Technical and Regulatory Guidance

Environmental Molecular Diagnostics

New Site Characterization and Remediation Enhancement Tools

April 2013

Prepared by
The Interstate Technology & Regulatory Council
Environmental Molecular Diagnostics Team

ABOUT ITRC

The Interstate Technology and Regulatory Council (ITRC) is a public-private coalition working to reduce barriers to the use of innovative environmental technologies and approaches so that compliance costs are reduced and cleanup efficacy is maximized. ITRC produces documents and training that broaden and deepen technical knowledge and expedite quality regulatory decision making while protecting human health and the environment. With private and public sector members from all 50 states and the District of Columbia, ITRC truly provides a national perspective. More information on ITRC is available at www.itrcweb.org. ITRC is a program of the Environmental Research Institute of the States (ERIS), a 501(c)(3) organization incorporated in the District of Columbia and managed by the Environmental Council of the States (ECOS). ECOS is the national, nonprofit, nonpartisan association representing the state and territorial environmental commissioners. Its mission is to serve as a champion for states; to provide a clearinghouse of information for state environmental commissioners; to promote coordination in environmental management; and to articulate state positions on environmental issues to Congress, federal agencies, and the public.

DISCLAIMER

EMD-2

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50 F Street NW, Suite 350, Washington, DC 20001

EMD-2 Revisions

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EXECUTIVE SUMMARY

Environmental molecular diagnostics (EMDs) is a collective term that describes a group of advanced and emerging techniques used to analyze the biological and chemical characteristics of environmental samples. Over the last decade, great advances have been made in adapting and applying EMDs for environmental site management. EMDs are becoming increasingly powerful, and standardized methods are being developed. As a result, their use is increasing rapidly, and a growing need exists for technical information and training on EMDs. EMDs provide additional and often unique information that supplements conventional data. The purpose of this technical and regulatory guidance document is to:

- Provide objective guidance on the best practices for using EMDs
- Demonstrate appropriate uses of EMDs, including their strengths and limitations
- Explain how to evaluate, apply, and interpret the results of EMDs.

The document provides detailed descriptions of each of the major EMDs, along with case studies of their uses and recommendations regarding the appropriate uses of these techniques. In addition, appendices are included to address the frequently asked questions regarding the underlying science, including stable isotope chemistry and fundamental molecular biology, so that interested project managers, stakeholders and regulators can easily find the information needed to understand the basis for each of the individual EMDs.

TYPES OF EMDS

EMDs can be classified into two major categories of analytical techniques: chemical techniques, specifically compound specific isotope analysis (CSIA), and a variety of molecular biological techniques (MBTs). CSIA measures the amounts of stable isotopes (typically carbon, hydrogen, or chlorine) in contaminants to determine the extent of specific chemical and biochemical reactions impacting the contaminant. As a contaminant degrades through natural or engineered processes, the relative amount of each stable isotope in the contaminant can change. In contrast, the isotopic composition of contaminants is largely unaffected by processes such as dilution that do not result in degradation of the contaminant. CSIA therefore can be useful for answering several important questions regarding a chemical's source, degradation mechanisms, and rate of degradation.

MBTs evaluate the types, abundance, and biochemical capabilities of microorganisms present in the environment. Often, the microorganisms responsible for the degradation of specific contaminants cannot be detected and quantified by conventional methods and MBTs can overcome these limitations. Several types of MBTs are available - some can be used to detect known microorganisms, others are also useful for quantification, some can be used to determine whether microorganisms are actively degrading specific contaminants, and some can identify currently unknown microorganisms involved in degradation.

To date, the most commonly used MBTs are polymerase chain reaction (PCR), quantitative PCR (qPCR) and reverse transcriptase-qPCR (RT-qPCR), and DNA microarrays. However other

MBTs have uses as well, including fluorescence in situ hybridization (FISH), enzyme activity probes (EAPs) and stable isotope probing (SIP). There are also several potentially useful microbial fingerprinting techniques, including phospholipid fatty acid (PLFA) analysis, denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (T-RFLP).

EMDs have application in each phase of environmental site management, including site characterization, remediation, monitoring, and closure activities (See Figure ES-1). EMDs can provide unique information valuable in conjunction with more conventional data.

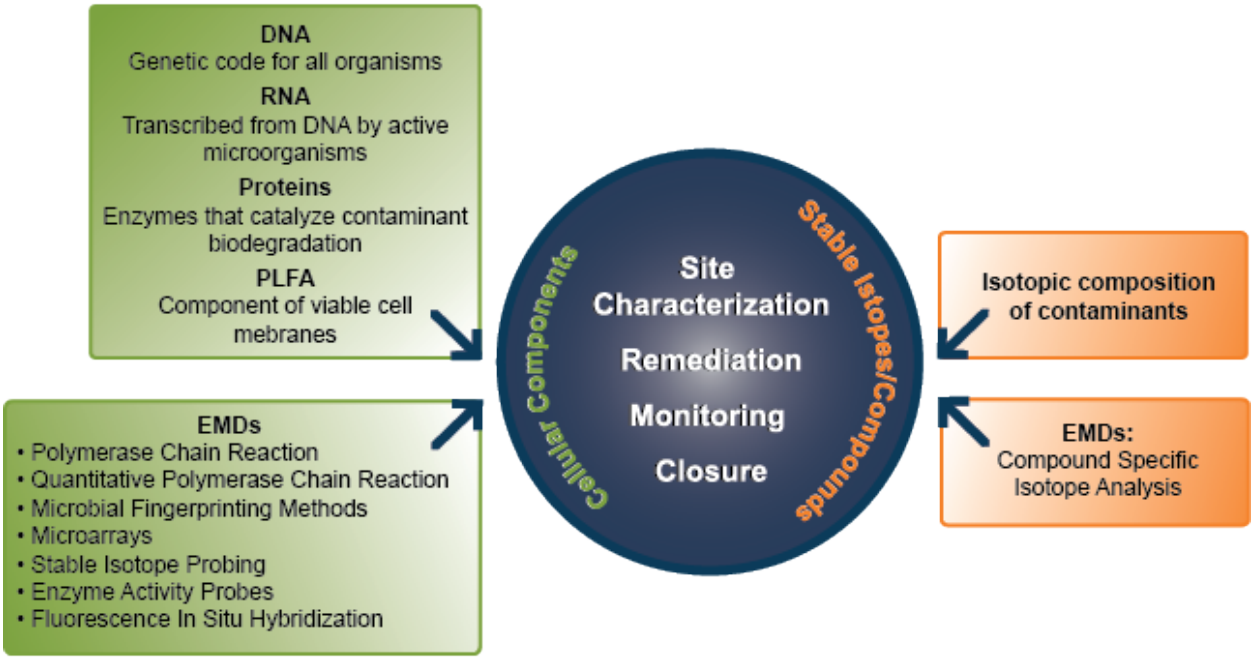

Figure ES-1. Overview of EMDs.

USING EMDS

EMDs have been used at hundreds of environmental cleanup sites in the US (and also at sites around the world). Figure ES-2 includes cumulative data for numbers of projects by state from 2009 to 2012. The data are from two commercial laboratories, so the data may not fully represent the actual number of EMD projects completed during that time. The states were assigned to the projects based on the information available to the laboratories. There is uncertainty in some of the project locations because of client confidentiality. The map is intended to provide a relative understanding of the usage of EMDs at this time, and not to provide definitive numbers of projects.

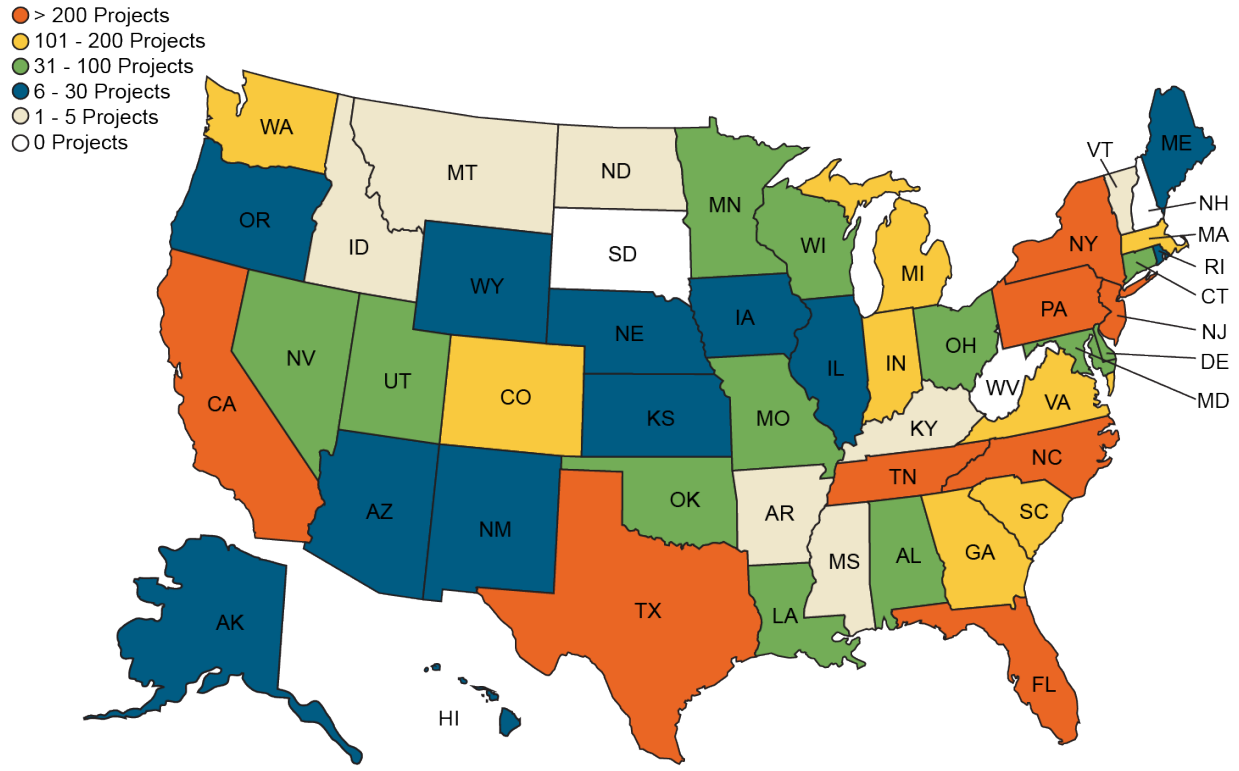

Figure ES-2. EMD projects by state.

EMDs can provide the following benefits:

- Improve the management of contaminated sites
- Determine whether biotic or abiotic degradation is occurring at a site
- Identify specific contaminant sources or reveal whether multiple sources of contamination are present (for instance, isotopic fractionation)
- Improve evaluation and decision making for remediation strategies
- Identify degradation pathways and their degree of completion
- Identify the need for enhancements such as chemical amendments or bioaugmentation
- Estimate degradation rates, for instance, CSIA using the Rayleigh equation (see Appendix C.12)
- Aid in monitoring program decisions
- Provide complementary data to support site closure and other site management decisions

Figure ES-3 describes potential uses of EMDs in each phase of site management.

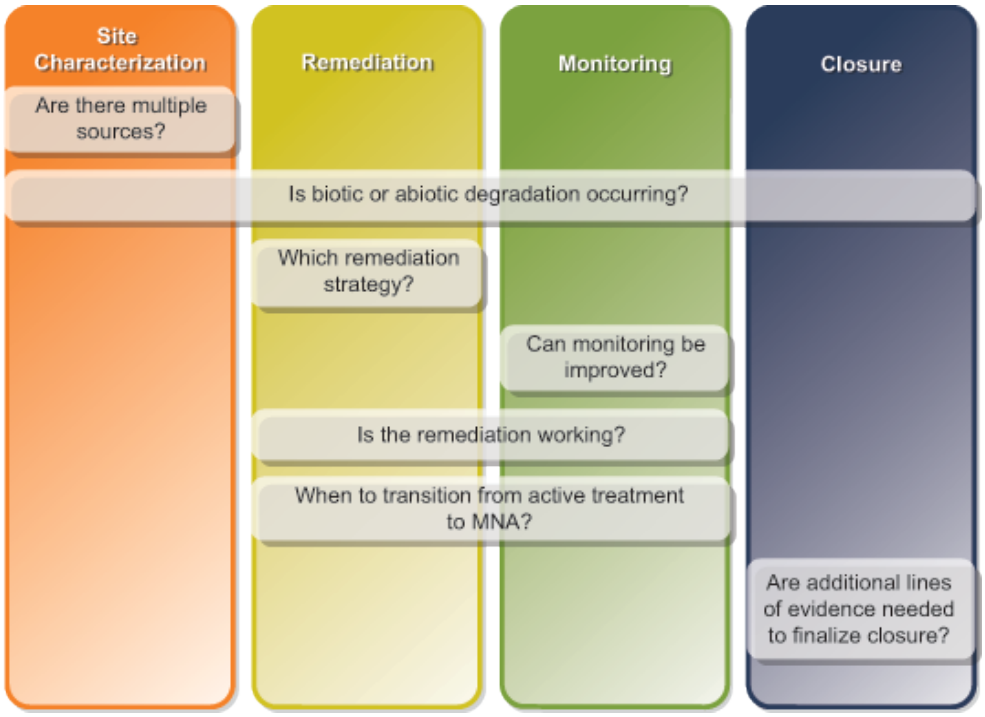

Figure ES-3. Potential uses of EMDs in site management.

The key information available from such tools includes:

- Microbial presence (and abundance in some cases)
- Microbial cellular activity (e.g., transcription)
- Biodegradation activity
- Direct evidence of contaminant biodegradation

To select the appropriate EMDs, it is important to understand the connections between the information provided by DNA-based analysis, RNA analysis, and stable isotope-based analysis. In general, the genes of microbes (and other organisms) are composed of deoxyribonucleic acid, or DNA. DNA can be transcribed into RNA, and ultimately translated into enzymes (and other proteins) that degrade the contaminant (see Figure ES-4). Thus DNA-based analyses (such as PCR, FISH, some fingerprinting methods, and microarrays) can determine if microorganisms with the potential to biodegrade target contaminants are present at a site, and in some cases (notably qPCR), the abundance of the target microorganisms. RNA-based analyses (such as RT-qPCR, FISH, microarrays, and some fingerprinting methods) or analyses that identify the end product of specific enzymes (such as EAPs) can show that biodegrading organisms are actively expressing biodegradation genes.

One of the most frequent uses of EMDs is to verify that natural or enhanced biodegradation can occur, or in fact is occurring, in situ. Some EMDs can be used to estimate biodegradation rates. CSIA in particular can be very useful for this purpose. Care must be taken, however, in extrapolating rates both spatially and temporally. Additionally, EMDs that indicate number of gene copies, or count the number of organisms in a given sample, could potentially be used to infer

degradation rates, or at least to conclude that useful rates are occurring or that native or added organisms are increasing in number over time. However, there is currently no method to calculate degradation rates from the number of organisms or gene copies in a sample.

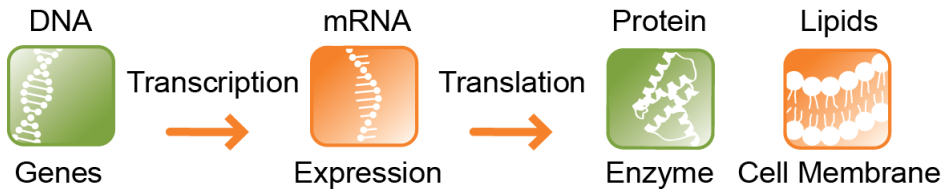

Figure ES-4. Flow of information within cells.

EMD ISSUES

Project managers, stakeholders and regulators must be concerned with the quality of EMD analyses and the proper interpretations of the results. This guidance includes recommendations on standard practices that have been developed recently to ensure that samples are collected and analyzed appropriately. Using the guidance presented here, project managers, regulators and stakeholders can evaluate plans for EMD analyses, the quality of EMD results, and the data interpretations.

EMD plans and analyses should be based on a sufficient number of samples, taken at appropriate locations and times, using appropriate techniques, and including appropriate documentation and QA/QC controls. The number of samples needed for a given site will be a function of site conditions including geology, hydrogeology, geochemistry, and contaminant distribution. A successful sampling program will incorporate these parameters in a site-specific sampling plan. Generally, vadose zone biology is more location-specific because microbial transport (and thus distribution) depends on excess water. With less water available, more samples may be required to characterize a smaller area. Since microbial diversity in soils and sediments can vary on a millimeter scale, homogenization and multiple samples are desirable.

Samples may be collected by either active or passive sampling techniques, from groundwater or solid materials. For some bacteria (e.g., *Dehalococcoides*, perchlorate degraders) active sampling of groundwater alone will be useful because a significant fraction of the bacteria can be found in the aqueous phase (see Section 10.4.2). However, other bacteria may be primarily attached to the aquifer solids, and groundwater analysis alone may not be appropriate. When sampling groundwater, most EMDs require filtration of the sample to collect concentrated biomass for analysis. Various biomass extraction/filtration approaches are available for collecting active microbial biomass from environmental media. Passive microbial sampling devices are groundwater sampling tools (for example, biofilm coupons, in situ microcosms, groundwater dialysis chambers, porous beads, BioTrap® samplers) that facilitate colonization of subsurface microorganisms onto a retrievable matrix. Passive microbial sampling devices may be very useful for assessing activity in situ, but the results may only be semi-quantitative, since it is difficult to relate microbial concentrations in the groundwater or aquifer matrix to those detected on the passive microbial sampling device (See EMD Sampling Methods Fact Sheet and Section 10.4.3).

