In comparison, acetate thresholds of 5.2  $\mu\text{M}$  were measured by Lovley and Phillips (1987b) in methanogenic sediments which supports the idea that the acetate threshold in the wetland sediment and groundwater evaluated in this study was controlled, at least in part, by methanogens.

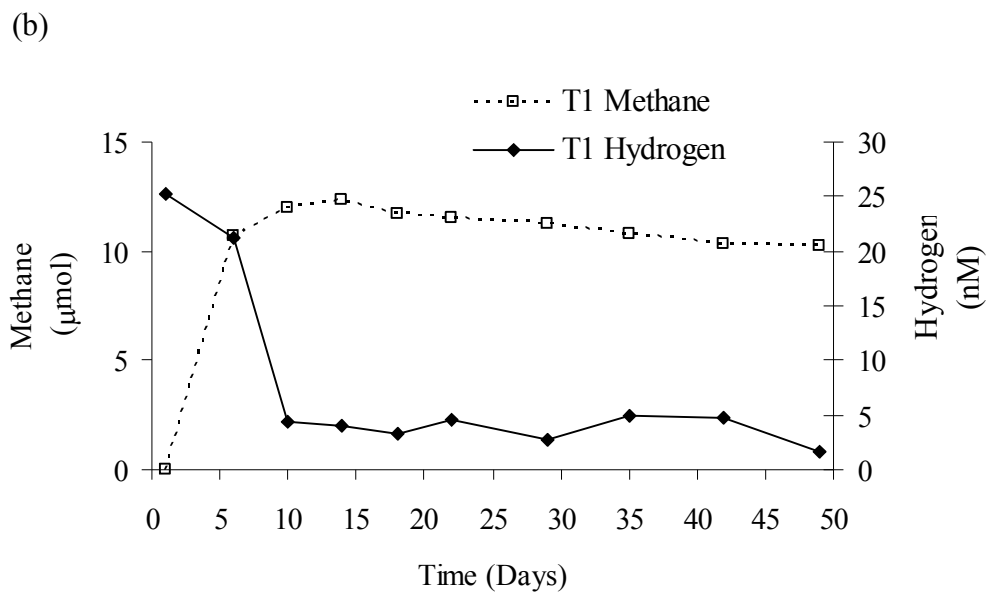
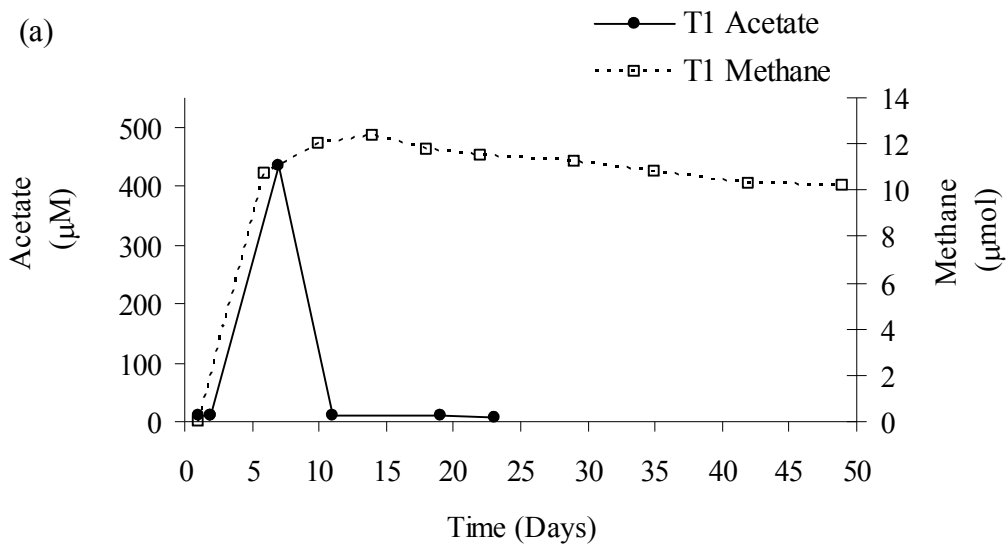


Figure 5.4. Concentrations in the T1 microcosms of methane and (a) acetate and (b) hydrogen. All data points represent the average of duplicate samples.

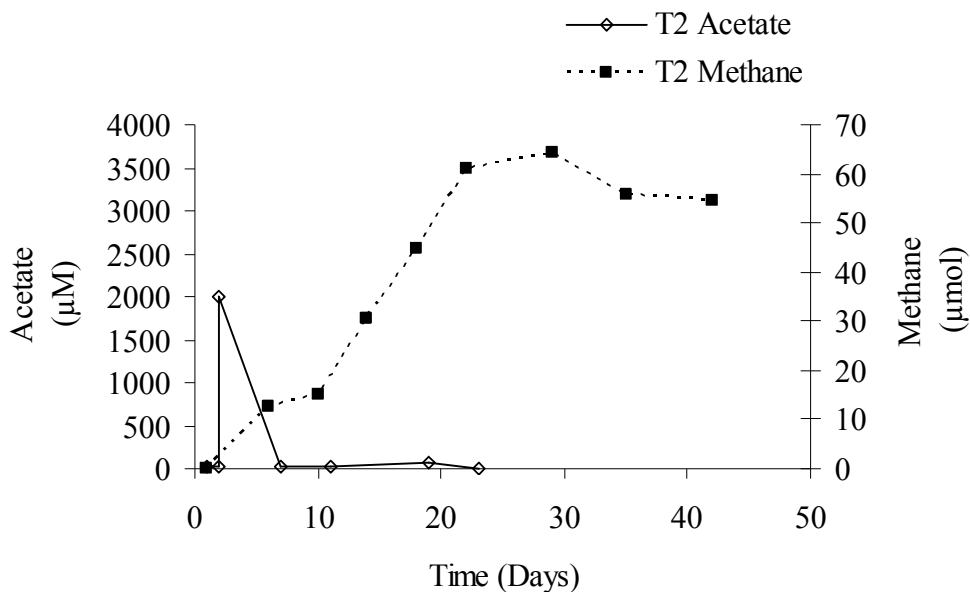


Figure 5.5. Acetate and methane concentrations in the T2 microcosms. Acetate (2mM) was added on day 2. Acetate and methane data points represent the average of duplicate samples.