SURVEY RESULTS

A survey of regulators, consultants and stakeholders indicated that a lack of standardized QA/QC guidelines is one of the primary concerns regarding use of these emerging techniques (see Appendix B Survey Results). Recent QA/QC guidance is available from USEPA for the use of CSIA, as well as for PCR-based methods. Much of this latter guidance also is applicable to other MBTs as well.

This ITRC document includes several QA/QC considerations that should be part of any plan for the use of EMDs. However, if EMDs are to be used at a site, it is important that the regulator be involved as early as possible, to allay such QA/QC concerns. During the initial meeting, a draft work plan should be available, and should include:

1. The current conceptual site model, based upon the results of existing conventional methods
2. The EMDs to be used, with an explanation of how EMD data can complement the existing data
3. The life cycle stage of the environmental cleanup process for which the EMD is to be used
4. The sample locations and frequencies
5. Specific data quality objectives.
6. Any permitting requirements necessary for the use of the EMD.

Finally, EMD team members and most of those surveyed agree that education is the key to more widespread use of EMDs in the environmental site management field. Figure ES-5 includes the results from the respondents to the survey when asked about their EMD experience.

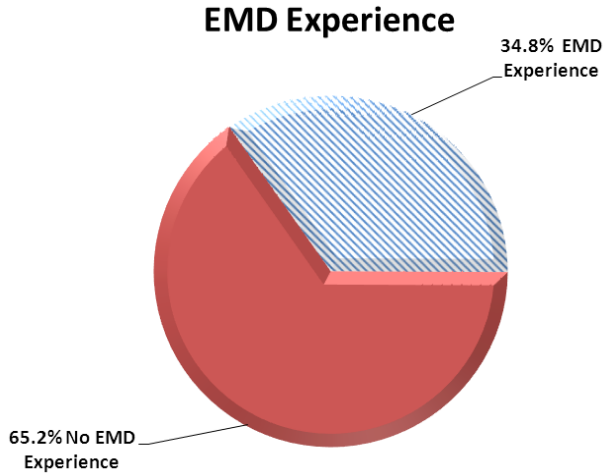

Figure ES-5. Experience with EMDs survey results.

As regulators become better educated and more comfortable with their use, guidance and regulations specific to EMD use will be developed. Until state documents are developed, this ITRC document and the related Fact Sheets developed by this team will serve as the most comprehensive resources available for regulators, consultants, and the general public.

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1.0 INTRODUCTION

Environmental molecular diagnostics (EMDs) is a collective term that describes a group of advanced and emerging techniques used to analyze biological and chemical characteristics of soils, sediments, groundwater, and surface water. Many of these analytical techniques were originally developed for applications in medicine, defense, and industry. However, over the last decade, great advances have been made in adapting and applying EMDs for environmental site management.

Based on the results of a survey conducted as part of development of this document, and on the experience of the EMD Team, there are hundreds of examples of current application of EMDs to environmental site management projects (see Appendix B, Table B-1, and Figure ES-2). EMDs can be classified into two major categories of analytical techniques: chemical techniques, specifically compound specific isotope analysis (CSIA), and molecular biological techniques (MBTs).

CSIA measures the stable isotopes (typically carbon, hydrogen, or chlorine) in contaminants. This information helps to determine the extent to which specific chemical and biochemical reactions impact the contaminant. As a contaminant degrades through natural or engineered processes, the relative amount of each stable isotope in the contaminant can change. In contrast, contaminant isotopic composition is largely unaffected by processes such as dilution that do not result in contaminant degradation. Questions pertaining to a chemical's source, degradation mechanism, and rate of degradation can be answered through CSIA.

MBTs, also referred to as molecular biology-based EMDs, are used to determine the biochemical capabilities of microorganisms present in the environment. In many cases, particular microorganisms are responsible for the degradation of specific contaminants. Some molecular biology–based EMDs can detect and quantify known microorganisms. Other molecular biology–based EMDs can determine whether microorganisms are actively degrading specific contaminants. These EMDs can also provide identification of currently unknown microorganisms involved in these processes. Questions pertaining to biochemical capabilities and activities of microorganisms, and changes in microbial population sizes in natural and engineered environments can be answered through these types of analyses.

EMDs have applications in each phase of environmental site management, including site characterization, remediation, monitoring, and closure activities. EMDs provide additional lines of evidence for making decisions during each phase of a project. The improved decision making that results from the application of EMDs is beginning to gain acceptance throughout the environmental community.

1.1 Purpose

The purpose of this technical and regulatory guidance document is to:

- provide objective guidance on the best practices for using EMDs for environmental site management

- demonstrate appropriate uses of EMDs, including their strengths and limitations
- explain how to evaluate, apply, and interpret the results of EMDs.

EMDs are becoming increasingly powerful, and standardized methods are being developed. As a result, their use is increasing rapidly, and a growing need exists for technical information and training on EMDs. As discussed in this document, these diagnostic tools provide the following benefits:

- determine whether biotic or abiotic degradation is occurring at a site, potentially including degradation pathways and rates
- reveal whether multiple sources of contamination are present
- contribute to decision making for remediation strategies, including monitored natural attenuation
- identify when enhancements such as chemical amendments or bioaugmentation are necessary
- aid in the advancement of monitoring program decisions
- provide complementary data to support site management decisions, including closure

This guidance and the companion internet-based training will foster the appropriate uses of EMDs and help regulators, consultants, site owners, and other stakeholders to better understand a site and to make decisions based on the results of EMD analyses.

1.2 Project Life Cycle

EMDs have applications throughout the life cycle of environmental cleanup projects. The terminology and regulatory framework for the stages of the project within its life cycle, however, often vary under different regulatory programs. For simplicity, this document organizes the discussion of site management around four main technical tasks:

- site characterization
- remediation
- monitoring
- closure

These tasks and their descriptions presented here correlate with the activities described in various regulatory programs (such as RCRA, CERCLA, State Voluntary Cleanup, and UST Site Cleanup). Although individual projects may vary in their progression through these stages, EMDs can support decision making regardless of how the project is defined. Figure 1-1 summarizes the correlations between the terms used in this guidance document and the terms used in several regulatory programs.

Project Lifecycle Stage	Site Characterization	Remediation			Monitoring		Closure
CERCLA/ Superfund	Remedial Investigation	Feasibility Study or EE/CA	ROD	Remedial Action Implementation/ Optimization	Remedial Action Monitoring	Response Complete	LTM/LTMgt / LTMO
RCRA	RCRA Facility Investigation	Corrective Measures Study	RCRA Permit	Corrective Measures Implementation	Compliance Monitoring	Certification of Remedy Completion or Construction Complete	Post Closure Care
UST/ LUST	Varies by Regulatory Authority						
State	Varies by State						

Figure 1-1. Correlation of regulatory terms.

Source: ITRC RRM IBT slide 2011.

1.2.1 Site Characterization

A typical site investigation has five main goals:

1. Identify the contaminants present.
2. Delineate the severity and spatial extent of contamination.
3. Develop the conceptual site model.
4. Provide information for the risk assessment.
5. Define baseline conditions for a preliminary evaluation of potential remedies, including monitored natural attenuation (MNA).

As part of the site investigation, soil samples may be acquired and monitoring wells installed to collect groundwater samples. The samples are used to identify the contaminants present, quantify contaminant concentrations, and delineate soil impacts and the size of a dissolved contaminant plume. Field measurements (such as dissolved oxygen and pH) and laboratory geochemical analyses (such as nitrate and sulfate) may also be performed to evaluate subsurface oxidation-reduction potential and assess potential biodegradation processes. Performing EMD analyses on a select subset of samples collected during site investigation aids in site characterization and preliminary assessment of potential remediation options.

1.2.2 Remediation

Remediation includes a number of significant tasks, such as:

- remedy selection
- design
- pilot testing or bench-scale testing
- sampling to develop a baseline before starting active remediation
- installing equipment (such as air sparging wells, groundwater pumps, and treatment systems)
- applying chemical amendments or reagents for bioremediation strategies (such as biostimulation and bioaugmentation).

The chemical, geochemical, and EMD data obtained from the site characterization will often lead to a limited number of remediation alternatives worthy of further consideration. This selection process may include bench-scale experiments, field microcosms (see passive sampling devices), or pilot studies. MNA is often considered a potential remediation alternative and may serve as a basis of comparison for evaluating enhanced remediation (that is biological, chemical, or physical) alternatives. Potential enhanced remediation technologies usually involve supplying an amendment (such as an electron donor or electron acceptor) to stimulate contaminant biodegradation or a reagent (such as a chemical oxidant) to promote abiotic degradation. EMDs can be used to supplement chemical and geochemical analyses to gain better insight into the most appropriate remedy. Additionally, EMDs can be used in conjunction with chemical and geochemical analyses as part of the baseline sampling before starting active remediation. Monitoring may be conducted as part of the remediation stage and remediation may be ongoing as a project moves into a monitoring stage.

1.2.3 Monitoring

Incorporating EMDs into monitoring efforts can provide an additional line of evidence to support chemical and geochemical data.The United States Environmental Protection Agency (USEPA 2004b) defines monitoring to be

> "… the collection and analysis of data (chemical, physical, and/or biological) over a sufficient period of time and frequency to determine the status and/or trend in one or more environmental parameters or characteristics. Monitoring should not produce a 'snapshot in time' measurement, but rather should involve repeated sampling over time in order to define the trends in the parameters of interest relative to clearly-defined management objectives. Monitoring may collect abiotic and/or biotic data using well-defined methods and/or endpoints. These data, methods, and endpoints should be directly related to the management objectives for the site in question."

Under the USEPA definition for monitoring, six types of monitoring can be identified that are used throughout both federal and state regulatory programs.

- Detection monitoring – parameters are measured and compared to background data, or regulatory thresholds, to determine if there is a statistical increase which would reflect that a release has occurred.

- Compliance monitoring – parameters are monitored to determine that chemical concentrations remain below an established regulatory threshold level.
- Characterization monitoring – parameters are monitored to determine the magnitude and migration of contamination.
- Remediation monitoring – parameters are monitored to determine the performance and effectiveness of the remedial action. This monitoring includes monitoring after a remedy has been installed, monitoring subsequent to serial applications of amendments, and the trend and performance analyses for remedies.
- Post-closure monitoring – parameters are monitored over the long term after a site has been remediated and closed to show that contaminant concentrations remain below regulatory threshold levels. Post-closure monitoring is typically used at sites being remediated under the Resource Conservation and Recovery Act (RCRA).
- Post-remediation monitoring – parameters are monitored to show that remediation has truly been accomplished and that contaminant concentrations do not rebound.

1.2.4 Closure

Site closure is determined by local, state, or federal regulatory agencies based upon applicable laws, regulations, and policies. Sites ideally are closed when cleanup goals are attained and impacts to environmental media (soil, groundwater, surface water, soil vapor, and air) no longer exist in concentrations that pose a threat to human health or the environment. EMDs can serve as additional lines of evidence for site closure. Examples of closure concerns and of how EMDs are used to complement traditional data are provided in each specific EMD section.

Site cleanup goals may be based upon state or federal regulatory levels (such as state standards for surface water or federal standards, established under the Safe Drinking Water Act) or site-specific risk-based levels. Meeting these goals is commonly accomplished through remedial actions. For a site, the remedial goals may include interim remedial goals or final remedial goals. The site cleanup goals and the remedial goals for a specific site may be the same or they may be different. Typically, the basis for considering closure is through traditional chemical data, that show downward trends in contaminant concentrations are occurring and that the plume is shrinking. In these cases, an evaluation of whether degradation will continue to occur using EMD data may be a line of evidence that assures that the residual concentrations will not pose a threat to human health or the environment following site closure.

In some programs or states, sites may be closed based upon a low-risk scenario. A low-risk scenario is where site-specific data or models show that concentrations of contaminants that are proposed to be left at a site (although above established state or federal regulatory levels) do not pose a threat to human health or the environment (either based on an assessment of the current and future

risks or the absence now or in the foreseeable future of receptors). Some concerns of the low-risk scenario are whether residual contamination will continue to degrade within a site-specific time-frame, and if so, whether contaminants will degrade to non-hazardous byproducts (in the absence of continued monitoring). To allay these concerns, additional information (over and above chemical and geochemical analyses) which could be provided by EMDs on the mechanisms of degradation (biological destruction versus physical factors like dilution or downgradient migration) also may be helpful to the closure assessment.

1.3 Individual EMD Definitions

The main sections of this document include information about CSIA and about a group of molecular biology-based EMDs. The main sections and the appendices also provide limited information about emerging technologies. For ease of use, definitions are presented for the primary methods that are discussed:

- **Compound Specific Isotope Analysis (CSIA)**

  Analyzes the relative abundance of various stable isotopes (such as ^{13}C:^{12}C, ^{2}H:^{1}H). Degradation processes can cause shifts in the relative abundance of stable isotopes of the contaminant; changes in isotopic ratios can be measured. See the CSIA Fact Sheet for more information.

- **Quantitative Polymerase Chain Reaction (qPCR)**

  A laboratory analytical technique for quantification of a target gene based on DNA. See the qPCR Fact Sheet for more information.

- **Reverse Transcriptase qPCR (RT-qPCR)**

  A laboratory analytical technique for quantification of a target gene based on RNA. See the qPCR Fact Sheet for more information.

- **Phospholipid Fatty Acid (PLFA) Analysis**

  A laboratory analytical technique that differentiates groups of microorganisms based on quantifying PLFA types. See the Microbial Fingerprinting Fact Sheet for more information.

- **Denaturing Gradient Gel Electrophoresis (DGGE)**

  Type of gel electrophoresis used to separate mixtures of PCR products based on the melting point which is reflective of the DNA sequence. DGGE is used to generate a genetic fingerprint of the microbial community and potentially identify dominant microorganisms. See the Microbial Fingerprinting Fact Sheet for more information.

- **Terminal restriction fragment length polymorphism (T-RFLP)**

  A nucleic acid (DNA or RNA)-based technique used to generate a genetic fingerprint of the microbial community and potentially identify dominant microorganisms. See the Microbial Fingerprinting Fact Sheet for more information.

- **Microarrays**

  Detects and estimates the relative abundances of hundreds to thousands of genes simultaneously. See the Microarrays Fact Sheet for more information.

- **Stable Isotope Probing (SIP)**

  A synthesized form of the contaminant containing a stable isotope (such as ^{13}C label) is added. If biodegradation is occurring the isotope will be taken up by the organism and detected in biomolecules (e.g., phospholipids, DNA). See the SIP Fact Sheet for more information.

- **Enzyme Activity Probes (EAPs)**

  Transformation of surrogate compounds (probes) resembling contaminants produces a fluorescent (or other distinct) signal in cells which is then detected using a microscope. See the EAP Fact Sheet for more information.

- **Fluorescence In Situ Hybridization (FISH)**

  Detects and localizes the presence of targeted genetic material in an environmental sample, which can be used to estimate the number of specific microorganisms or groups of microorganisms. See the FISH Fact Sheet for more information.

1.4 Document Organization

This guidance is organized in the following topics:

Additional appendices provide in-depth supporting information:

2.0 USING EMDS IN SITE MANAGEMENT

EMDs can provide unique information at each stage of the project life cycle. In many circumstances, conventional data (including current and historical contaminant and geochemical data) are insufficient to make a planned technical decision in the project life cycle. Often, decision makers pursue traditional strategies because they lack sufficient data to support alternatives that could result in remediation of equal or greater efficiency at a lower cost and in equal or less time. EMDs provide additional and often unique information that supplements conventional data.

This section provides an overview of EMDs and guidance on the following topics:

- using EMDs at a site throughout the project life cycle
- clarifying the connection between conventional and EMD data
- identifying questions EMDs may answer better than conventional data
- deciding which EMD to use to answer primary questions identified for EMDs (by comparing the data each EMD provides)

While the exact number and names of the stages may vary in different states or under different regulatory programs, most activities fall into one of four stages: site characterization, remediation, monitoring, and closure (see Section 1.2). Figure 2-1 introduces questions that EMDs can help to answer, and thereby improve project decision making over relying only on conventional data. The questions included in Figure 2-1 are only examples of the types of questions that EMDs can help to answer by providing supplemental information to the site-specific characterization data and information. The project life cycle stages are depicted as linear steps for simplicity. Often at sites the stages overlap; for example, some tasks under monitoring are conducted during what might be considered the remediation phase and some characterization tasks continue throughout the life of an environmental cleanup project.

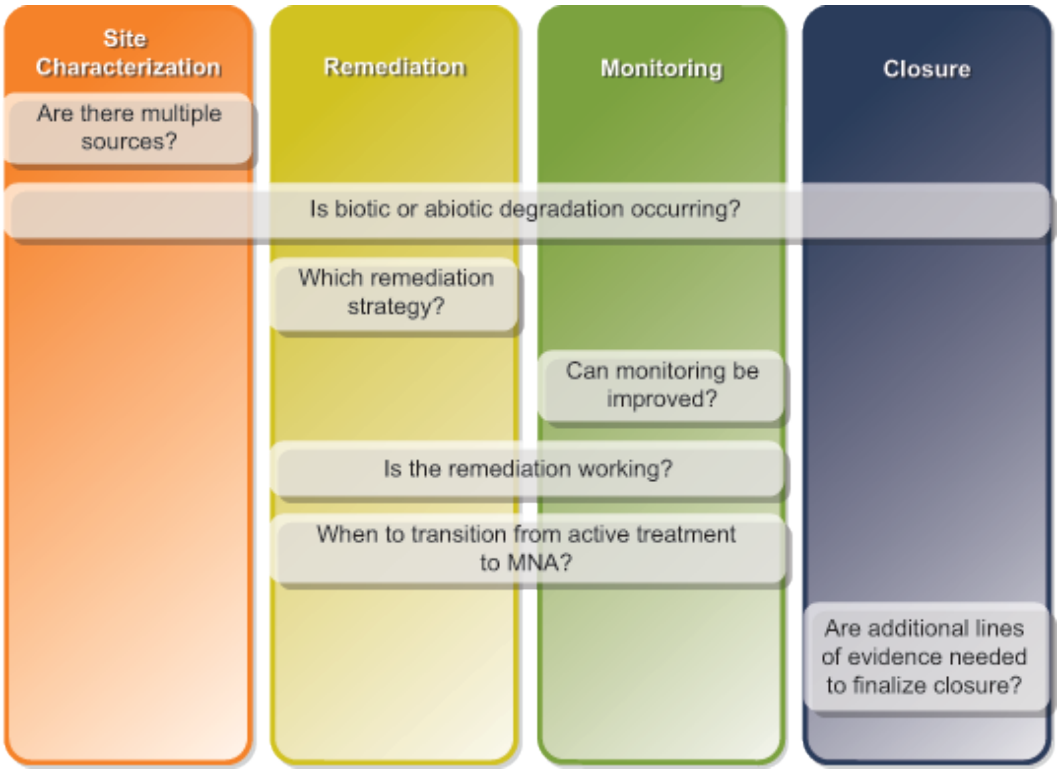

Figure 2-1. EMDs provide supplemental data for answering site management questions.

EMDs do not replace conventional data, but complement them by providing additional lines of evidence that help to explain the degradation mechanisms. While EMD data are necessary for some site management decision making (for instance, whether it is necessary to implement bioaugmentation), all site management decisions, and in particular site closure decisions, still require traditional data gathering and interpretation. A thorough conceptual site model (CSM) should take into account all existing data (such as contaminant monitoring, geochemistry, and EMD data) and is important for understanding and interpreting the results generated by EMD methods. The CSM should reflect a current understanding of the site and uncertainties in the CSM should be explicitly acknowledged, so that proper interpretation of the EMD results can be made.

2.1 Introduction to Using EMDs

Table 2-1 summarizes the connections between conventional data and EMD data. Conventional data (for example, hydrogeological data, chemical, and geochemical analyses) often provide only indirect data regarding the mechanisms and rates of key attenuation or treatment processes. EMDs can complement these data by providing direct measurements of the organisms, genes, or enzymes involved in contaminant biodegradation, as well as relative contributions of abiotic and biotic processes and relative rates of various degradation processes. In addition, EMDs can be used to identify the source of contamination when several sources are suspected.The information that EMDs provide can improve estimates of attenuation rates and capacities and improve remedy performance assessments and optimization efforts. Improved understanding of the biological and non-biological degradation processes also can lead to greater confidence in MNA or closure decisions.

Table 2-1. How EMDs can complement conventional data

Conventional data	Potential data gaps	Complementary EMD data
Site Characterization		
• Evaluate concentrations and distributions of contaminants. • Understand geochemistry and field parameters that influence remedy selection and design. • Formulate a CSM.	• Weak assessment of microbial biodegradation potential for some compounds. • Does not differentiate among various sources of the same contaminant. • Little information related to degradation mechanism(s).	• Improves understanding of the microbial community and its capability to degrade contaminants. • Can identify different contaminant sources • Identifies abiotic processes able to degrade contaminants • Update CSM with EMD results
Remediation		
• Evaluate trends in concentrations and mass discharge of contaminants and byproducts to ensure selected remedy will adequately address these. • Evaluate field attenuation and geochemistry parameters to appropriately design the remedial technology. • Switch from active to passive treatment, optimize remedy, or identify that a different remedy is needed.	• Limited ability to confirm that the targeted degradation mechanism is occurring. • Limited ability to confirm anticipated degradation rates of abiotic and biotic processes.	• Provides details related to the presence and rate of abiotic and biotic degradation. • Supports lines of evidence of abiotic and biotic treatment. • Provides strong lines of evidence to support a switch in remedy.
Monitoring		
• Evaluate trends in concentrations and mass discharge of contaminants and byproducts to assess remedy performance or monitoring return towards background conditions. • Evaluate field attenuation and geochemistry parameters as conditions change during, or after, a remedy.	• Little ability to assess the relative contributions of abiotic and biotic processes for some compounds. • Inconclusive or contradictory monitoring results. • Limited ability to confirm anticipated degradation rates of abiotic and biotic processes.	• Provides direct data on abiotic and biotic responses to treatment. • Provides details related to the presence and rate of abiotic and biotic degradation.
Closure		
• Assess concentrations of contaminant to determine if site cleanup goals (such as MCLs) have been met. • Complete CSM.	• Little ability to assess the relative contributions of abiotic and biotic processes to the performance of the remedy implemented. • Limited ability to confirm or project degradation rates of abiotic and biotic processes.	• Increases the number of lines of evidence and understanding how abiotic and biotic processes are degrading contaminants. • Provides better evidence of biodegradation which may support the estimation of time frame to meet remedial goals.

Table 2-2 describes the benefits and limitations of the most common EMDs and how they may complement each other. One limitation among most EMDs is the limited standardization of QA/QC protocols; this is discussed more in Section 3.3 and Section 10.0. Table 2, Introduction to EMDs Fact Sheet, provides a comparison of the EMDs, particularly in relation to availability and relative cost. The results and interpretation for each EMD method are presented in the individual method sections of this document. At times, different EMDs can provide similar information, so use care in selecting the most appropriate and cost efficient method (see Section 2.3 and the individual EMD method sections for more information).

Table 2-2. Benefits, limitations, and complementary EMDs

EMD (and Complementary EMDs)	Description	Potential benefits	Potential limitations
CSIA (qPCR, SIP)	• Analytical method that quantifies the relative abundance of stable isotopes (such as $^{13}C/^{12}C$) in contaminants.	• Commercially available. • Provides direct evidence of biological or chemical degradation of contaminants. • Useful for generating attenuation rates and mechanisms. • Provides information to identify multiple sources.	• Need fractionation factors for key contaminants and degrading organisms. • Multiple samples are required to generate attenuation rates. • More commercial labs would be beneficial.

Table 2-2. Benefits, limitations, and complementary EMDs (continued)

EMD (and Complementary EMDs)	Description	Potential benefits	Potential limitations
qPCR (CSIA)	• Chemical process that generates copies of specific DNA sequences in a quantifiable and repeatable manner. • Used to calculate the number of copies of a specific DNA sequence that are present in a sample.	• Readily commercially available for some key organisms and biodegradation associated genes. • Confirms presence and determines abundance of target microbes and genes. • Estimates of total microbial numbers possible. • Allows monitoring of population growth and distribution of microbes involved in bioremediation.	• Does not confirm bioremediation is actually occurring, but only that the right microorganisms are present and they are in sufficient quantity for bioremediation potentially to occur.
RT-qPCR (qPCR, CSIA)	• Provides indirect evidence of microbial activity by detecting expression of biodegradation associated genes.	• Provides indirect evidence of biodegradation gene expression and thus biodegradation activity.	• Sampling and preservation challenging for RNA • Not quantitative • Not widely commercially available

Table 2-2. Benefits, limitations, and complementary EMDs (continued)

EMD (and Complementary EMDs)	Description	Potential benefits	Potential limitations
Fingerprinting (qPCR, CSIA, SIP)	• Group of methods that provide indirect evidence of microbial community diversity, structure and overall biomass based on analysis of microbial DNA or cell structures such as phospholipids.	• Provides basic information on community diversity and changes in structure over time. • Allows tracking individual organisms over time and space within a community, in combination with other EMDs. • Allows tracking of microbial groups within a community over time and during bioremediation.	• Some techniques are unable to identify individual organisms (such as *Dehlococcoides*), identify groups of organisms (Archaea), or are not quantitative. • Abundant microbes can dominate profiles. • Not widely commercially available. • Does not confirm bioremediation.
Microarrays (qPCR, CSIA, RT-qPCR)	• Quickly determines the presence or absence of a large number of targeted DNA fragments in an environmental sample.	• Provides basic information on community diversity and changes in structure over time. • Allows tracking individual organisms over time and space within a community, in combination with other EMDs.	• Not widely commercially available. • Guidance is needed on interpretation of the results. • Does not confirm bioremediation activity, but only its potential.

Table 2-2. Benefits, limitations, and complementary EMDs (continued)

EMD (and Complementary EMDs)	Description	Potential benefits	Potential limitations
SIP (qPCR, CSIA, Fingerprinting)	• Suite of techniques that provides directed evidence of biodegradation by detection of incorporation of stable isotopes from isotopically-enriched contaminant into microbial cell structures or generation of terminal products containing stable isotopes from the added compounds.	• Provides direct evidence of biological degradation of contaminants. • Identifies degrading microorganisms; this information can be applied to future analyses.	• Not widely commercially available. • Other methods required to identify biodegrading microorganisms, or certain organisms classes (such as *Archaea*). • Isotopically labeled compounds can be expensive to synthesize.
EAPs (qPCR, FISH)	• Chemical analysis that detects the presence of enzymes through a reaction of the enzyme with chemical surrogates.	• Provides direct evidence of enzyme activity and thus indirect evidence of biodegradation of contaminants.	• Limited commercial availability. • Analyses are not available for many organisms. • Limited cross-laboratory validation for certain analyses.

Table 2-2. Benefits, limitations, and complementary EMDs (continued)

EMD (and Complementary EMDs)	Description	Potential benefits	Potential limitations
FISH (CSIA)	• Provides direct evidence of microbial presence and allows for quantification of desired organisms. • Provides indirect evidence of microbial activity by detecting expression of biodegradation associated genes. • Allows monitoring of population growth and distribution of microbes.	• Provides direct measurement of presence and indirect measurement of activity of organisms of interest • Provides visual information of the spatial distribution of organisms and cultures; • Allows for total biomass determination	• Not widely commercially available • Probes not available for a wide range of organisms

2.2 Comparison of EMDs

In order to select among the various EMD tools available to assess site management activities and answer primary questions identified for the use of EMDs (see Section 2.3), decision makers must understand the difference between analyses that delineate the following:

- biotic and abiotic attenuation mechanisms
- microbial presence (and enumeration in some cases)
- cellular activity (such as transcription)
- historic and potential biodegradation activity
- evidence of contaminant biodegradation

In particular, an understanding of the direct connection between the information provided by a DNA-based analysis, RNA analysis, and stable isotope-based analysis is necessary to select appropriate EMD technologies.

All microbes contain DNA which is organized into genes. Each gene contains the information a microbe requires to make a single type of protein. Many proteins have catalytic activities and are called enzymes. The degradation of a specific contaminant can often be attributed to the activity of a specific enzyme and consequently, a specific gene. Some genes associated with biodegradation processes can be found in many different microorganisms. However, in some instances, the distribution of specific genes is limited to a very small range of microorganisms.

DNA-based analyses (such as qPCR, FISH, some fingerprinting methods, microarrays) detect the presence of specific genes and can determine if microorganisms capable of degrading a contaminant are present at a site. In many cases elevated numbers of specific genes can be a strong line of supporting evidence for biodegradation, although it is difficult to estimate the rates of biodegradation from DNA data alone.

Before the information in DNA can be used by the microbe to produce proteins and enzymes, the information in individual genes must be copied (transcribed) into short-lived RNA. RNA-based analyses (such as RT-qPCR, FISH, microarrays, and some fingerprinting) can therefore show that microorganisms at a site are actively expressing (transcribing) specific genes associated with specific biodegradation processes. In many cases elevated levels of gene transcription (for instance, increased RNA levels) can be a strong line of supporting evidence for contaminant biodegradation. Again, it is difficult to estimate rates of contaminant biodegradation from RNA data alone.

A few methods, such as EAPs, detect the presence of active enzymes at a site. However, these methods indirectly measure the activities of specific contaminant-degrading enzymes using surrogate compounds that are selectively transformed by contaminant-degrading enzymes into detectable products. While these methods can establish that contaminant-degrading enzymes are present and active at a site, they still do not provide unequivocal evidence for contaminant biodegradation.

The only methods currently available that can provide definitive evidence that biodegradation of a specific contaminant is occurring at a site include analyses that determine the stable isotope composition of contaminants themselves, such as CSIA, or analyses that determine whether stable isotopes derived from contaminants have been incorporated into microbial structures such as lipids or DNA (SIP).

2.3 Comparison of EMDs in answering primary questions

The first step in determining if EMDs can benefit an environmental management project is to determine if the conventional chemical and geochemical data leave a data gap that an EMD method can fill. Figure 2-2 provides an initial decision framework for beginning this evaluation and leads the user to explore additional options. Specifically, Figure 2-2 points to a section of Table 2-3 based on the phase of the project (site characterization, remediation, monitoring, or closure). Within the project phase sections of Table 2-3, several primary questions often arise as part of these project phases. An "X" indicates which EMDs can be used as a primary line of evidence to answer a particular question. However, the information generated by these tools is most often used as part of a lines-of-evidence approach to understanding the site.

For eight of the 27 questions in Table 2-3, CSIA is the primary EMD used to answer the question. However, for the remaining 19 questions more than one EMD may answer the question depending on the site-specific needs and constraints. It is not necessary to use all of the EMDs identified in the table to answer a given question. Figures 2-3 through 2-9 (linked in Table 2-3 and included at the end of this Section 2) are decision trees that will help you to compare and decide which EMD would be the most beneficial at a particular site. In some cases, different EMDs may answer different parts of the overall question listed in Table 2-3. In those situations, supplemental questions in Figures 2-3 through 2-9 are asked to provide the user with additional information on which to base their decision. Furthermore, information regarding the commercial availability and state of development of particular EMDs are considered as well.

In addition to these supplemental decision framework figures, examples of how each question could be answered using a given EMD is presented in each EMD method section along with the details of the method, the requirements for data quality, and sampling plans with respect to the specific site requirements. You can review all of the information provided for the questions and methods of interest and determine based on their site-specific needs and constraints which method would be most suitable. The table and decision charts are intended to point you in the right direction, but may not be applicable to all sites in all circumstances. In addition, advances in EMD methods are occurring at a rapid pace, so consult with the analytical laboratory in making a site-specific decision.

In summary, for site decision making, begin with Figure 2-2 to determine if EMDs may provide information to augment conventional data. From that decision framework figure the user is directed the applicable section in Table 2-3. For 19 of the questions more than one EMD may be appropriate for the site. In these cases, consult the supplemental decision framework figure linked to the question in Table 2-3 for more information. Finally, consider the information presented in the applicable EMD method section.

Table 2-3. Summary comparison of EMDs for primary questions

Questions	Figure	CSIA	qPCR	RT-qPCR	Fingerprinting	Microarrays	SIP	EAP	FISH
Site Characterization									
A) Are contaminant-degrading microorganisms present?	2-3		X	X	X	X	X	X	X
B) Are contaminant-degrading microorganisms active?	2-4	X		X		X	X	X	X
C) Are the microorganisms capable of complete degradation?	2-5		X	X		X	X		X
D) Is biodegradation occurring?	2-6	X					X	X	
E) Is the contaminant attenuating abiotically?	-	X							

Table 2-3. Summary comparison of EMDs for primary questions (continued)

Questions	Figure	CSIA	qPCR	RT-qPCR	Fingerprinting	Microarrays	SIP	EAP	FISH
F) Are multiple sources contributing to the contamination?	-	X							
G) If there is a potential for multiple sources, can the sources be distinguished?	-	X							
Remediation	**Figure**	**CSIA**	**qPCR**	**RT-qPCR**	**Fingerprinting**	**Microarrays**	**SIP**	**EAP**	**FISH**
H) Are numbers of contaminant-degrading microorganisms and/or genes changing?	2-3		X	X	X	X	X	X	X
I) Is the remediation strategy affecting the numbers or types of contaminant-degrading microorganisms?	2-3		X	X	X	X	X	X	X
J) Is there a biological basis for intermediates accumulating?	2-7		X	X		X			X
K) Are intermediates being degraded?	-	X							
L) Is there evidence of abiotic transformation?	-	X							
M) Is biodegradation occurring?	2-6	X					X	X	
N) What is the rate of biodegradation?	2-6	X					X	X	
Monitoring	**Figure**	**CSIA**	**qPCR**	**RT-qPCR**	**Fingerprinting**	**Microarrays**	**SIP**	**EAP**	**FISH**
O) Does the microbial community composition support the remediation strategy?	2-3		X	X	X	X	X	X	X
P) Do contaminant-degrading microorganisms continue to be sufficiently abundant?	2-3		X	X	X	X	X	X	X
Q) Are contaminant-degrading microorganisms remaining active?	2-4	X		X		X	X	X	X
R) Is there a biological basis for intermediates accumulating?	2-7		X	X		X			X
S) Are intermediates being degraded?	-	X							

Table 2-3. Summary comparison of EMDs for primary questions (continued)

Questions	Figure	CSIA	qPCR	RT-qPCR	Fingerprinting	Microarrays	SIP	EAP	FISH
T) Is there evidence of abiotic transformation?	-	X							
U) Is biodegradation occurring?	2-6	X					X	X	
V) What is the rate of biodegradation?	2-6	X					X	X	
Closure	**Figure**	**CSIA**	**qPCR**	**RT-qPCR**	**Fingerprinting**	**Microarrays**	**SIP**	**EAP**	**FISH**
W) Is contaminant degradation likely to continue?	2-8	X	X	X	X	X	X	X	X
X) Are intermediates being degraded?	-	X							
Y) Is biodegradation occurring?	2-6	X					X	X	
Z) What is the rate of biodegradation?	2-6	X					X	X	
AA) Does the microbial community composition suggest that sufficient contaminant degradation has occurred?	2-9				X	X			

2.4 Common Examples of EMD Uses

The following are a few examples of common situations where EMDs can be applied to supplement existing chemical and geochemical data to provide additional insight into site conditions and additional lines of evidence for site management decisions. Additional, detailed examples are located within each of the EMD sections.