### 5.1.6 Hydrogen Concentrations

Aqueous hydrogen concentrations in the four sets of microcosms were monitored because they are frequently used as indicators of the dominant TEAPs in sediment or groundwater and, thus, a significant amount of H<sub>2</sub> data is available in the literature for comparison. The H<sub>2</sub> data are shown in Figure 5.6. Initially, relatively high concentrations of hydrogen ( $\geq 25$  nM) were noted in all four sets of microcosms. At least two potential explanations for these data exist. First, it is possible that slow-growing H<sub>2</sub> utilizers like the methanogens were temporarily inhibited during microcosm preparation. For example, small amounts of air could have been introduced and inhibited methanogens. If H<sub>2</sub>-producing organisms continued to be active immediately following microcosm preparation, then H<sub>2</sub> would accumulate until the hydrogenotrophs recovered and started growing again. In addition, the anaerobic chamber in which the microcosms

were prepared contained approximately 5% H<sub>2</sub>. Therefore, the initial atmosphere in the microcosms contained a similar amount of H<sub>2</sub>, and no attempt was made to flush it out. However, had hydrogenotrophic populations in the microcosms been active immediately following their preparation in the anaerobic chamber, H<sub>2</sub> concentrations would presumably have decreased very rapidly because the H<sub>2</sub> turnover rate in active anaerobic environments has been calculated to be 176 μmol/L·h (Goodwin et al., 1988). Thus, it is quite likely that a combination of factors (the H<sub>2</sub>-containing atmosphere used in preparation of the cultures and perturbed hydrogen-consuming populations) contributed to the initially high H<sub>2</sub>-levels.

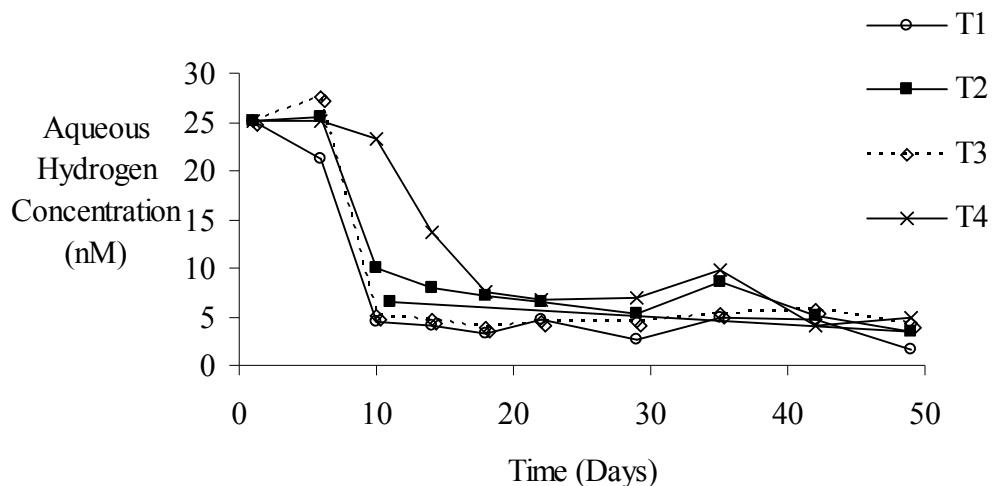


Figure 5.6 Headspace hydrogen levels in the four characterization study microcosm treatments: T1 = controls, T2 = acetate, T3 = molybdate, T4 = acetate + molybdate. Acetate (2mM) was added to T2 and T4 on day 2 and molybdate (2mM) was added to T3 and T4 on day 2. Data represents the average of duplicate samples.

H<sub>2</sub> began declining in all of the microcosms by day 3. Aqueous hydrogen concentrations in the T1 and T3 microcosms decreased particularly quickly and reached apparent thresholds (4.5 nM) by day 10. Presumably, the utilization of hydrogen by



hydrogenotrophic methanogens to produce methane (Figure 5.4) in the T1 and T3 microcosms explains why the hydrogen threshold was rapidly achieved in these treatments. The aqueous hydrogen concentrations in the T2 and T4 microcosms decreased at a slightly slower rate, compared with the T1 and T3 microcosms. This could theoretically be attributed to the addition of acetate to the T2 and T4 microcosms. That is, it makes sense that in the presence of an alternative electron donor, the demand for H<sub>2</sub> would not be as high as in microcosms that were not amended with acetate. The final threshold H<sub>2</sub> concentrations measured in the T2 and T4 microcosms were approximately 6.5 nM. Similar H<sub>2</sub> threshold concentrations were measured in previous studies involving methanogenic sediments (Lovley et al., 1994c), as discussed in Chapter 3.

Thus, the converging lines of evidence provided by measurement of TEAs and/or their reduction products, along with acetate and hydrogen thresholds, all lead to the same conclusion about the dominant TEAPs in the wetland sediment. That is, the wetland source sediment used in this study appears to be slightly sulfate-reducing, but methanogenesis appears to occur to a greater extent. The results of this experiment agree with previous characterization of the Aberdeen Proving Ground sites WB23 and WB24, which as illustrated in Figure 4.1, found that in the layers just below the ground surface, methanogenesis was the dominant TEAP (Lorah et al., 1997).

## **5.2 Methanogenic Acetate Threshold Experiment**

Because methanogenesis was found to be the dominant naturally-occurring TEAP in the sediment in this and previous studies, it made sense to carefully measure the acetate threshold under methanogenic conditions in the wetland sediment. Therefore, this was the focus of the first acetate threshold experiment. This was convenient because it

was expected that the acetate thresholds under methanogenic conditions would be in the  $\mu\text{M}$  range and higher than the thresholds measured under more favorable TEAPs. This was important because acetate thresholds of less than  $1\mu\text{M}$  could not be reliably quantified using the enzymatic-HPLC method and may have occurred under Fe(III)-reducing or dehalorespiring conditions (Sung et al., 2006). The following paragraphs summarize the results of the experiment designed to determine the acetate threshold when methanogenesis was the dominant TEAP.

The methane threshold experiment was conducted over 60 days in duplicate 2000 ml reactors (R1 and R2). The initial conditions measured in each reactor, on day 1 of the experiment are summarized in Table 5.1. Acetate was added to both reactors on day 2 to yield 2 mM.