- Two nearby sites have groundwater releases (plumes) of methyl tertiary-butyl ether (MTBE). In addition to traditional data (e.g., concentration trends, groundwater flow data), CSIA could be used to differentiate which plume is affecting a downgradient receptor (see Section 3.2).
- A groundwater plume of chlorinated solvents is being evaluated for MNA. qPCR can be used to quantify whether *Dehalococcoides* bacteria are present in sufficient numbers to pursue MNA or whether active remediation with biostimulation or bioaugmentation will be required (see Section 4.0).
- An electron donor was added to stimulate biodegradation of chlorinated solvents (for instance, TCE) in groundwater. qPCR can be used to determine if complete degradation of vinyl chloride to ethene can occur and to monitor the effectiveness of the treatment approach (see Section 4.0).

- In a case where MNA of a groundwater gasoline plume is being evaluated for closure. CSIA can be used to estimate the degradation rate of the contaminants and also provide an estimated cleanup time (see Section 3.2).
- Aniline is one of many infrequent contaminants that can be a remedial driver. Aniline can be biodegraded via multiple mechanisms (i.e., aerobically or anaerobically). SIP can be used to determine the dominant degradation mechanism, thus leading to effective remedial decision making (see Section 7.2).

Dehalococcoides: Recent Developments

The biodegradation of chlorinated contaminants in the environment is an active area of research. The first Dehalococcoides isolate capable of complete dechlorination of PCE,'Dehalococcoides ethenogenes' strain 195, is capable of reductive dehalogenation of mono- and poly-chlorinated and brominated aromatic compounds, alkanes, and alkenes. (Maymó-Gatell et al. 1997).

Many Dehalococcoides strains have been isolated from geographically distinct freshwater locations (such as river sediments and aquifer materials), and exhibit differing dechlorination abilities, but share greater than 98% 16S rRNA gene sequence similarity (the cutoff typically used to classify two organisms as the same species). Specific reductive dehalogenase genes distinguish these different strains and confer distinct dechlorination capabilities among the strains.

Dehalococcoides mccartyi (Dhc) was recently published as the type species of the genus Dehalococcoides, which includes all characterized strains including strains 195, BAV1, CBDB1, FL2, GT and VS (Löffler et al. 2013). This species is the only known species with strains capable of complete dechlorination of tetrachloroethene (PCE) to ethene and inorganic chloride. More than a single Dhc strain may be included in commercially available bioaugmentation consortia. In this document, "Dhc" refers to Dehalococcoides mccartyi, including all of the strains that reductively dechlorinate chlorinated ethenes to environmentally benign ethene and inorganic chloride.

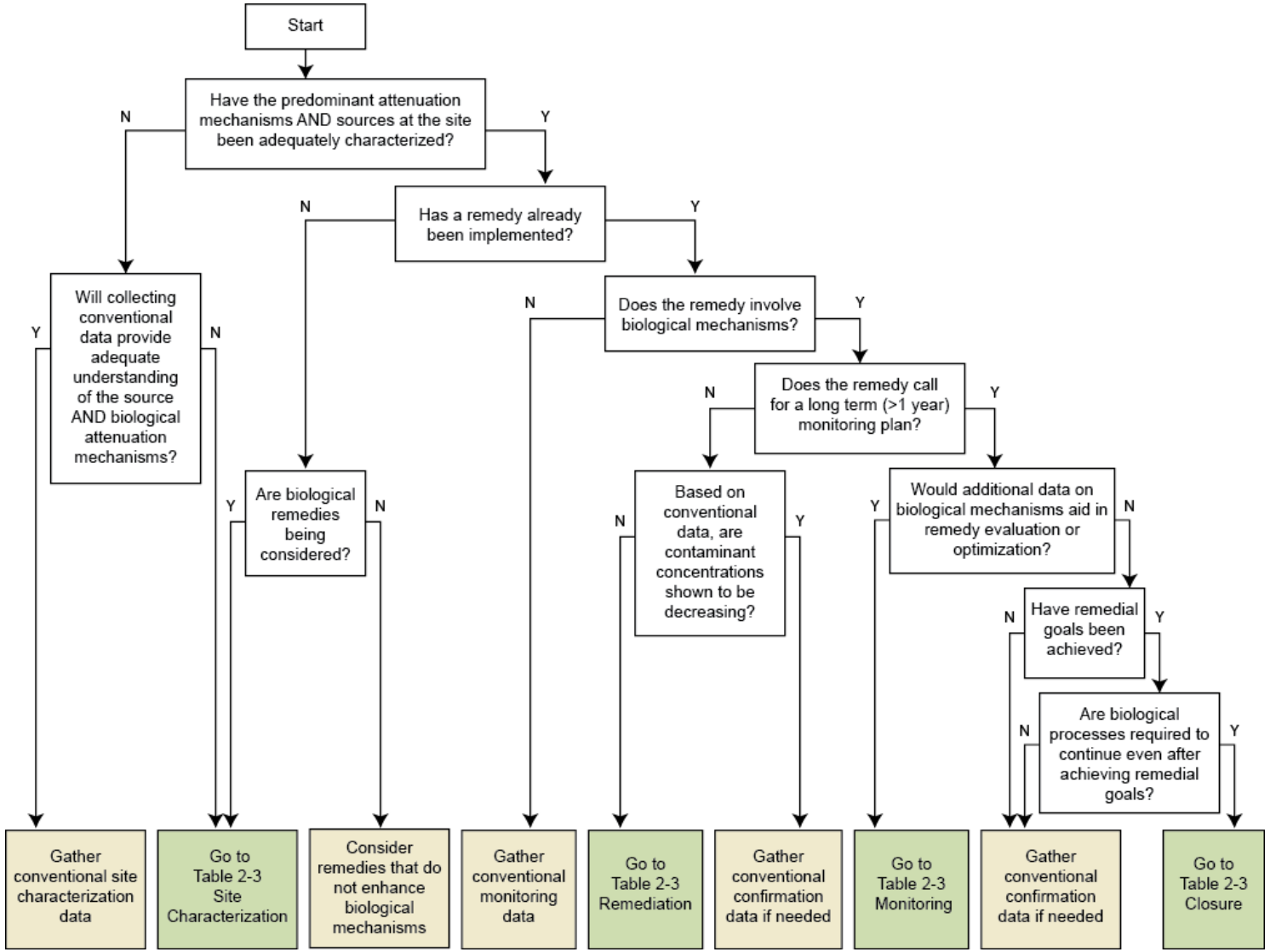

Figure 2-2. Decision tree.

Table 2-3 Questions Addressed:
A) Site Characterization - Are contaminant-degrading microorganisms present?
H) Remediation - Are numbers of contaminant- degrading microorganisms and/or genes changing?
I) Remediation - Is the remediation strategy affecting the numbers or types of contaminant degrading organisms?
O) Monitoring - Does the microbial community composition support the remediation strategy?
P) Monitoring - Do contaminant-degrading micro- organisms continue to be sufficiently abundant?

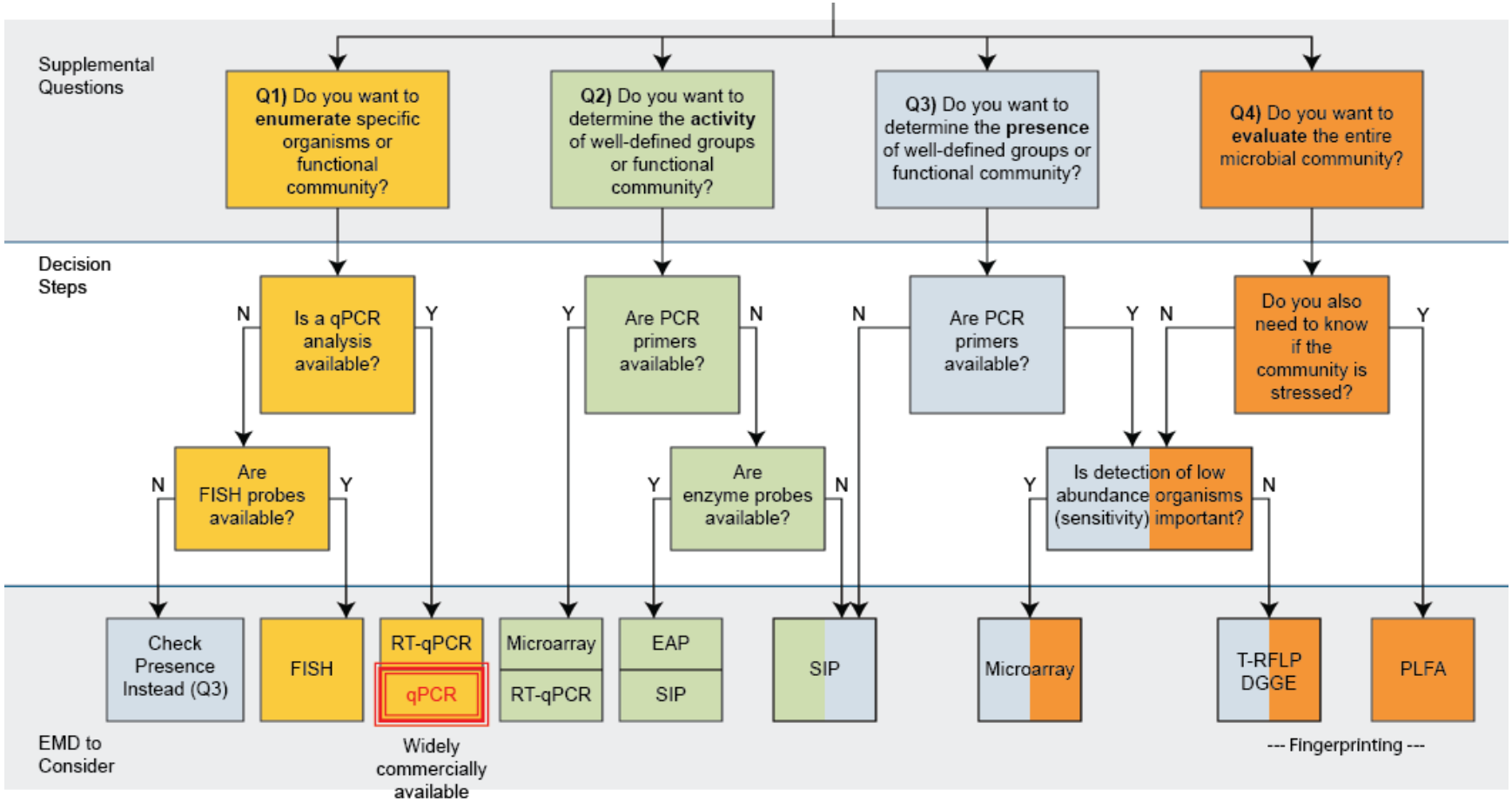

Figure 2-3. Decision tree.

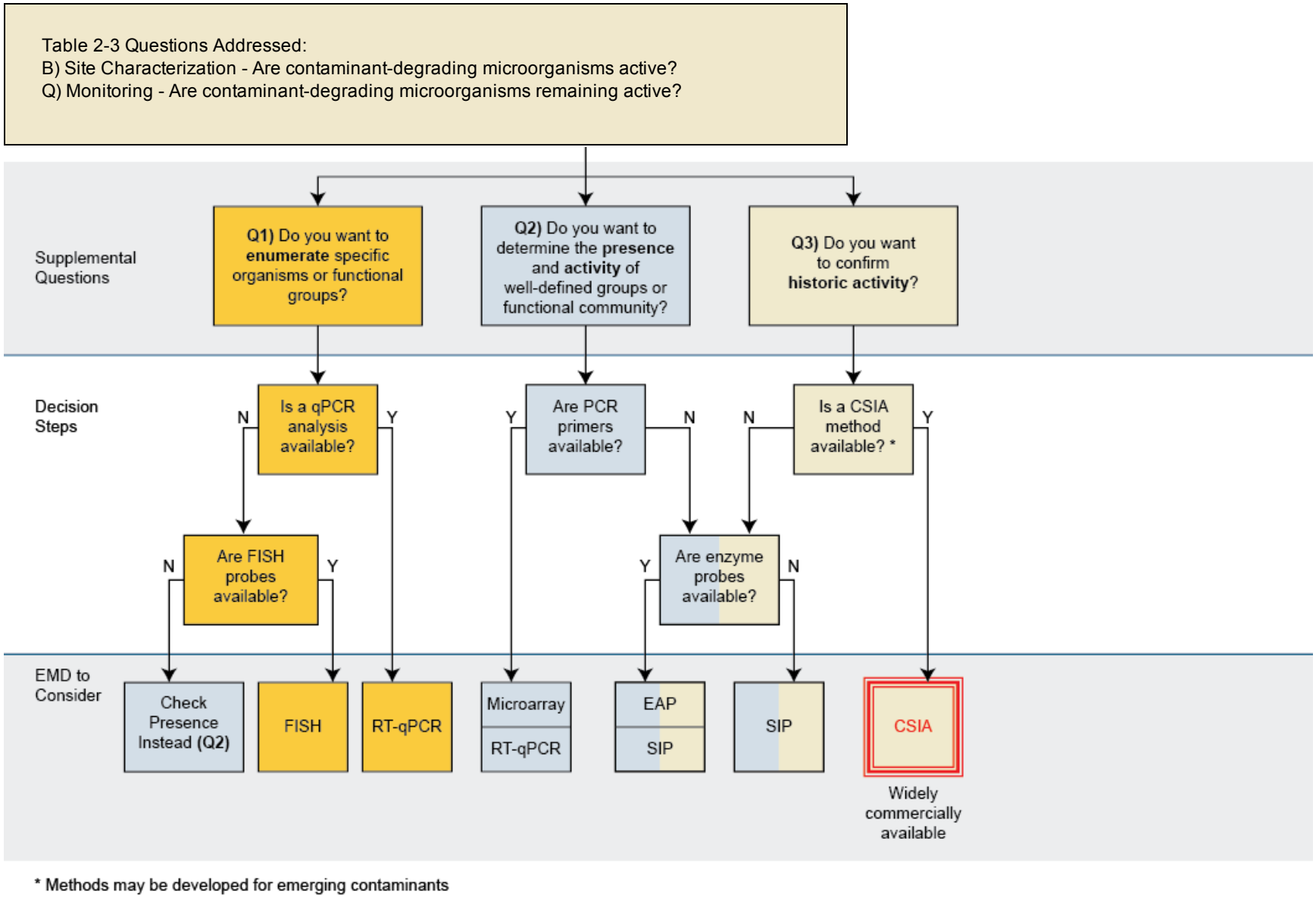

Figure 2-4. Decision tree.

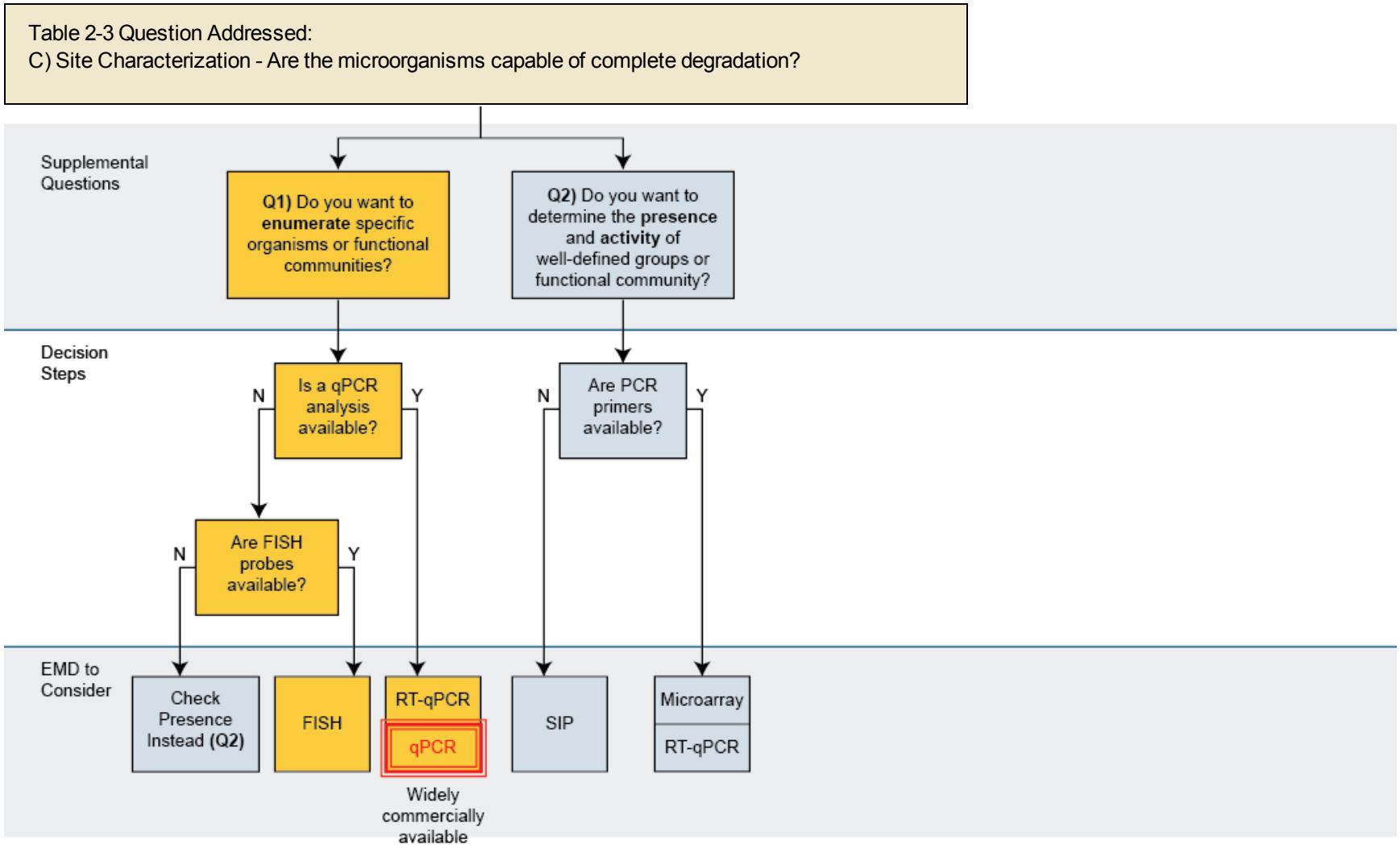

Figure 2-5. Decision tree.