Table 5.1. Background levels measured on day 1 of the methanogenic threshold experiment.

	<b>R1</b>	<b>R2</b>
<b>Total Pressure (psi)</b>	1	1
<b>Acetate (<math>\mu\text{M}</math>)</b>	80	106
<b>CH<sub>4</sub> (<math>\mu\text{mol}</math>)</b>	1057	975
<b>H<sub>2</sub> (nM)</b>	12	8

Figure 5.7 displays methane and acetate levels in the duplicate reactors. Acetate concentrations of  $4259\mu\text{M}$  and  $3644\mu\text{M}$  were measured in the reactors on day 7. Thus, it seems likely that there was some fermentative or homoacetogenic production of acetate in the reactors, in addition to the 2mM acetate amendment made on day 2 of the experiment.

Methane production was consistent through day 27 based on Figure 5.7. At least some of this methane was the result of metabolism of acetate, which decreased between day 8 and day 27. Acetate levels then appeared to level off after 27 days. Methane production also leveled off during this period. To evaluate whether acetate concentrations had reached a threshold concentration, a straight line was fitted to the five data points obtained between days 27 and 36 and the slope of this line was compared statistically to zero. The p value of the statistical slope analysis ( $\alpha/2=0.025$ ) was determined to be 0.389. Therefore, the slope of the line did not appear to be statistically different from zero, and the average acetate concentration from day 27 through day 36 (40  $\mu\text{M}$ ) was assumed to reflect the acetate threshold. However, this concentration was substantially higher than the final acetate concentration measured under methanogenic conditions in the characterization study.

Therefore, to evaluate whether 40  $\mu\text{M}$  represented the true acetate threshold, a second dose of acetate (0.89 mM) was added to the two reactors on day 36. Subsequently, acetate concentrations began declining and actually decreased below the presumptive threshold concentration observed between days 27 and 36. By day 46, the acetate concentration appeared to be at a threshold concentration. The threshold concentration was determined in the same way as for the first acetate spike, using the acetate data for days 46 to 56, and giving an average value of 10  $\mu\text{M}$  in this case. The methane increased slightly after day 42, presumptively due to acetate metabolism.

After spiking the reactors a second time with acetate, there was a slight spike in the hydrogen concentration (Figure 5.8) was observed. It is possible that reverse homoacetogenesis, which converts acetate to  $\text{H}_2$  and  $\text{CO}_2$  contributed to this  $\text{H}_2$  spike.

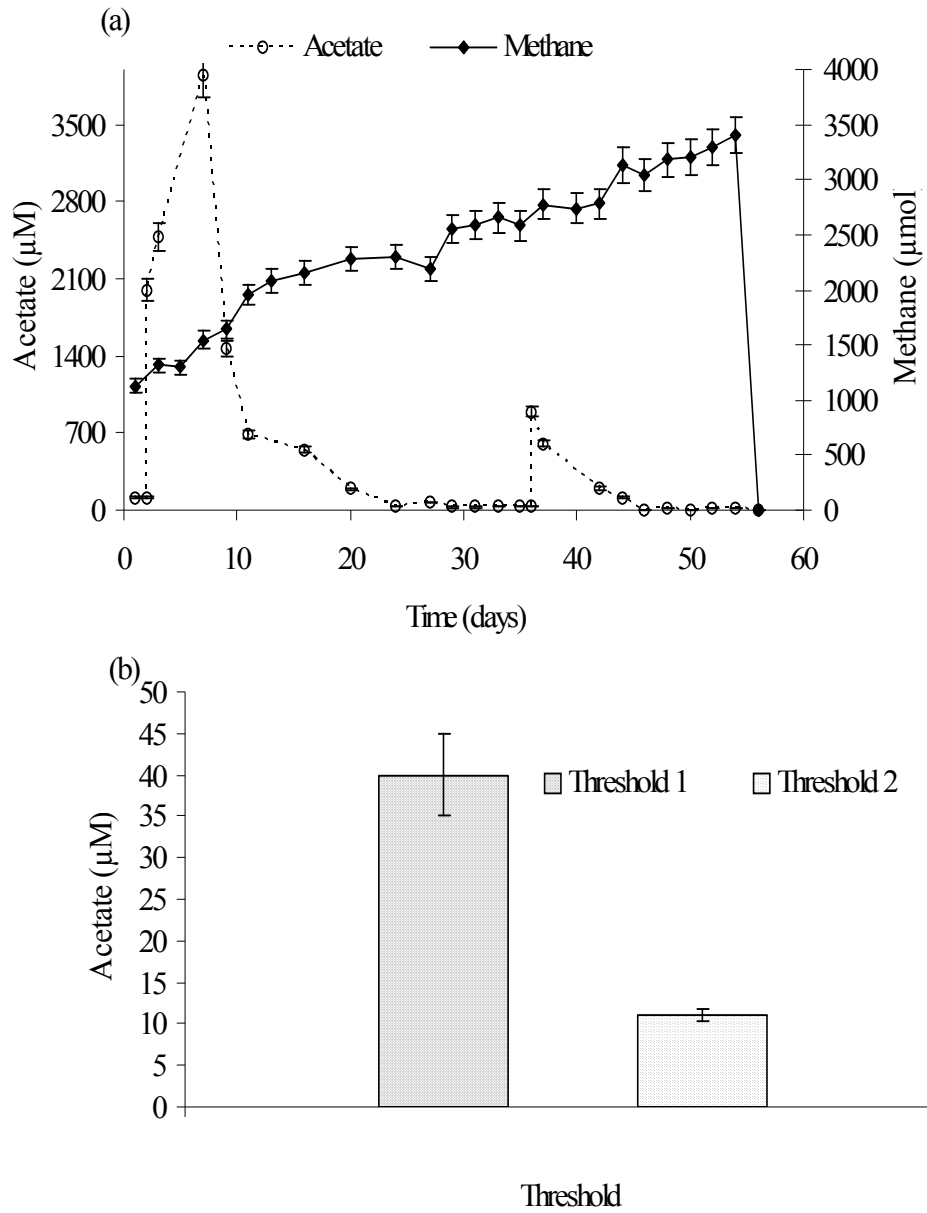


Figure 5.7. Acetate and methane data from the methanogenic acetate threshold experiments (a) Average acetate and methane levels for the duplicate reactors (R1 and R2). The acetate concentration on day 2 is an assumed value equal to the acetate spike of 2 mM. (b) Average acetate threshold concentrations for each threshold determined. Error bars represent  $\pm$  one standard deviation.

Reverse homoacetogenesis becomes thermodynamically feasible when H<sub>2</sub> concentrations are very low. For example, Lee and Zinder (1988) calculated that at standard temperature, oxidation of acetate to H<sub>2</sub> and CO<sub>2</sub> is thermodynamically favorable when H<sub>2</sub> ≤ 24 μM. Thus, it is conceivable that this process was at least temporarily stimulated by the combination of high acetate and low H<sub>2</sub> levels. It is also possible that the acetate spike on day 36 caused a shift in the fermentative processes by temporarily increasing the flow of electrons from organic products like acetate to H<sub>2</sub>.

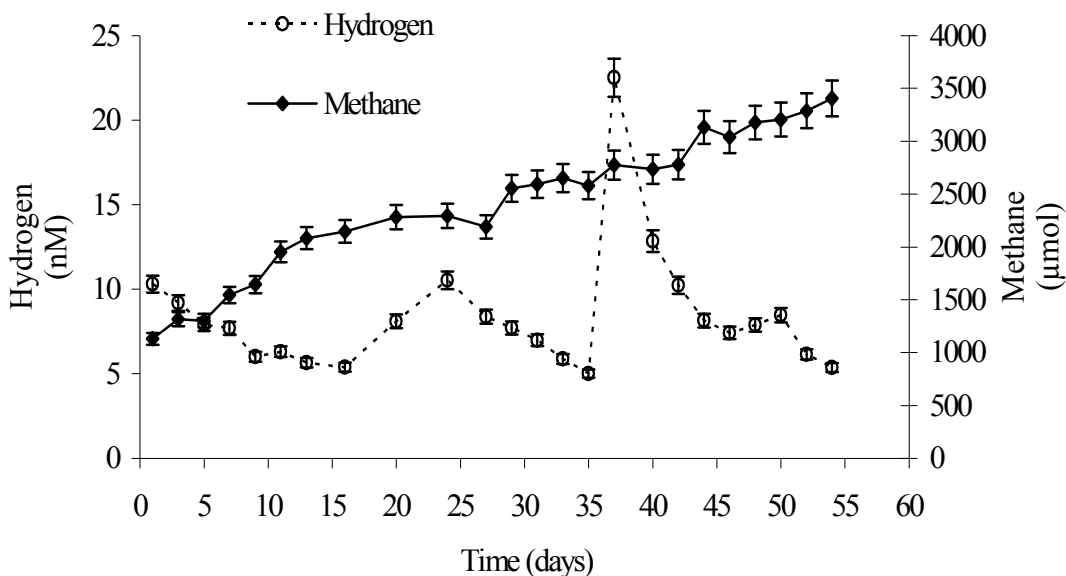


Figure 5.8. Methane and hydrogen levels in the methanogenic acetate threshold experiment. Data plotted is the average in duplicate reactors (R1 and R2) and error bars represent one standard deviation.

The acetate thresholds for methanogenic zones of contaminated sediment found in this study (40 μM and 10 μM) are lower than the acetate threshold concentrations measured in previous studies involving pure cultures of acetoclastic methanogens, which ranged from approximately 70 μM up to 1.2 mM (Westermann et al., 1989; Jetten et al.,

1992). However, the experimental approach used in some of these investigations was quite different than that used in the current study. For example, in some cases, the thresholds were measured in non-growing cells. Presumably, differences in the experimental methods used and/or kinetic characteristics of the organisms studied explain the differences in the thresholds measured in the pure cultures and current studies. A better point of comparison may be the previously mentioned study of methanogenic sediment conducted by Lovley and Phillips (1987b) in which the measured acetate thresholds (5.8  $\mu\text{M}$ ) were very similar to those determined in this study.

### **5.3 Dehalorespiration Acetate Threshold Experiment**

Although no evidence of ongoing dehalorespiration was obtained in the characterization experiment, other studies had shown that organisms capable of dehalorespiration were present in at least some regions of the wetland sediment. Therefore, it seemed reasonable to expect that the addition of chlorinated VOCs to the wetland sediment microcosms would stimulate dehalorespiring populations and allow assessment of acetate thresholds controlled by dehalorespiration. The following paragraphs summarize the results of the two-part experiments designed to determine the acetate thresholds in wetland sediment under dehalorespiring conditions. The dehalorespiring experiments were conducted as described in Section 4.2.2.4. The experiment that did not go to completion is analyzed first, followed by the dehalorespiring threshold experiment completed with sediments from site WB35.

The first dehalorespiring threshold experiment was conducted over 11 days using duplicate 160-ml microcosms. The initial samples were taken on day 1, as noted in section 4.2.2.4. However, the acetate analyses for those samples was not completed until

day 6 of the experiment due to complications with the HPLC. In the meantime, all of the amendments described in Table 4.2 were added on day 2 when the initial, background levels were still unknown. The microcosms were then sampled again on day 3, and the results of those analyses are presented in Table 5.2. As can be seen in Table 5.2, there were apparently high levels of endogenous acetate in the microcosms (see C1 and PCE1). As a result, the desired conditions, which were to limit acetate and force the thresholds to occur were not attained, precluding this experiment from occurring as planned. One option for re-creating acetate-limiting conditions would have been to add more PCE. However, previous experience with these sediments indicated that the PCE levels that would have been necessary were above what had been shown to be toxic to the indigenous microorganisms. Therefore, to avoid toxicity to the microcosms, PCE could not be re-amended to excess concentrations, and the PCE and acetate had to be completely degraded prior to amending the microcosms again. Unfortunately, the time available for this study was not sufficient to allow that to occur.