Table 2-3 Questions Addressed:	
D) Site Characterization – Is biodegradation occurring?	U) Monitoring - Is biodegradation occurring?
M) Remediation - Is biodegradation occurring?	V) Monitoring - What is the rate of biodegradation?
N) Remediation - What is the rate of biodegradation?	Y) Closure - Is biodegradation occurring?
	Z) Closure - What is the rate of biodegradation?

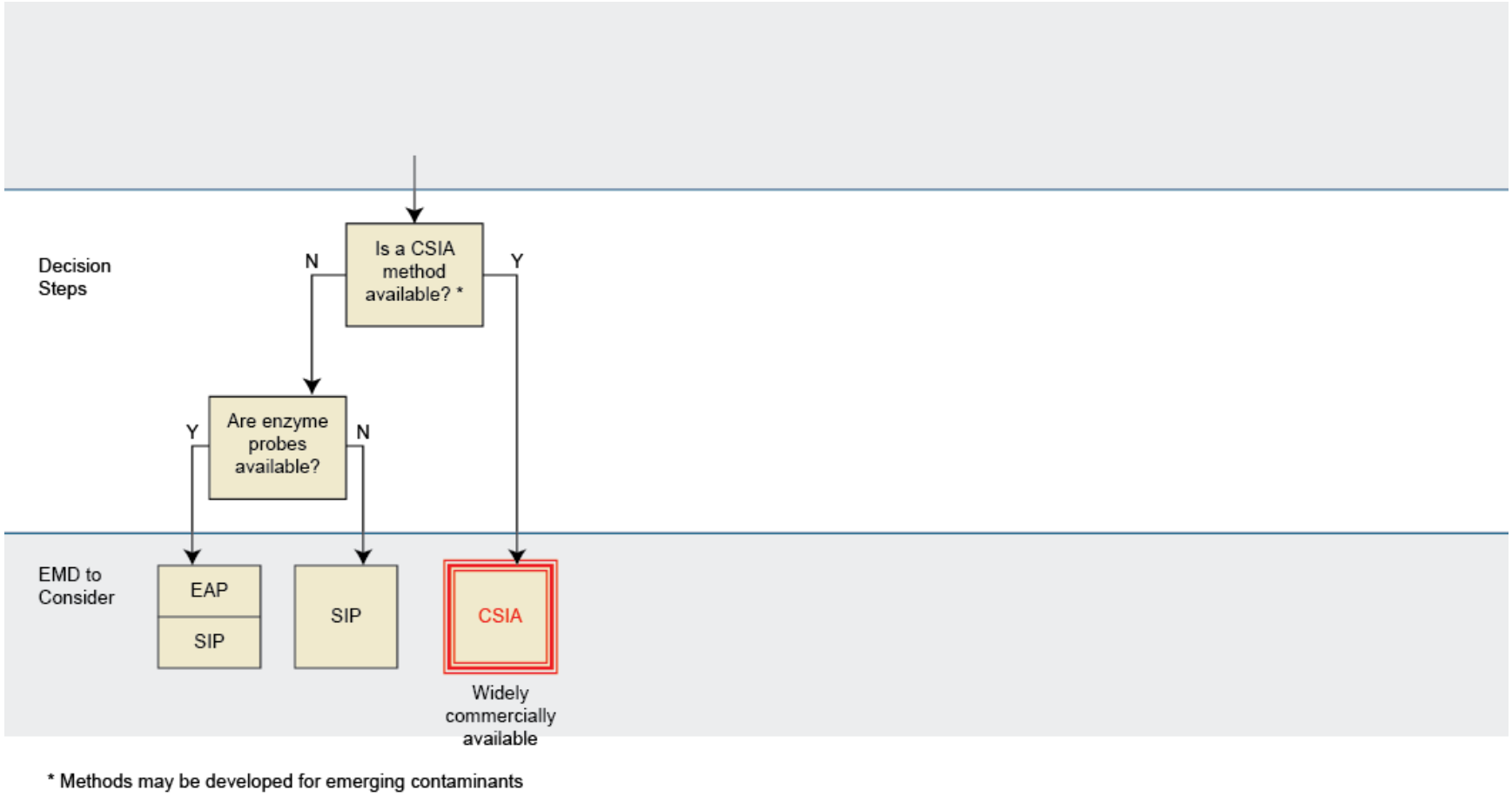

Figure 2-6. Decision tree.

Table 2-3 Questions Addressed:
J) Remediation - Is there a biological basis for intermediates accumulating?
R) Monitoring - Is there a biological basis for intermediates accumulating?

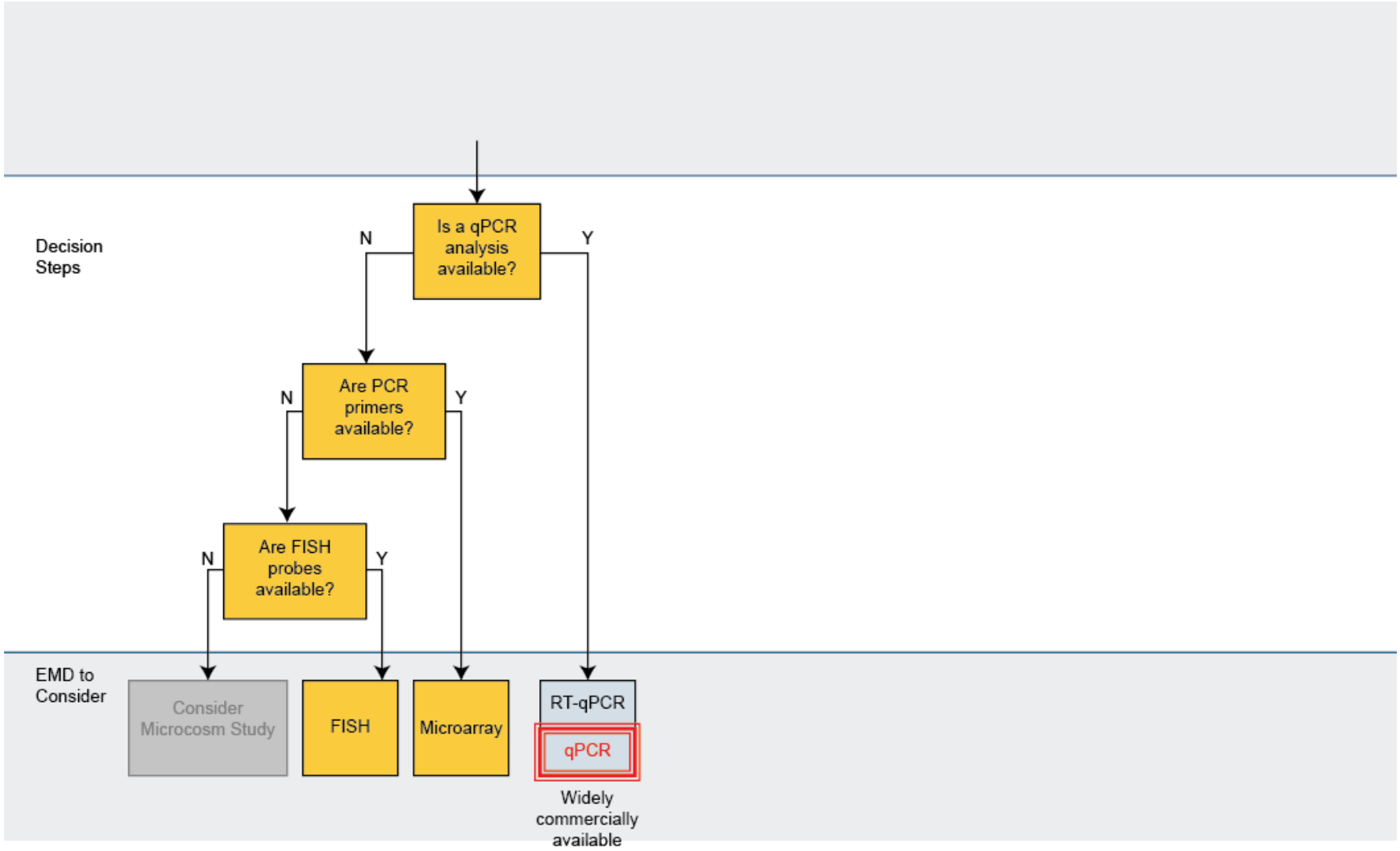

Figure 2-7. Decision tree.

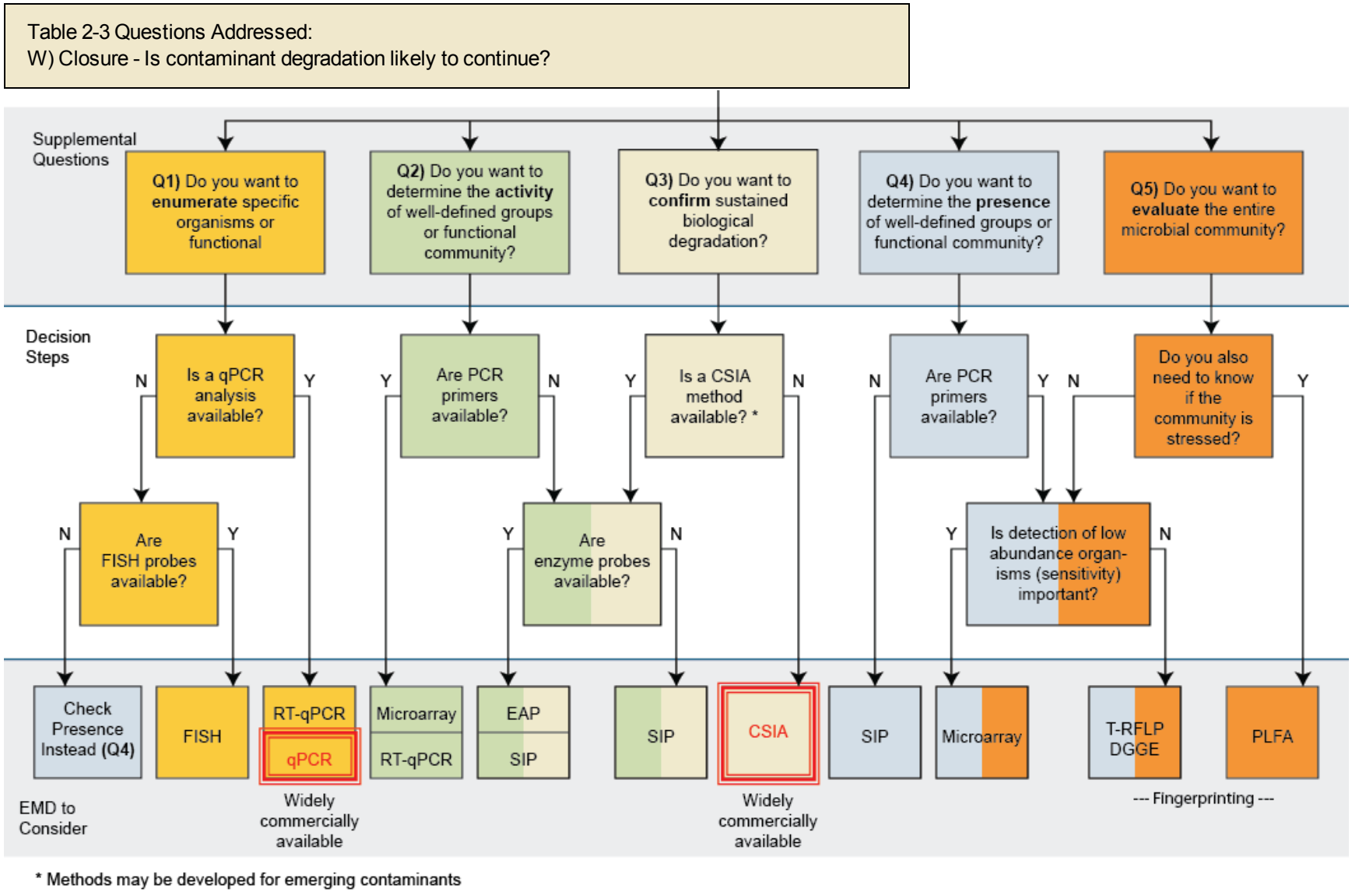

Figure 2-8. Decision tree.

Table 2-3 Question Addressed:
AA) Closure - Does the microbial community composition suggest that sufficient contaminant degradation has occurred?

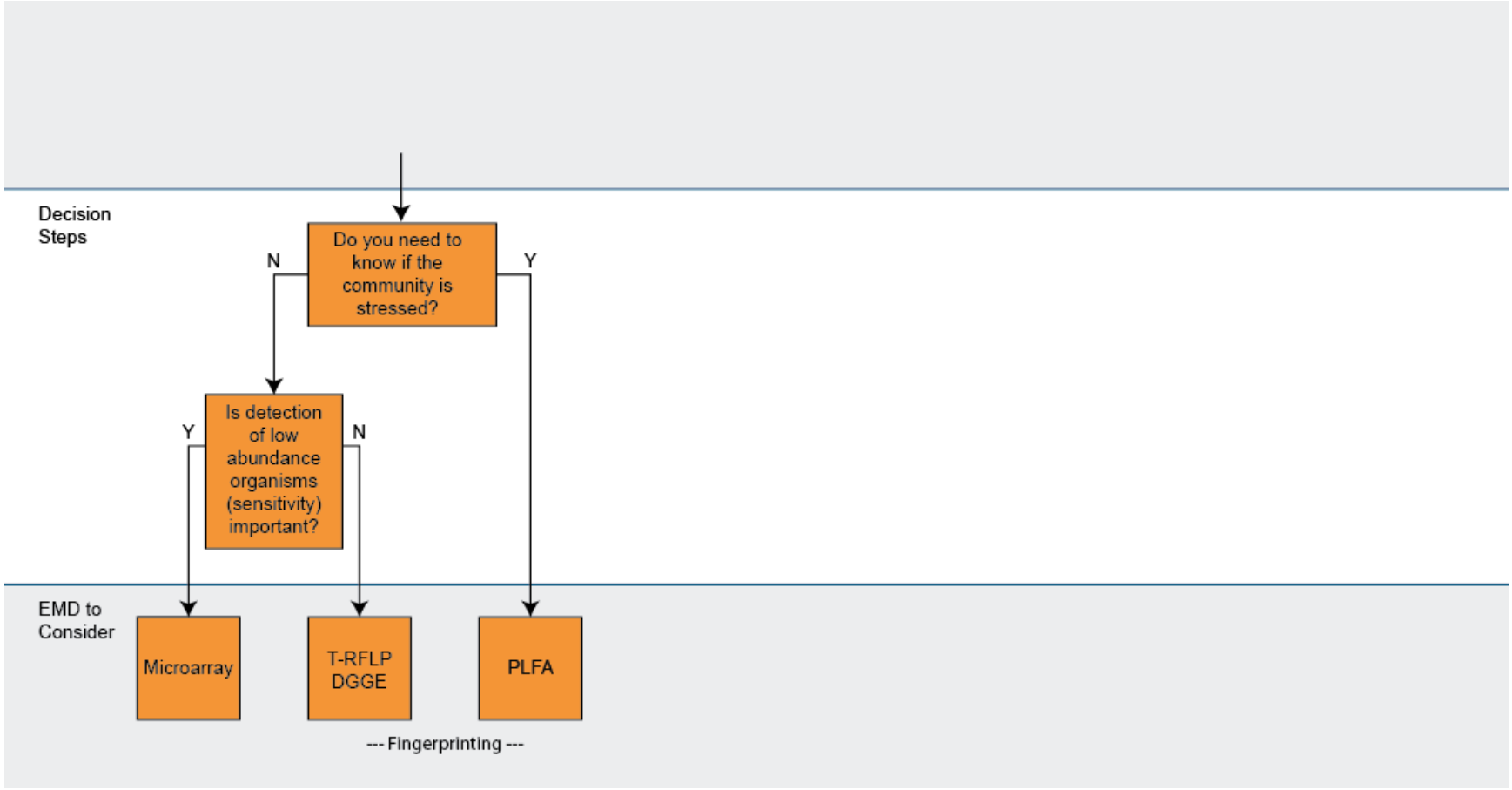

Figure 2-9. Decision tree.

3.0 COMPOUND SPECIFIC ISOTOPE ANALYSIS

3.1 Summary of CSIA

Each contaminant is made up of elements (for example TCE contains carbon, chlorine, and hydrogen; MTBE contains carbon, oxygen, and hydrogen). Each element within the contaminant has a distinct isotopic ratio (which is the ratio of heavy to light isotopes, such as $^{13}C/^{12}C$ in carbon within TCE). This ratio is called delta (δ) and is expressed in "per mil" (parts-per-thousand or ‰) relative to some internationally recognized standard. The compound specific isotope analysis (CSIA) method measures the isotopic ratio precisely relative to a standard, so those differences can be documented and interpreted. The isotopic ratio is initially determined by the natural material of origin and the manufacturing process, but then altered through degradation processes. CSIA can be used to measure degradation of a compound because the isotopic ratio changes as the compound is degraded.