Representative PCE and TCE data for this experiment are presented in Figure 5.9 for the Mix microcosms which were amended with both acetate (80  $\mu$ M, nominal concentration) and PCE (40  $\mu$ M, nominal concentration). As illustrated in Figure 5.9, PCE removal was occurring, as demonstrated by the declining PCE and TCE production. However, the PCE removal was proceeding at an extremely slow rate, even in the presence of excess amounts of acetate. For future studies, this experiment will be continued once the PCE and acetate have sufficiently degraded that the microcosms can be re-spiked with PCE and acetate to meet the desired experimental ratio.

Table 5.2. Average acetate, CH<sub>4</sub>, H<sub>2</sub>, PCE and TCE for the duplicate microcosms in samples taken on day 3 of the experiment. C1 are the controls, with the other microcosms amended on day 2 as follows; Ac-1 with 80 μM acetate, PCE1 with 40 μM PCE and Mix1 with both 80 μM acetate and 40 μM PCE

	<b>C1</b>	<b>Ac1</b>	<b>PCE1</b>	<b>Mix1</b>
<b>Acetate (μM)</b>	687	780	703	778
<b>CH<sub>4</sub> (μmol)</b>	51	55	57	56
<b>H<sub>2</sub> (nM)</b>	5	4	5	5
<b>PCE (μmol)</b>	0	0	40	239
<b>TCE (μmol)</b>	0	0	0	0

Figure 5.9. PCE removal and TCE production data for the Mix1 microcosms, in the presence of excess amounts of acetate (~10 fold). Data plotted is the average of duplicate samples and error bars represent one standard deviation.

The second set of dehalorespiring experiments was conducted using sediment slurry microcosm prepared with sediment from site WB35 and groundwater from site WB24. Once no measurable level of a given chlorinated solvent was present, single headspace and slurry samples were collected from the 160-ml microcosms for analysis of H<sub>2</sub>, CH<sub>4</sub> and aqueous acetate levels. A summary of these data is represented in Table 5.3.

Sung et al. (2006) reported an acetate threshold of  $3.0 \pm 2.1$  nM under PCE-respiring conditions of a bacterial isolate, *Geobacter lovleyi* sp. strain SZ. Based on this information, acetate levels were expected to go below the lowest concentration (1 μM)



that could reliably be quantified in this study using the enzymatic-HPLC method (King, 1991).

Table 5.3. Acetate, H<sub>2</sub> and CH<sub>4</sub> levels measured in wetland sediments collected from site WB35 following the dechlorination of PCE and CT in the absence of any added electron donors.

Parameter	Chlorinated VOC Added	
	PCE	CT
Acetate (μM)	8.6	12
H <sub>2</sub> (nM)	6.9	16.9
CH <sub>4</sub> (μmol)	0.812	0.34

However, the acetate concentrations measured to date in the microcosms amended with chlorinated compounds fell within the range of the enzymatic-HPLC method and, in fact, were similar to the threshold concentrations detected in the methanogenic sediment during the characterization (10 μM) and threshold experiments (10 μM). In contrast, the thermodynamic framework outlined in Chapter 3, predicts instead higher thresholds under methanogenic conditions than under dehalorespiring conditions. There are several potential explanations for the relatively high acetate thresholds detected in the microcosms amended with chlorinated VOCs. One possibility is that kinetic constants like  $K_s$  (e.g., equation 3.3) were more important than reaction thermodynamics in controlling the acetate thresholds in the sediment microcosms.  $K_s$  values in particular vary significantly among different bacterial strains and are not related to the TEAP. It is also possible that the chlorinated VOCs were not transformed by metabolic TEAPs, in which case, the concept of thresholds may not be applicable. In particular, organisms that respire CT, have not been identified, so it is not known whether or not this compound

can serve as TEA. In fact, CT was converted at similar rates in viable and sterile sediment controls (Devilleir, personal communication), which suggests that CT transformation occurred abiotically. Further, lesser chlorinated methanes did not accumulate to a significant extent, implying that reductive dehalogenation processes did not play a major role in CT removal. On the other hand, dehalorespiration using PCE has been observed in several bacterial strains and presumably was feasible in the wetland sediment. However, it is possible that acetate did not serve as the electron donor for dehalorespiration of PCE (or other chlorinated VOCs). For example, several PCE-respiring bacteria can use H<sub>2</sub> as an electron donor. In this case, the acetate concentration in the chlorinated VOC-amended microcosms would not have been determined by dehalorespiration and instead may have been controlled by the dominant acetotrophic TEAP, which could have been methanogenesis or sulfate reduction.

## CHAPTER 6

### SUMMARY AND CONCLUSIONS

This research project examined the effectiveness of using acetate concentrations as a global indicator of the dominant TEAPs in contaminated sediment. The overall goal of this study was to evaluate if characteristic acetate threshold concentrations exist for various TEAPs and, therefore, changes in *in situ* acetate can provide an additional piece of evidence that contaminant biodegradation is occurring in the subsurface.

This evaluation was performed using contaminated sediment and groundwater microcosms and reactors. In general, the results of these experiments demonstrated that characteristic acetate thresholds do exist for methanogenic and dehalorespiring conditions. However, for the experimental condition studied here the methanogenic conditions exhibited similar acetate threshold levels to that of dehalorespiring conditions. Thus, the results obtained under dehalorespiring conditions point out that a complication of interpreting acetate thresholds may arise if acetate is not the primary electron donors sustaining a given TEAP.

The results of this study provide some additional insight into the potential for using acetate thresholds as a TEAP indicator for *in situ* bioremediation applications. However, it is apparent that the use of acetate as a microbial footprint is subject to challenges of interpretation and application as noted for the other footprints currently used in the field. For example, interpretation of acetate thresholds may be complicated by TEAPs in which acetate is not the primary electron donor as noted above. With regard to application, difficulties were encountered in terms of acetate quantification,

similar to the application of H<sub>2</sub> measurements used for the same purpose. Specifically, in this study, the enzymatic-HPLC analytical method described by King (1991) proved to be challenging to perform with respect to selection of appropriate conditions for variables such as reaction pH, elution phase and gradients, and reagent concentrations.

Nonetheless, the overall results of this research suggest that characteristic acetate thresholds may potentially be useful for defining predominant TEAPs in contaminated sediments and it is suggested that further research be performed with such sediments to identify the acetate thresholds under other TEAPs and provide a more complete assessment of the technique.

## **APPENDIX**

**Appendix A.1**  
**Characterization Experimental Results**

Methane-Average of duplicates in ( $\mu\text{mol}$ )				
Time (days)	T1	T2	T3	T4
1	0.0	0.0	0.0	0.0
6	10.7	12.4	11.2	13.2
10	12.0	17.4	13.0	18.9
14	12.3	30.0	12.9	36.6
18	11.7	43.7	11.8	52.8
22	11.5	59.6	17.1	66.3
29	11.2	59.7	11.7	64.3
35	10.8	48.5	12.0	58.7
42	10.3	51.9	12.7	61.5
49	10.2	37.0	10.9	60.4

Aqueous Hydrogen-Average of duplicates (nM)				
Time (days)	T1	T2	T3	T4
1	25.2	25.1	25.1	25.2
6	21.2	25.6	27.6	25.2
10	4.4	10.0	5.2	23.3
14	4.0	8.0	4.6	13.8
18	3.4	7.2	3.9	7.5
22	4.6	6.5	4.5	6.7
29	2.7	5.4	4.5	6.9
35	5.0	8.5	5.2	9.9
42	4.8	5.1	5.7	4.1
49	1.7	3.6	4.2	4.9

Appendix A.1. Methane production and hydrogen consumption during the characterization experiment. Data shown is the average of duplicate samples.

## Appendix A.2

### Characterization Experimental Results

(a)

<b>Bioavailable Ferrous and Ferric Iron-Average of duplicates (<math>\mu\text{mol}</math>)</b>								
	T1		T2		T3		T4	
Time (days)	Fe(II)	Fe(III)	Fe(II)	Fe(III)	Fe(II)	Fe(III)	Fe(II)	Fe(III)
<b>1</b>	245	200	170	150	79.75	665.25	97.5	322.5
<b>7</b>	147.5	72.5	22.5	172.5	72.5	947.5	0	0
<b>11</b>	320	150	295	125	287.25	257.75	270	50
<b>15</b>	47.5	197.5	72.5	97.5	47.5	72.5	0	170
<b>19</b>	0	70	22.5	72.5	159.75	485.25	147.5	197.5
<b>23</b>	0	0	0	0	0	0	0	0

(b)

<b>Aqueous Acetate-Average of duplicates (<math>\mu\text{M}</math>)</b>				
Time (days)	T1	T2	T3	T4
<b>1</b>	100	117	276	76
<b>7</b>	256	1011	44	647
<b>11</b>	49	68	29	61
<b>19</b>	72	490	107	61
<b>23</b>	6	6	10	7

Appendix A.2. (a) The bioavailable ferrous and ferric iron levels measured during the characterization experiment. Data shown is the average of duplicate samples. (b) Represents the aqueous acetate levels measured during the experiment. Data shown is the average of duplicate samples.

## Appendix B.1

<b>Methane Threshold Experimental Data</b>											
	Cumulative Methane (umol/volume headspace)				Aqueous Hydrogen (nM)				Aqueous Acetate (uM)		
<b>Day</b>	<b>R1</b>	<b>R2</b>	<b>Average</b>		<b>R1</b>	<b>R2</b>	<b>Average</b>		<b>R1</b>	<b>R2</b>	<b>Average</b>
<b>0</b>	0	0	0		0	0	0				
<b>1</b>	1058	975	1017		12	8	10		76	150	113
<b>2</b>									76	150	113
<b>3</b>	1305	1148	1227		10	8	9		2000	2000	2000
<b>5</b>	1381	1134	1258		8	8	8		2076	2875	2475.5
<b>7</b>	1654	1360	1507		8	8	8		4259	3644	3951.5
<b>9</b>	1759	1452	1606		6	6	6		930	2012	1471
<b>11</b>	1965	1741	1853		6	7	6		740	633	686.5
<b>13</b>	2132	1862	1997		6	6	6		451	637	544
<b>16</b>	2191	1919	2055		5	5	5		122	273	197.5
<b>20</b>	2276	2046	2161		8	8	8		42	35	38.5
<b>24</b>	2307	2058	2182		11	11	11		76	71	73.5
<b>27</b>	2527	2297	2412		10	7	8		31	24	27.5
<b>29</b>	2527	2297	2412		8	7	8		31	25	28
<b>31</b>	2553	2333	2443		7	7	7		44	31	37.5
<b>33</b>	2418	2388	2403		5	6	6		25	34	29.5
<b>35</b>	2351	2774	2563		4	6	5		25	34	29.5
<b>37</b>	2775	2774	2775		31	14	23		890	890	890
<b>40</b>	2679	2735	2707		15	11	13		224	980	602
<b>42</b>	2730	2779	2755		11	9	10		106	293	199.5
<b>44</b>	3015	3131	3073		9	8	9		147	86	116.5
<b>46</b>	3021	3057	3039		8	7	8		5	8	6.5
<b>48</b>	3156	3198	3177		8	8	8		13	14	13.5
<b>50</b>	3137	3274	3206		8	9	9		4	4	4
<b>52</b>	3208	3366	3287		6	7	6.5		5	24	14.5
<b>54</b>	3306	3502	3404		4	6	5		12	18	15
<b>56</b>	3369	3623	3496		6	7	6.5		7	8	7.5

Figure B.1. Data from methanogenic acetate threshold experiment.



## Appendix B.2

Total Suspended Solids		Total Pressure		
Reactor	Total Solids (g/L)	Day	R1	R2
R1	38	3	1	1
R2	32	5	1.5	1.2
		7	2	2
		9	4	4
		11	3	2
		13	2	1
		16	2	1
		20	4	3.9
		27	4	4
		29	3.5	3.8
		33	3.8	3.5
		35	3.6	3.6
		37	3.5	3.2
		40	3.8	3.2
		42	4	3.8
		44	4.2	4
		50	4	4
		52	3.8	4.1
		54	4	3.8

Figure B.1. Data from methanogenic acetate threshold experiment. (a) Represents total suspended solids in each reactor. (b) Represents the total pressure monitored at the beginning of each sampling event. This table is not all inclusive of each sampling event, rather a partial table of various pressures monitored throughout the experiment.