Additional information about atomic structure and the CSIA process is provided in the CSIA Fact Sheet and in Appendix C Isotopic Chemistry.

Compound specific isotope analysis (CSIA) is used to directly examine individual contaminants to learn both about their original isotopic composition and about any degradation the compound has undergone. CSIA is unique among the EMD tools discussed in this document in that it is not biologically based. This technique also provides unique information concerning chemical fate because the data from CSIA analyses can be used to document contaminant degradation even in the absence of intermediate products or end products. CSIA can provide useful information at sites where there are multiple degradation mechanisms, including both biodegradation (by one or more pathways) and abiotic degradation (such as in situ chemical oxidation or reduction). For example, CSIA can be used to document contaminant degradation and to distinguish dominant mechanisms at a site where there is reductive dechlorination near the source, co-metabolism down gradient and abiotic degradation across the site. Further, under many conditions, CSIA can be used for forensic purposes to distinguish mixed sources of a single contaminant at a site or to determine the likely original source of a contaminant, such as perchlorate (which has both synthetic and natural origin as described in Case Study A.1). As discussed in the accompanying CSIA Fact Sheet, the utility of CSIA can be further increased for both forensic and environmental fate applications, by analyzing multiple isotopes in a given molecule (for example, both $^{13}C/^{12}C$ and $^{37}Cl/^{35}Cl$ in TCE).

3.2 Applications

CSIA has applications in environmental site assessment and remediation because environmental contaminants are composed of atoms of various elements and most elements have measurable stable isotopes. Both the forensic identity and the degradation of those contaminants can be traced based on the isotopic composition of one or more of these component elements. Applications of CSIA for evaluating contaminant degradation and distinguishing sources are discussed in more detail below.

3.2.1 Degradation

Physical processes such as sorption, dilution, diffusion, and volatilization do not significantly change the isotopic ratio of a compound in groundwater. In contrast, the isotopic ratio of an element (for example, $^{13}C/^{12}C$) changes in a predictable fashion when bonds between the elements are broken. As the dissolved mass of a contaminant is degraded, the portion of that contaminant that contains a heavy isotope increases. This increase occurs because the bond strength between elements with a heavy isotope (such as the ^{13}C-^{35}Cl bond) is slightly greater than that between elements composed only of light isotopes (^{12}C-^{35}Cl bond). As a result, the reaction rates of molecules with the heavy isotopes are slightly slower, resulting in an enrichment of the parent molecules with a heavy isotope during degradation processes. This "fractionation" process is illustrated in Figure 3-1 for a chemical with a C-Cl bond. Thus, the processes that cause molecular destruction can be isotopically distinguished from physical loss.

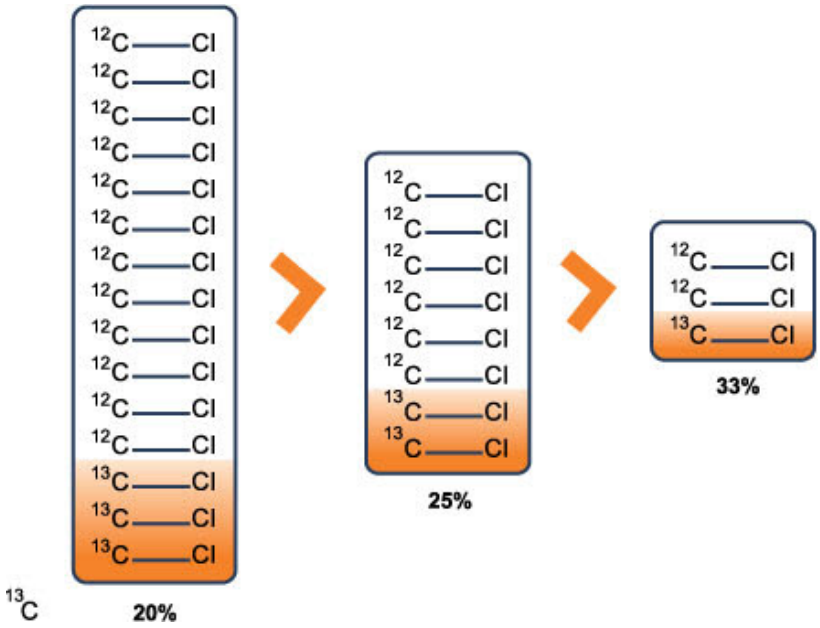

Figure 3-1. Example of ^{13}C enrichment as biodegradation of a compound with a C-Cl bond proceeds (left to right).

Source: Microseeps, Inc. 2011, used with permission.

CSIA allows measurement of isotopic ratios. Knowledge of the change in isotopic ratios (that is, fractionation or enrichment) allows the data measured through CSIA to be linked to fractionating processes such as biodegradation. As an example, consider the CSIA data collected from multiple measurements of benzene in microcosms. The data produced by those measurements are provided in Figure 3-2. Of course, there is a limit to the precision of CSIA, but CSIA is generally precise enough to measure the changes in the isotopic ratio that biodegradation introduces. The typical precision for carbon CSIA is ± 0.5 ‰, and USEPA recommends a conservative doubling of that range as an indication that biodegradation is occurring (USEPA 2008a). This 2 ‰ range is indicated by the vertical arrow in Figure 3-2. In addition, the initial conditions are at the far right and as

degradation proceeds the fraction of benzene remaining decreases, so the relevant data proceeds to the left.

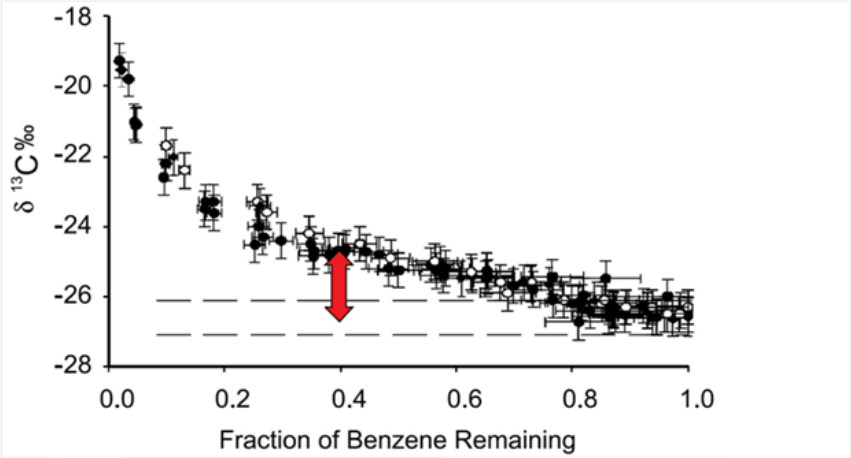

Figure 3-2. Figure showing the isotopic ratio of benzene (expressed as $\delta^{13}C$ and in units of per mil, or ‰) as the fraction changes.

Source:USEPA 2008a.

CSIA can be used both to measure the extent of degradation and to provide information on the pathway or mechanism of degradation. Multiple degradation processes can often be distinguished based on the extents of fractionation of isotopes of a given element (such as the change in $^{13}C/^{12}C$ ratio as a function of degradation extent). Mechanisms can also be inferred from the relative extents of fractionation of multiple elements (for example, H and C in MTBE), which can differ significantly when degradation occurs by different mechanisms. This inference is demonstrated in Figure 3-3 for data collected from multiple microcosm studies of MTBE. The data show the difference in the relative extents of fractionation for several mechanisms of MTBE loss. Note that in this case, however, the fractionation of C and H in MTBE by aerobic bacteria shows significant variability, possibly due to different initial reaction mechanisms (Rosell et al. 2012).

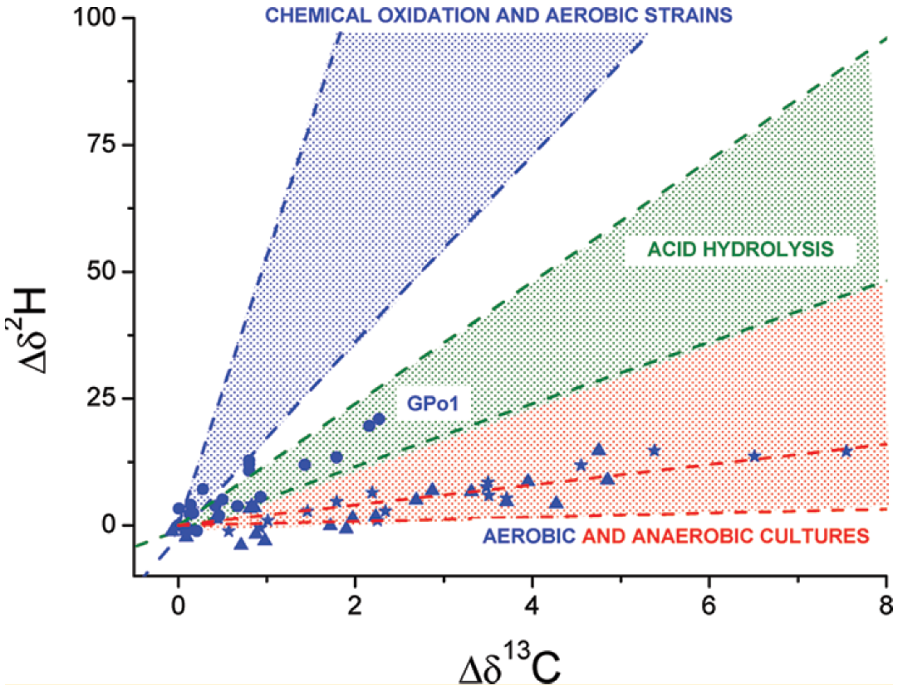

Figure 3-3. Plot of the δ²H vs. δ¹³C for MTBE under different experimental conditions. The authors used Δδ = δ – δo, presumably to eliminate variation in the initial isotopic ratio, δo, between the data sets (δ is a measure of isotopic ratio).

Source: Reprinted with permission from Rosell, M., R. Gonzalez-Olmos, T. Rohwerder, K. Rusevova, A. Georgi, F.D. Kopinke, and H.H. Richnow. 2012. Critical evaluation of 2D-CSIA scheme for distinguishing fuel oxygenate degradation reaction mechanisms. Environmental Science & Technology 2012, 46(9) pp 4757-4766. Copyright 2012 American Chemical Society.

CSIA can also be used to investigate both biodegradation and abiotic degradation of contaminants in groundwater. Further, it can be used to measure the contributions of degradation to attenuation versus those of nondegrading mechanisms such as dilution. Some applications of CSIA for examining contaminant loss are provided in the CSIA Fact Sheet. NAVFAC also provides a CSIA tool among its ER Technology Transfer resources. Additional examples are listed in Table 3-1.

3.2.2 Environmental Forensics

In addition to the evaluation of contaminant fate, CSIA is often used in environmental forensics applications to distinguish between contaminant sources at a site and to identify the most likely source of a contaminant in a specific well. The isotopic ratio of elements in a compound are determined by the source materials that the manufacturers use to make that compound as well as by the manufacturing process used to produce it. The source materials for a specific compound may vary from lot-to-lot or may be very consistent over time. In addition, the manufacturing process may change, therefore a chemical made by Process A may be isotopically distinguished from that made by Process B. Consequently, the isotopic ratios of elements in a chemical may differ over time (due to process or lot variability), even though the chemical is made by the same manufacturer. Initially, researchers did not recognize this variability for chlorinated ethenes, and failed in attempts to use CSIA to identify manufacturers of specific released materials (Beneteau et al. 1999; van

Warmerdam et al. 1995). The lot-to-lot variability (and often a lack of good information on the isotopic characteristics of the starting materials), prevents CSIA from specifically identifying a given manufacturer (Shouakar-Stash, Frape, and Drimmie 2003). CSIA can, however, be used to distinguish one source or release of a chlorinated ethene or ethane from another at a given site, particularly when the materials originate from different locations (which can be sampled to determine source isotopic ratios), and then become co-mingled (Smallwood, Philp, and Allen 2002; Slater-,2003; Blessing et al. 2009). Additionally, the contribution of each of the multiple sources can be calculated as long as the isotopic composition of each source can be determined and compared to the isotopic composition of the environmental samples.

In some instances, the isotopic ratio of a single element within a contaminant provides enough evidence to forensically distinguish one source from another at a site. For example, the Aviation Plaza data described in Case Study A.3, in which the CSIA of carbon in TCE provided sufficient evidence of multiple sources to justify a membrane interface probe (MIP) investigation which both confirmed the conclusions of the CSIA and provided other valuable information for the conceptual site model. However, results using CSIA for one element are often not as clear as in this example. In many cases, it is desirable to determine the isotopic ratio of multiple elements within a contaminant, rather than just a single element for forensic evaluations. This way, characteristic differences in isotopic ratios among all relevant isotopes can be compared, effectively providing multiple lines of evidence for contaminant differences. One example of this approach is the evaluation of the isotopic ratios of both oxygen ($\delta^{18}O$) and nitrogen ($\delta^{15}N$) in nitrate to distinguish possible sources (atmospheric production, septic systems, manure, and chemical sources such as nitric acid).

Figure 3-4 includes an example of using isotopic data to evaluate nitrate sources with a dual-isotope plot of $\delta^{18}O$ and $\delta^{15}N$. The data indicate that the source nitrate was derived from septic systems rather than atmospheric sources (precipitation), nitric acid, or degradation of nitrogen-containing explosives (RDX). The arrow in the figure (denitrification trend line) shows the expected isotopic fractionation of nitrate derived from septic sources if nitrate was biodegrading through denitrification. The data from downgradient wells clearly follow the denitrification trend line.

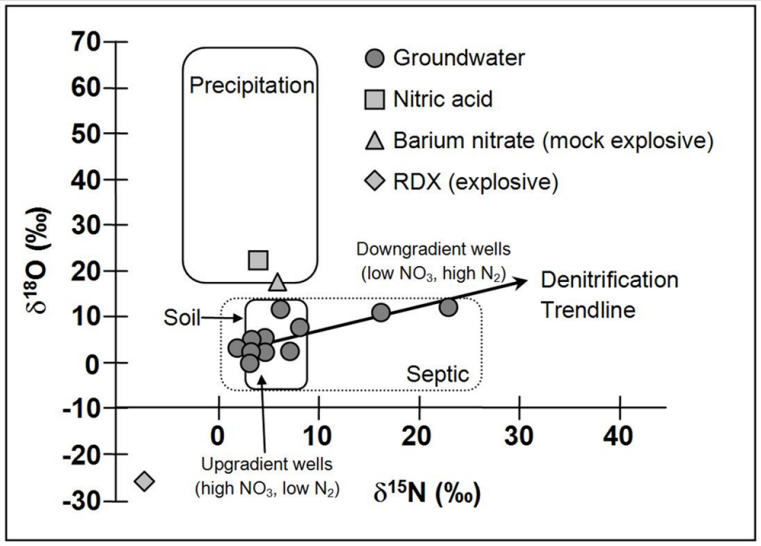

Figure 3-4. Plot of δ ^{18}O vs. δ ^{15}N for nitrate, showing the benefit of using CSIA data from multiple elements in forensics.

Source: Clu-in's Tech-Trends, Issue 46, February 2010.

In another example, using perchlorate (ClO_4^-), isotopic ratios of Cl ($^{37}Cl/^{35}Cl$) and O ($^{18}O/^{16}O$ and $^{17}O/^{16}O$) each provide unique evidence concerning the source of this compound in groundwater and soils (Bohlke et al. 2009; Jackson et al. 2010; Sturchio et al. 2012). In this case, synthetic sources from fireworks, flares, and others, can be readily distinguished from natural sources derived from nitrate fertilizers or natural atmospheric processes by comparing data derived from multiple isotopes (see Case Study A1).

Both of the previous cases show data for dissolved contaminants, but CSIA can also be applied to gaseous samples, such as indoor air where vapor intrusion is a suspected source of chlorinated solvent contamination. The basis of the application is presented in Figure 3-5. As indicated in this figure, a comparison of the $\delta^{13}C$ value of TCE in groundwater underlying a residence, with the $\delta^{13}C$ value of TCE in air within the structure can provide important evidence concerning whether vapor intrusion is the likely contaminant source. Values for $\delta^{37}Cl$ can also be used in conjunction with $\delta^{13}C$. As an example, McHugh et al. (2011) (see Case Study A.2) analyzed $\delta^{13}C$ in five residences where chlorinated ethenes were present in the indoor air, and in four of those cases, they also measured $\delta^{37}Cl$. In one residences, where only $\delta^{13}C$ was measured, the results indicated the contaminant source and no confirmation was needed. In two other residences both $\delta^{13}C$ and $\delta^{37}Cl$ were analyzed and the contaminant source was found to be consumer products stored within the home. In all three cases, confirmation of these conclusions were not necessary. In the remaining two residences, the CSIA results did not identify a specific source. While CSIA does not always provide clear answers, it shows promise for vapor intrusion applications.