## REFERENCES

- APHA (1989). Standard Methods for the Examination of Water and Wastewater. Washington D.C., American Public Health Office.
- Barcelona, M. J. (1980). "Dissolved organic carbon and volatile fatty acids in marine sediment pore waters." *Geochimica et Cosmochimica Acta* 44: 1977-1984.
- Becker, J. G. (1998). Characterization of anaerobic microbial communities that adapt to 3-chlorobenzoate and 2-chlorophenol: an integrated approach of chemical measurements and molecular evaluations. Ph.D. Dissertation. Civil and Environmental Engineering. Evanston, Illinois, Northwestern University.
- Becker, J. G., E. A. Seagren and H. Montas (2005). Theoretical and experimental evaluation of acetate thresholds as a monitoring tool for in situ bioremediation, University of Maryland.
- Chapelle, F. H., S. K. Haack, P. Adriaens, M. A. Henry and P. M. Bradley (1996). "Comparison of E(h) and H<sub>2</sub> measurements for delineating redox processes in a contaminated aquifer." *Environmental Science & Technology* 30(12): 3565-3569.
- Chapelle, F. H. and D. R. Lovley (1992). "Competitive-Exclusion of Sulfate Reduction by Fe(III)-Reducing Bacteria - a Mechanism for Producing Discrete Zones of High Iron Groundwater." *Ground Water* 30(1): 29-36.
- Chapelle, F. H., P. B. McMahon, N. M. Dubrovsky, R. F. Fujii, E. T. Oaksford and D. A. Vroblesky (1995). "Deducing the Distribution of Terminal Electron-Accepting Processes in Hydrologically Diverse Groundwater Systems." *Water Resources Research* 31(2): 359-371.
- Conrad, R. (1996). "Soil microorganisms as controllers of atmospheric trace gases (H<sub>2</sub>, CO, CH<sub>4</sub>, N<sub>2</sub>O, and NO)." *Microbiological Reviews* 60(4): 609.
- Cozzarelli, I. M., M. J. Baedecker, J. M. Fischer and C. S. Phinney (1994). "Groundwater Contamination by Petroleum-Hydrocarbons - Natural Biodegradation in a Dynamic Hydrologic and Geochemical System." *Abstracts of Papers of the American Chemical Society* 208: 80-ENVR.
- Cozzarelli, I. M., R. P. Eganhouse and M. J. Baedecker (1990). "Transformation of Monoaromatic Hydrocarbons to Organic-Acids in Anoxic Groundwater Environment." *Environmental Geology and Water Sciences* 16(2): 135-141.

- Cozzarelli, I. M., J. S. Herman and M. J. Baedeker (1995). "Fate of Microbial Metabolites of Hydrocarbons in a Coastal Plain Aquifer: The Role of Electron Acceptors." *Environmental Science & Technology* 29(2): 458-469.
- Goodwin, S., Conrad, R., Zeikus and J. G. (1988). "Influence of pH on Microbial Hydrogen Metabolism in Diverse Sedimentary Ecosystems." *Applied and Environmental Microbiology* 54(2): 590-593.
- Gossett, J. M. (1987). "Measurement of Henry's Law Constants for C1 and C2 Chlorinated Hydrocarbons." *Environmental Science & Technology* 21(2): 202-208.
- He, Q. and R. A. Sanford (2004). "Acetate threshold concentrations suggest varying energy requirements during anaerobic respiration by *Anaeromyxobacter dehalogenans*." *Applied and Environmental Microbiology* 70(11): 6940-6943.
- Hoehler, T. M., M. J. Alperin, D. B. Albert and C. S. Martens (1998). "Thermodynamic control on hydrogen concentrations in anoxic sediments." *Geochimica Et Cosmochimica Acta* 62(10): 1745-1756.
- Iannotti, E. L., D. Kafkewit, M. J. Wolin and M. P. Bryant (1973). "Glucose Fermentation Products of *Ruminococcus-Albus* Grown in Continuous Culture with *Vibrio-Succinogenes* - Changes Caused by Interspecies Transfer of H<sub>2</sub>." *Journal of Bacteriology* 114(3): 1231-1240.
- Jetten, M. S. M., A. J. M. Stams and A. J. B. Zehnder (1992). "Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanotrix soehngenii* and *Methanosarcina spp.*" *FEMS Microbiology Reviews* 88: 181-198.
- Kampbell, D. H., Wiedemeier, T. H., Hansen and J. E. (1996). "Intrinsic bioremediation of fuel contamination in ground water at a field site." *Journal of Hazardous Materials* 49(2-3): 197-204.
- Keating, E. H. and J. M. Bahr (1998). "Reactive transport modeling of redox geochemistry: Approaches to chemical disequilibrium and reaction rate estimation at a site in northern Wisconsin." *Water Resources Research* 34(12): 3573-3584.
- King, G. M. (1991). "Measurement of Acetate Concentrations in Marine Pore Waters by Using an Enzymatic Approach." *Applied and Environmental Microbiology* 57(12): 3476-3481.
- Lee, M. J. and S. H. Zinder (1988). "Hydrogen Partial Pressures in Thermophilic Acetate-Oxidizing Methanogenic Coculture." *Applied and Environmental Microbiology* 54(6).
- Löffler, F. E., J. M. Tiedje and R. A. Sanford (1999). "Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halo-respiratory physiology." *Applied and Environmental Microbiology* 65(9): 4049-4056.

Lorah, M. M., L. D. Olsen, B. L. Smith, M. A. Johnson and W. B. Fleck (1997), Natural Attenuation of Chlorinated Volatile Organic Compounds in a Freshwater Tidal Wetland, Aberdeen Proving Ground, Maryland, WRIR-97-4171, D. o. Interior, United States Geological Survey

Lovley, D. R. (1985). "Minimum Threshold for Hydrogen Metabolism in Methanogenic Bacteria." *Applied and Environmental Microbiology* 49(6): 1530-1531.

Lovley, D. R. (1997a). "Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers." *Journal of Industrial Microbiology & Biotechnology* 18(2-3): 75-81.

Lovley, D. R., F. H. Chapelle and J. C. Woodward (1994c). "Use of Dissolved H<sub>2</sub> Concentrations to Determine Distribution of Microbially Catalyzed Redox Reactions in Anoxic Groundwater." *Environmental Science & Technology* 28(7): 1205-1210.