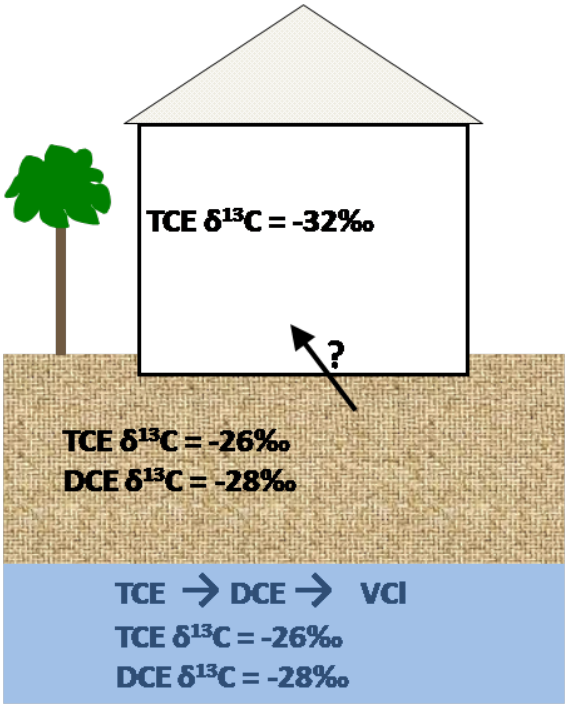

Figure 3-5. Schematic showing reductive dechlorination in the groundwater elevating $\delta^{13}C$ of the TCE, causing an isotopic ratio distinguishable from that of undegraded TCE in the breathing space.

Source: Microseeps, Inc. 2012, used with permission.

3.2.3 CSIA Applications Summary

CSIA can be used for monitoring contaminant degradation and for forensic studies. The application of CSIA for monitoring contaminant degradation was previously discussed in Section 3.2.1, with specific examples provided in Figure 3-2 and Figure 3-3. Forensic applications are discussed in the previous section, and specific examples of CSIA at sites contaminated with perchlorate or PCE/TCE are presented in Table 3-1, as well as in the case studies noted. It is beyond the scope of this section to present detailed discussions of each unique application of CSIA for environmental forensics and contaminant biodegradation, however, the following brief summary identifies some common applications and provides references for further information.

- **PCE/TCE**. Manufactured TCE can be often distinguished from TCE derived from PCE biodegradation via reductive dechlorination based on hydrogen isotopic composition (Shouakar-Stash et al. 2003). Several TCE manufacturing processes produce TCE characterized by a δ^2H of 100 ‰ or higher. In contrast, the hydrogen atom of TCE produced by the reductive dechlorination of PCE is derived from groundwater. Since the δ^2H of groundwater is generally 0 ‰ or lower, observation of TCE with a δ^2H of 100 ‰ or more is clear evidence of manufactured TCE. Recently, however, TCE manufacturing processes that produce TCE with a δ^2H value near or below 0 ‰ have been identified (personal communication, R Philp and T. Kuder 2012). As a result, while TCE with a δ^2H of 100 ‰ is clearly manufactured, TCE with a δ^2H near 0 ‰ is not necessarily derived solely from PCE biodegradation. Additional lines of evidence should be used in conjunction with CSIA for such determinations. For monitoring biodegradation of chlorinated ethenes, fractionation of carbon ($\delta^{13}C$) is most frequently measured (USEPA 2008a).
- **Perchlorate**. Isotopic characteristics of Cl and O ($\delta^{15}Cl$, $\delta^{18}O$, and $\delta\ ^{17}O$) in perchlorate have been used successfully for forensic evaluation of the occurrence of synthetic versus natural sources in groundwater (Bohlke et al. 2009; Sturchio et al. 2012). Isotopic fractionation of Cl and O also provides clear evidence of the biodegradation of this contaminant in the laboratory (Sturchio et al. 2007) and in the field (Hatzinger et al. 2011; Hatzinger et al. 2009). The use of multiple isotopes is critical for both source discrimination and evaluation of perchlorate biodegradation via CSIA.
- **Nitrate**. Extensive research has been conducted to evaluate nitrate sources (for example from septic systems, manure, nitrification, and synthetic fertilizer) in groundwater and soils through CSIA using $\delta^{15}N$ and $\delta^{18}O$. A recent review of this topic is provided by Aravena and Mayer (2010). Also, Jackson et al. (2010) have successfully used this technique to determine the origin of natural nitrate and perchlorate in southwestern U.S soils, mineral deposits, and groundwater. The isotopic fractionation of $\delta^{15}N$ and $\delta^{18}O$ in nitrate during deni-

trification has also been extensively studied, and fractionation factors have been reported in laboratory and field experiments (such as Mariotti 1986; Chen and MacQuarrie 2004).

- **MTBE**. CSIA can be used to track ISCO of MTBE as well as to track the biodegradation of MTBE by many, but not all, organisms. Observation of isotopic enrichment is evidence of MTBE degradation, but absence of that enrichment is not evidence of the absence of MTBE biodegradation.
- **Sulfate**. Much like nitrate, sulfate from anthropogenic production and deposition, sea spray, sulfide oxidation, detergents, and other natural and man-made sources can often be distinguished using CSIA (Aravena and Mayer 2010). In this case, values of $\delta^{34}S$ and $\delta^{18}O$ in sulfate are often compared for forensic evaluation. Transformation mechanisms of sulfur, including fractionation of $\delta^{34}S$ and $\delta^{18}O$ during sulfate reduction, have also been extensively studied.
- **Methane**. CSIA of deuterium ($\delta\ ^{2}H$) and carbon ($\delta\ ^{13}C$) in methane has been widely used to determine the origin of the gas, including whether or not the source of methane in groundwater is biogenic (biological origin) or thermogenic (fossil methane from thermal degradation processes), as discussed in Hornibrook and Aravena's review (2010). This technique can be useful in discriminating between dissolved methane derived from shale gas and methane derived from the biodegradation of material on or near the ground surface.
- **Petroleum hydrocarbons**. CSIA has been widely used to evaluate the original source of petroleum hydrocarbons, including crude oils from different regions of the world and gasoline derived from those oils (see review, Philip 2007).
- **Polycyclic aromatic hydrocarbons (PAHs)**.Examples of $\delta^{13}C$ analysis being used to evaluate different sources of PAHs in the environment are discussed in Philip (2007). For this application, chemical fingerprinting of PAHs is used in conjunction with CSIA for forensic evaluation.
- **Biodiesel**. Identification of the source of biodiesel-derived carbon in motor oil through molecular and isotopic analysis has been reported (Peacock et al. 2010).
- **PCBs**. Although less common, CSIA has proven effective for forensic delineation of PCB sources. Specifically, Jarman et al. (1998) used CSIA analysis for a series of commercial PCB formulations including Aroclors, Clophens, Kaneclors, and Phenoclors. They showed that a wide range of $\delta^{13}C$ ratios exist between PCB mixtures and individual congeners, which could be used for source identification.
- **1,2-dibromoethane (EDB)**. CSIA of $\delta^{13}C$ has recently been used by Henderson et al. (2008) to document the anaerobic biodegradation of this fuel oxygenate. Additional information on CSIA for EDB can be found in USEPA 2008b.

Table 3-1. Example applications of CSIA

Title and location	General information	Contaminants	EMDs	Project life cycle stage
M Canal Site (see description below)	Document in situ degradation in actively pumped area	PCE, TCE, *cis*-DCE and VC	CSIA	Site characterization

Table 3-1. Example applications of CSIA (continued)

Title and location	General information	Contaminants	EMDs	Project life cycle stage
Gasoline Site, CT (see description below)	Forensics: use of CSIA – Hydrogen and statistics to differentiate LNAPLs and "raw" gasoline	LNAPL, gasoline	CSIA	Site Characterization
New Jersey ISCO (see description below)	Use of CSIA to assess performance of ISCO and direct future ISCO applications	PCE, TCE, *cis*-DCE and VC	CSIA	Remediation
England AFB, LA (see description below)	Documenting degradation	TCE, *cis*-DCE, VC	CSIA	Monitoring
Pensacola NAS, FL (see description below)	Monitor biodegradation in areas previously remediated with ISCO	TCE, *cis*-DCE and VC	CSIA	Monitoring
CC Site, CA (see description below)	Documenting degradation	PCE, TCE, *cis*-DCE VC	CSIA	Remediation
CSIA of Perchlorate,NY (Case Study A.1)	Evaluate sources of perchlorate in groundwater	Perchlorate	CSIA	Site Characterization
CSIA for Vapor Intrusion, UT (Case Study A.2)	Use of CSIA to assess the source of vapor intrusion	PCE, TCE, DCE	CSIA	Site Characterization
Aviation Plaza, NJ (Case Study A.3)	Forensics: multiple sources of PCE	PCE, TCE, *cis*-DCE, VC	CSIA	Site Characterization
qPCR Case Study – Seal Beach, CA (Case Study A.5)	Monitor biodegradation and extent of dechlorination at Seal Beach Naval Weapons Station	PCE, TCE, *cis*-DCE and VC	qPCR, CSIA	Monitoring
EAP Case Study - Gaseous Diffusion Plant, Paducah, KY (Case Study A.7)	Evaluated degradation of TCE via aerobic co-metabolism	TCE	EAP, CSIA, qPCR	Site characterization and remediation

For some of the entries in Table 3-1, a full case study is provided in Appendix A. For others, a brief description of the setting, problem, role of CSIA, and the results are provided below.

M Canal Site

The M Canal site is an example of the use of CSIA to better understand the in situ fate of PCE and TCE at a site and to use that understanding to develop a remediation strategy that provided significant cost and energy savings. At a facility on the edge of a large river in the Southwest US, a mixture of wastes that included PCE and TCE were released. As part of the remedial effort, a pump and treat system was installed. In order to be protective, the pump and treat system was intensive and involved pumping the contaminated water from the river into the treated land versus from the land to the river. Although costly, the system could not be shut down unless there was sufficient evidence that natural attenuation was occurring. Samples were taken from the treated area and CSIA was performed on the PCE, TCE, *cis*-DCE, and VC samples. These samples proved that an active pathway to complete dechlorination existed, allowing shutdown of the pump and treat system.

Gasoline Site, CT

The challenge for this project was to differentiate one LNAPL from another. In an attempt to prove

and test the method, seven samples were collected. One of those samples was a duplicate and one was a gasoline sample taken directly from a pump. CSIA–Hydrogen was performed on the samples for 12 compounds. Hydrogen was chosen because there is more range in the $\delta^{2}H$ values of petroleum product components than in $\delta^{13}C$, even when considering the higher precision of $\delta^{13}C$ measurements (typically ±0.5 ‰) to that of $\delta^{2}H$, typically ±2 ‰ (USEPA 2008a). Two statistical techniques were used to aid in the interpretation: sample pair standard deviation and dissimilarity coefficient (DC).

The DC for the CSIA data was plotted against the DC calculated using the concentrations as shown in Figure 3-6. The difference between the far left group and DEP-6 is obvious. It is less obvious that DEP-4 and DEP-5 are not one group, but the standard deviation for the pairing DEP-4 and DEP-5 is one of the largest standard deviations for any pair. These data indicate that four of the samples, DEP-1, DEP-2, DEP-3 and DEP-7 (a duplicate of sample DEP-1), were from one source. The fresh gasoline sample (DEP-6) was from a second source, and the two remaining samples (DEP-4 and DEP-5) were either from different sources or perhaps contained a combination of two sources. The first two observations were a match to what was known before the test and the duplicate produced excellent results, so the conclusions about DEP-4 and DEP-5 were accepted.

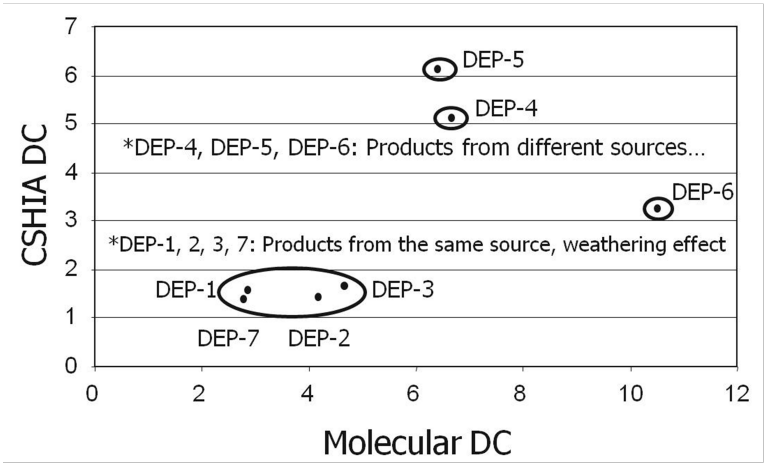

Figure 3-6. 2-D plot of dissimilarity coefficient (DC) for δ²H from CSHIA vs. Molecular DC calculated from the concentrations indicated multiple gasoline sources.

Source: Dr. Yi Wang at ZymaX Forensics Isotope, DPRA Inc., 2012. Used with permission.

ISCO Site, NJ

At the New Jersey ISCO site, in situ chemical oxidation (ISCO) via potassium permanganate was the chosen remedial strategy for groundwater containing PCE and TCE. There were five ISCO applications. Carbon CSIA data for the chlorinated ethenes were compared pre-treatment and post-treatment for the initial ISCO applications. The CSIA data through four ISCO applications revealed that the oxidant was effectively destroying the chlorinated ethenes, but significant rebound was occurring in the treated area due to delivery complications in complex hydrogeology. Accordingly, hydraulic fracturing was performed prior to the fifth ISCO treatment. The hydraulic fracturing made the final application much more effective and eliminated any rebound in the concentration. The use of CSIA informed the remedial decisions and allowed the project manager to optimize the remedial effectiveness while controlling costs.

England AFB

Monitored Natural Attenuation (MNA) was the selected remediation strategy for TCE at England AFB. Presumably, the dominant attenuation mechanism was reductive dechlorination. A small amount of ethene was observed, but far less than would be expected if the loss mechanism for TCE was predominantly reductive dechlorination. For many years, the concentrations of TCE, *cis*-DCE, and VC were all declining. However, during recent monitoring it was discovered the concentrations of *cis*-DCE and VC increased. These observations led project managers to pursue the use of Carbon CSIA to evaluate the dominant attenuation mechanisms at the site. Three questions were asked: 1) Is the *cis*-DCE degrading? 2) If vinyl chloride (VC) is observable, is it being degraded? and 3) Has there been any degradation of chlorinated ethenes to anything other than ethene? CSIA was used to answer each of these questions. The CSIA data showed that both *cis*-DCE and VC at the site were biodegrading and there was evidence of degradation of the chlorinated ethenes to compounds other than ethene such as acetylene or carbon dioxide (Shaw 2010). Without the observation of end products or any assumptions about the degradation mechanism, it

was shown that a portion of the TCE present in the initial release has been completely detoxified.

Pensacola NAS

At Naval Air Station (NAS) Pensacola, TCE and sulfuric acid were released into a sandy aquifer. Pump and treat was used from 1986-1997, an ISCO treatment with Fenton's reagent was performed in 1998-1999, and MNA was used beyond the source area. Biostimulation was investigated as a means to accelerate remediation. The complicated release and remediation history posed challenges for monitoring remediation progress, so CSIA was added to the suite of performance monitoring parameters to help understand the remedial processes. For the biostimulation, both changes to chlorinated volatile organic compound concentrations and $\delta^{13}C$ data from selected wells were used to evaluate performance. At some wells, the concentration of TCE decreased, but so did the $\delta^{13}C$ of the TCE. Further, in some wells, the concentration of *cis*-DCE rose far beyond what would be expected by the stoichiometric conversion of the TCE previously found in that well. The only explanation for these observations is degradation accompanied by the introduction of residual material.

CC Site, CA

At the CC site near San Francisco, CA, waste barrels with chlorinated ethenes were stored over a period of years. Chlorinated ethenes leaked from the barrels and eventually entered the groundwater. The contaminants entering the groundwater were from multiple sources and released over a period of several years. Concentration data suggested that a *cis*-1,2-DCE plume present from the partial reductive dechlorination of the parent compounds (PCE and TCE) was stable, but evidence was required to show that there was an active pathway to complete dechlorination of these compounds to ethene. CSIA of PCE, TCE, and dechlorination intermediate products provided g evidence to show that complete biological reduction of PCE and TCE to ethene occurred at this site.

3.3 Data Reporting, Validation, and Interpretation

CSIA data are reported as differences from isotopic standards and discussed in more detail in Section 3.3.1. A discussion of the data quality issues particular to CSIA is presented in Section 3.3.2. Finally, in Section data interpretation is covered explicitly as it relates to the commonly asked questions discussed in Table 2-3.

3.3.1 How are CSIA data reported?

CSIA is a sensitive technique, which reveals much through the observation of small changes in the isotopic ratio of an element. Because the differences in isotopic ratios are so small, it is more convenient to express the ratios relative to some standard and in "per mil" (parts-per-thousand or ‰) notation. This is accomplished by using the delta formula. The R_{std} refers to the isotopic ratio of an internationally agreed upon standard, for example $R_{std} = 0.01118$, is the standard for carbon. The delta formula, in "per mil" is:

Equation 3-1:

$$\delta_x = 1000 \times \frac{R_x - R_{Std}}{R_{Std}}$$

where the R_x is the isotopic ratio of sample "x" and δ_x (called "delta" of sample "x") is linearly related to the isotopic ratio. Therefore, if the $\delta(^{13}C)$ for a TCE sample is "-31 per mil", (a typical value for undegraded TCE), the $^{13}C/^{12}C$ in the sample is 31 per mil, or 3.1 percent, lower than in the standard. The only significance of the negative sign is the implication that the isotopic ratio in the sample is less than in the standard.

Additional information about the data reporting is included in Section C.9.

3.3.2 Data Quality Issues for CSIA

The QA/QC program used for CSIA sampling is largely dependent upon the application. To assist with the correct application of CSIA, USEPA has published *A Guide for Assessing Biodegradation and Source Identification of Organic Ground Water Contaminants using Compound Specific Isotope Analysis* (USEPA 2008a). This document discusses many of the technical aspects about this technology, including how to best design a CSIA study to address a particular question. Further, NELAC (http://www.nelac-institute.org/index.php) has general standards for sample handling, data manipulation, training, documentation, and reporting, all of which are important issues in acquiring CSIA services but which are not often covered in technical methods or in method specific SOPs. Because there are currently no USEPA-certified CSIA methods, QA/QC procedures should be discussed with the laboratory performing the analysis, the project manager, the applicable regulating authorities, and any other stakeholders prior to the collection of samples for CSIA.