Lovley, D. R. and S. Goodwin (1988). "Hydrogen Concentrations as an Indicator of the Predominant Terminal Electron Accepting Reactions in Aquatic Sediments." *Geochemica et Cosmochimica Acta* 52: 2993-3003.

Lovley, D. R. and E. J. Phillips (1987a). "Rapid Assay for Microbially Reducible Ferric Iron in Aquatic Sediments." *Applied and Environmental Microbiology* 53(7): 1536-1540.

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

Lu, X. X., S. Tao, T. Bosma and J. Gerritse (2001). "Characteristic hydrogen concentrations for various redox processes in batch study." *Journal of Environmental Science and Health Part a-Toxic/Hazardous Substances & Environmental Engineering* 36(9): 1725-1734.

Madsen, E. L. (1997). Methods for determining biodegradability. Manual of Environmental Microbiology. C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach and M. V. Walter. Washington D.C., ASM Press.

McCarty, P. L. (1972). Energetics of organic matter degradation. Water Pollution Microbiology. R. Mitchell. New York, Wiley-Interscience: 91-118.

McGuire, J. T., E. W. Smith, D. Long, D. W. Hyndman, M. J. Klug and M. A. Velbel (2000). "Temporal variations in parameters reflecting terminal-electron-accepting processes in an aquifer contaminated with waste fuel and chlorinated solvents." *Chemical Geology* 169(3-4): 471-485.

- Novelli, P. C., A. R. Michelson, M. I. Scranton, G. T. Banta, J. E. Hobbie and R. W. Howarth (1988). "Hydrogen and acetate cycling in two sulfate-reducing sediments: Buzzards Bay and Town Cove, Mass." *Geochimica et Cosmochimica Acta* 52: 2477-2486.
- NRC (1993). *In situ* Bioremediation: When does it work? Washington D.C., National Academy Press.
- NRC (2000). Natural Attenuation for Groundwater Remediation. Washington D.C., National Academy Press.
- Patidar, S. K. and V. Tare (2004). "Effect of molybdate on methanogenic and sulfidogenic activity of biomass." *Bioresource Technology* 96: 1215-1222.
- Plummer, L. N., J. F. Busby, R. W. Lee and B. B. Hanshaw (1990). "Geochemical Modeling of the Madison Aquifer in Parts of Montana, Wyoming, and South-Dakota." *Water Resources Research* 26(9): 1981-2014.
- Postma, D., Jakobsen and R. (1996). "Redox zonation: Equilibrium constraints on the Fe(III)/SO<sub>4</sub><sup>2-</sup> reduction interface." *Geochimica et Cosmochimica Acta* 60(17): 3169-3175.
- Reeburgh, W. S. (1983). "Rates of Biogeochemical Processes in Anoxic Sediments." *Annual Review of Earth and Planetary Sciences* 11: 269-298.
- Rittmann, B. E. and P. L. McCarty (2001). *Environmental Biotechnology: Principles and Applications*. New York, The McGraw-Hill Companies.
- Rittmann, B. E., E. Seagren, B. A. Wrenn, A. J. Valocchi, C. Ray and L. Raskin (1994). *In situ* Bioremediation. Park Ridge, Noyes Publications.
- Salanitro and J. P. (1993). "The Role of Bioattenuation in the Management of Aromatic Hydrocarbon Plumes in Aquifers." *Ground Water Monitoring and Remediation* 13(4): 150-161.
- Seagren, E. A. and J. G. Becker (2002). Review of natural attenuation of BTEX and MTBE in groundwater. *Practice of Hazardous, Toxic, and Radioactive Waste Management*. 6: 156-172.
- Shaw, D. G. and D. J. McIntosh (1990). "Acetate in Recent Anoxic Sediments: Direct and Indirect Measurements of Concentration and Turnover Rates." *Estuarine, Coastal and Shelf Science* 31: 775-788.
- Smith, R. L. (2002). Determining the Terminal Electron-Accepting Reaction in the Saturated Subsurface. *Manual of Environmental Microbiology*: 743-752.

Stookey, L. (1970). "Ferrozine - a New Spectrophotometric Reagent for Iron." *Analytical Chemistry* 42(7): 779-&.

Stumm, W. and J. J. Morgan (1996). *Aquatic Chemistry*, John Wiley & Sons, Inc.

Sung, Y., Fletcher, K. F., Ritalaliti, K. M., Apkarian, R. P., Ramos-Hernandez, N., Sanford, R. A., Mesbah, N. M., Löffler and F. E. (2006). "*Geobacter lovleyi* sp nov strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium." *Applied and Environmental Microbiology* 72(4): 2775-2782.

Vroblesky, D. A., P. M. Bradley and F. H. Chapelle (1997). "Lack of correlation between organic acid concentrations and predominant electron-accepting processes in a contaminated aquifer." *Environmental Science & Technology* 31(5): 1416-1418.

Westermann, P., B. K. Ahring and R. A. Mah (1989). "Threshold Acetate Concentrations for Acetate Catabolism by Aceticlastic Methanogenic Bacteria." *Applied and Environmental Microbiology* 55(2): 514-515.

Wiedemeier, T. H., Swanson, M. A., Wilson, J. T., Kampbell, D. H., Miller, R. N., Hansen and J. E. (1996). "Approximation of biodegradation rate constants for monoaromatic hydrocarbons (BTEX) in ground water." *Ground Water Monitoring and Remediation* 16(3): 186-194.

Wiedemeier, T., J. T. Wilson, D. H. Kampbell, R. N. Miller and J. E. Hansen (1999), Technical Protocol for Implementing Intrinsic Remediation with Long-Term Monitoring for Natural Attenuation of Fuel Contamination Dissolved in Groundwater, Volumes I,II,III, U. S. D. o. Defense, Air Force Center for Environmental Excellence

Wilhelm, E., R. Battino and R. J. Wilcock (1977). "Low-Pressure Solubility of Gases in Liquid Water." *Chemical Reviews* 77(2): 219-262.

Zogorski, J. S., J. M. Carter, T. Ivahnenko, W. W. Lapham, M. J. Moran, B. L. Rowe, P. J. Squillance and P. L. Toccalino (2006), Volatile Organic Compounds in the Nation's Ground Water and Drinking-Water Supply Wells, Circular 1292, U. S. D. o. Interior, U.S. Geological Survey