3.3.2.1 Understanding Reporting Limits for CSIA

One important issue that has emerged is the use of CSIA values only for sufficiently large contaminant concentrations. Determining a detection limit for CSIA can be complex. Recognizing that background noise can be mistaken as a legitimate detection, the method detection limit (MDL) is intended to be the "the minimum concentration of a an analyze (substance) that can be measured and reported with 99% confidence that the analyte concentration is greater than zero as determined by the procedure set forth in appendix B " (40 CFR 136.2). USEPA recommends (USEPA 2008a):

> "…the operational detection limit be defined as that concentration of the compound in the water sample below which the accuracy and reproducibility of the value for $\delta^{13}C$ deteriorate beyond acceptable limits. The criterion for "acceptable limits" depends on the use of the data, and is dependent on the methods and the instruments used."

To demonstrate how CSIA data can be unreliable at low concentrations, USEPA prepared the graph shown in Figure 3-7. Multiple $\delta^{13}C$ analyses were conducted for various concentrations of benzene in water. The standard deviation of the triplicate samples increases as the concentration decreases to less than 0.2 µg/l which is the method detection limit. Below the method detection limit the standard deviation and therefore the precision (indicated by the height of the red bars) becomes unacceptably high. The role of CSIA is not to measure contaminant concentration; enough contaminant mass must be present in a sample to provide reproducible CSIA results.

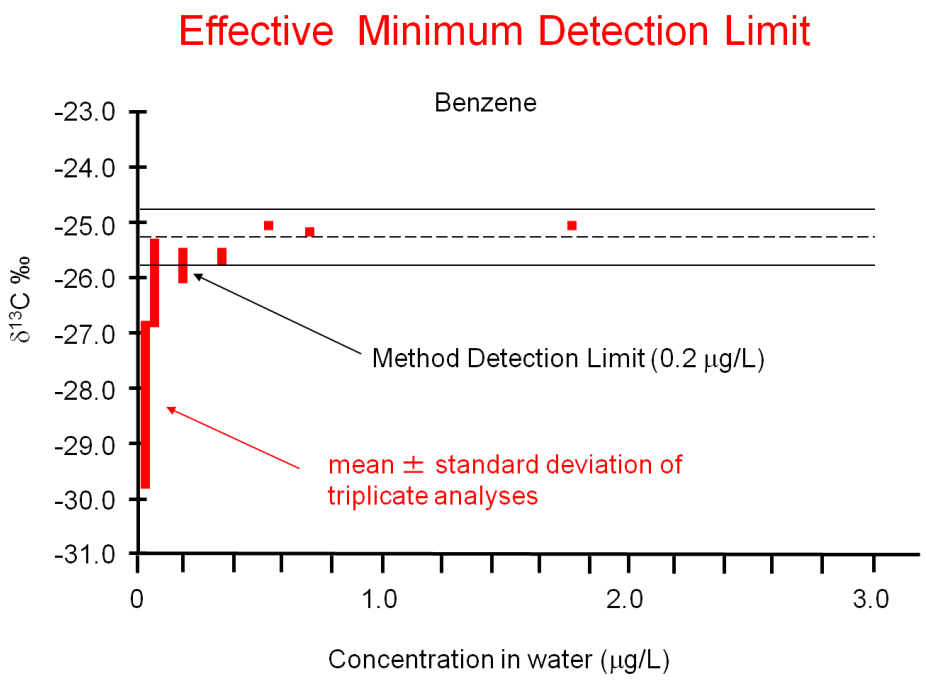

Figure 3-7. Graph showing decrease in precision of $\delta^{13}C$ of benzene at low concentrations. Note the very large standard deviation on the analyses as the concentration goes below 0.2 µg/l.

Source: John Wilson, USEPA, data from USEPA 2008a, used with permission.

The exact protocol for a study like the one depicted in Figure 3-7 is beyond the scope of this section, but it is important that the laboratory conducting CSIA analysis carry out similar detection limit studies before the project is initiated so that resources are not spent acting on non-representative data. This detection limit can be thought of as a practical quantitation limit (PQL). Typically there is a small but significant range below the PQL where the results are usable, but do not have the precision and accuracy that is typical of the method. In that case, "J flags" may be used to qualify the peak area. The "J flag" indicates that the measurement is less precise than a measurement at a concentration above the PQL, but still sufficiently accurate and precise to be useful.

3.3.2.2 CSIA Analytical Techniques

The quality control program used for CSIA partially depends on the specific analytical technique used. As such, an overview of the most common isotopic methods is presented below. Note that the analytical equipment used varies among the methods, so the quality control program for one method may be different from the quality control program for another method.

Stable isotopes of an element can be quantified in two basic ways: "bulk" and "compound" specific isotopic ratio analysis. "Bulk" stable isotope analysis measures the isotopic ratio of all compounds in a sample. In CSIA, the compounds are separated and the isotopic ratio of the specific contaminant is measured. This allows CSIA to focus on the individual contaminants. For relatively

small elements, such as carbon, chlorine, oxygen, nitrogen, and hydrogen, an isotope ratio mass spectrometer (IRMS) is used to measure the isotopic ratios of small, simple gases such as CO_2 for carbon or H_2 for hydrogen. The bulk method uses an IRMS with an offline process to convert the analyte in the sample to the appropriate gas. This is called "offline sample preparation." The second method uses online sample preparation in which a gas chromatograph (GC) is used to separate compounds of interest prior to isotopic analysis. This instrument is known as a GC-IRMS. It is this online sample preparation that makes CSIA possible.

New techniques are being developed to analyze the stable isotopic ratios of larger elements and those that are not readily converted to a gaseous form. Though they are not ordinarily compound specific, these techniques further extend the range of applications of isotopic analysis. For example, traditional inductively coupled plasma–mass spectrometry (ICP-MS) has been used to determine the concentrations of metals in environmental media and to estimate stable isotopic ratios, but with a relatively large error. The recent development of a multicollector ICP-MS (MC-ICP-MS) allows much more sensitive and precise measurement of the stable isotopic ratios of metals, such as Fe, Pb, Zn, Cu, Mo, and others (Romanek et al. 2010). Each of these techniques is described in more detail in the subsequent subsections.

IRMS with off-line sample preparation - Bulk

This approach has been used for more than 60 years, and involves the initial conversion of the sample of interest to the measured compound, which must be in the gas phase (for example, CO_2 for carbon). The converted sample is then introduced into a dual inlet mass spectrometer simultaneously with the relevant isotopic standard. Depending on the sample preparation method, this analysis is often not compound specific. However, it is still powerful and has been applied for many different elements and compounds. This method can be used for pure compounds or for complex mixtures without pre-separation of individual compounds. However in the case of mixtures, only one isotopic ratio is obtained (e.g., bulk isotopic ratio of $\delta^{13}C$ in crude oil) and this approach generates a weighted average of the isotopic composition of all the individual compounds in the mixture. An example of bulk isotopic data is provided in Figure 3-8. This type of data has been used in petroleum exploration for many years.

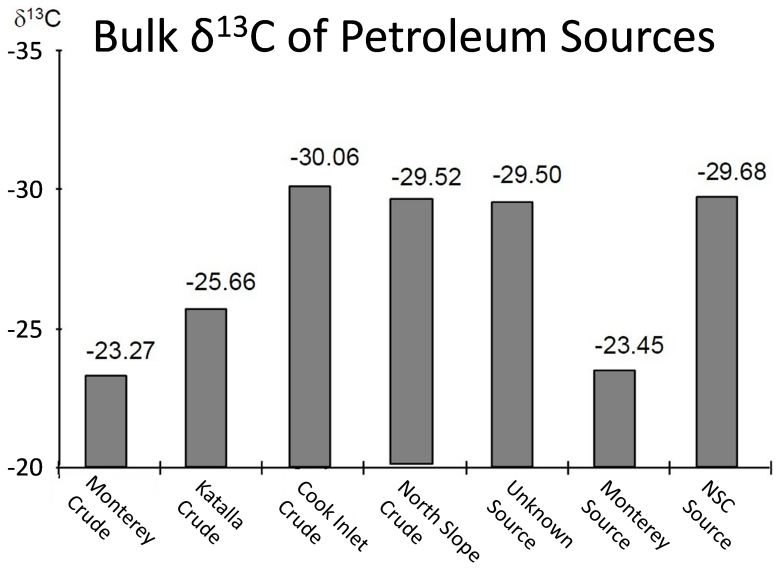

Figure 3-8. Bulk isotopic analysis of various petroleum sources.

Source: Adapted from US Navy 2008.

IRMS with off-line sample preparation – analyte isolation

It is possible to isolate the specific contaminant off-line, convert it after isolation, and then use an IRMS to measure the isotopic ratio of the analyte. This method is a form of CSIA and is the most common technique currently used to perform CSIA of Cl and O in perchlorate (for instance, Bohlke et al. 2005; 2009, Gu et al. 2011). The main disadvantage of this technique is that it requires a larger sample mass than similar online techniques. With a contaminant such as perchlorate, which is often present at low concentrations (for example, 10 µg/l) many liters of water may need to be processed to get enough mass for IRMS analysis. This mass is collected by pumping water through special ion exchange cartridges in the field that trap the perchlorate, and then shipping the cartridges to a laboratory for elution and purification of the trapped perchlorate prior to IRMS analysis.

GC-IRMS

An IRMS technique has been developed in the past 25 years that couples a gas chromatograph to an IRMS (GC-IRMS). GC-IRMS makes CSIA possible for many volatile (boiling point 40-180°C) and semi-volatile (boiling point 150-250°C) organic compounds. The ability to separate compounds and then determine the isotopic ratio of multiple separated compounds provided a significant advance in isotopic analysis for environmental applications by allowing stable isotopic ratios to be readily determined for individual compounds in complex mixtures.

In a typical GC-IRMS, all analytes are converted online to light gases prior to introduction into the IRMS for analysis; for example analytes are combusted into CO_2 for carbon analysis and pyrolized into H_2 for hydrogen analysis. A schematic of a GC-IRMS is provided in Figure 3-9. That

particular system is configured to use a purge and trap concentrator to analyze aqueous concentrations of environmental interest and to measure the isotopic ratio of carbon by converting analytes to CO_2 and measuring mass 44 ($^{16}O^{12}C^{16}O$), 45 ($^{16}O^{13}C^{16}O$ and $^{17}O^{12}C^{16}O$) and 46 ($^{18}O^{12}C^{16}O$). Details of how δ is calculated from these three signals are presented in C.7 What is actually measured?.

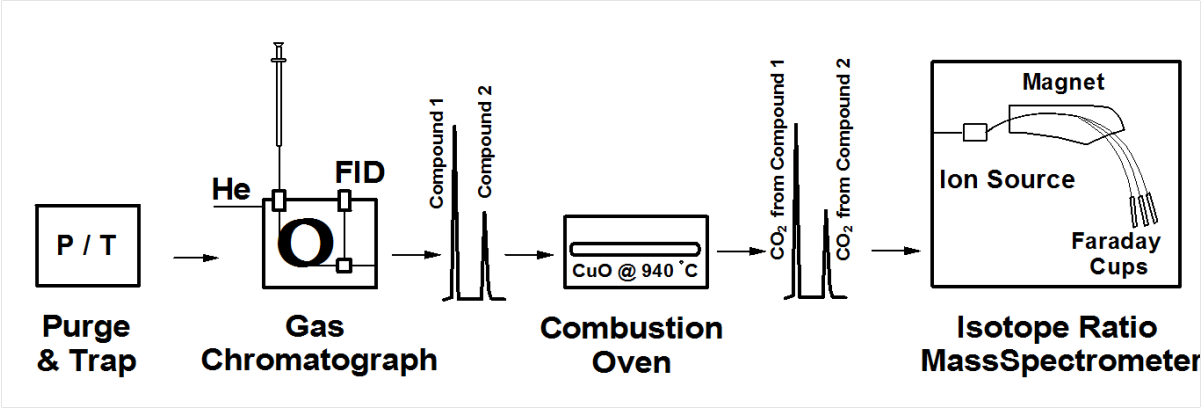

Figure 3-9. Schematic of a GC-IRMS configured to measure CO_2 from carbon at masses 44, 45, and 46.

Source: Microseeps, Inc. Used with permission.

GC-MS

There are some CSIA studies being conducted using a standard laboratory GC-MS operated in single ion monitoring (SIM) mode. This technique has recently been applied for analysis of Cl isotopes in VOCs and pesticides (Sakaguchi-Soder et al. 2007; Aeppli et al. 2010a). Using this instrument, the mass spectrometer provides both isotopic separation and positive compound identification since the analyte is not converted into a small gas molecule but analyzed by the mass spectrometer directly. As such, for this type of CSIA work, co-elution issues are not as critical.

ICP-MS and MC-ICP-MS

As previously noted, ICP-MS has been used for the analysis of stable isotopic ratios in specific metals, and a newer technique, MC-ICP-MS has been developed to improve the sensitivity and precision of the method. Additional details on stable isotope analysis using techniques and other new approaches can be found in Romanek et al. 2010. Isotope analysis has been used to determine the $\delta^{206}Pb$ (presumably in tetra-ethyl lead (TEL) derived from leaded gasoline). In this application, the $\delta^{206}Pb$ increases appreciably as an inverse function of the age of the gasoline, from the mid-1960's to the mid-1980's. The increasing $\delta^{206}Pb$ value is hypothesized to represent a slow shift in the source of the Pb used to produce TEL during this timeframe and is interpreted as linking the lead to leaded-gasoline (Hurst et al. 1996; Murphy and Morrison 2002). These techniques are included to

show that a number of different traditional and emerging approaches can be applied for specific forensic evaluation.

3.3.2.3 CSIA Reports

The key elements for CSIA report differ somewhat from application to application and target analyte to target analyte. Table 3-2 presents requirements that must be addressed for each application. While an attempt was made to be inclusive, for some applications (for example, perchlorate in groundwater) there are key parameters missing from the table as well as some unnecessary restrictions. It is critical to discuss these issues with the laboratory performing the CSIA measurements prior to the collection of samples. Note that these criteria concur with the guidelines set forth in the USEPA guidance (USEPA 2008a) for samples collected from groundwater.

Table 3-2. Example CSIA report requirements

Applicable isotopes	Description of information	Criteria	Frequency
All	Laboratory name and address	Must be present	Per report
All	ID of primary standard	Must be present	Per report
All	Minimum of signal [that is, a practical quantitation limit (PQL) for the peak area]	Must be present and traceable to a standard operating procedure and a referenced method	Per target analyte
Carbon, hydrogen, oxygen, sulfur, nitrogen and GC-IRMS chlorine	Yes/no for co-elution	Must be present	Once for every target analyte in every sample
Carbon, hydrogen, chlorine or oxygen of VOCs in water or air	Dilution factor (necessary to validate peak co-elution evaluation)	Must be present	Once for every target analyte in every sample
All	Area response (necessary to validate peak co-elution evaluation)	Must be present	Once for every target analyte in every sample
Carbon, hydrogen, chlorine or oxygen of VOCs in water or air	Concentration as per GCMS (necessary to validate peak co-elution evaluation)	Must be present	Once for every target analyte in every sample
All	Two control samples each containing the target analytes from a single source but at different concentrations	1) Acceptance criteria should be supplied and met. Those acceptance criteria should have been established through previous replicate analyses. 2) The spike concentration should be given to facilitate the validation of the co-elution determination.	Once per 10 samples or once per batch
All	Sample blank	No target analytes should be detected in the blank with an area greater than 50% of the PQL.	Once per 10 samples or once per batch

Table 3-2. Example CSIA report requirements (continued)

Applicable isotopes	Description of information	Criteria	Frequency
All	Duplicate sample	Should duplicate the original sample to within the expressed precision of the measurement (±0.5 ‰ for carbon, ±5.0 ‰ for hydrogen, ±0.5 ‰ for chlorine). If this criterion is not met the measurement should be repeated, but if it is still not met the problem is most-likely inherent in the sample matrix and outside of the laboratory's control	Once per 5 samples or once per batch
All	Analysis date	Must be present	Once for every target analyte in every sample
All	SOP reference	Must be present	Once per target analyte
All	Field and Laboratory sample IDs	Must be present	Once for every target analyte in every sample
All	Case Narrative	A brief description and evaluation of the data in which any exceptions to the SOP are explained, as are any potential impacts to the data.	Once per report

3.3.2.4 Sample collection, preservation and holding times for CSIA samples

In most instances, sample collection techniques, preservation methods, and holding times have not specifically been established by regulatory agencies for CSIA analyses. Site managers must discuss sample collection and preservation techniques with the responsible regulator at a site as well as with the analytical laboratory performing the CSIA measurements. In many cases, these laboratories will have SOPs for sample collection and preservation that should be followed to ensure data quality. Moreover, specialized techniques are occasionally required when collecting an analyte for isotopic analysis, as is the case with perchlorate where groundwater is passed through an ion exchange resin column to trap the perchlorate in sufficient quantity for analysis (Bohlke et al. 2009).

As with collection and preservation, there are no USEPA-established holding times for CSIA samples. Often, CSIA analyses will require more time to complete than traditional measurements of contaminant concentration, so using the same holding times for these methods can result in samples being analyzed beyond the recommended holding time for these traditional methods. With proper preservation and storage, and in the absence of any losses to evaporation, sorption, or other physical processes, isotopic ratios in analytes can be stable for several weeks to several months, (USEPA 2008a; Blessing et al. 2008; Hammer et al. 1998). Blessing et al. (2008) reported no substantial fractionation of groundwater containing BTEX or chlorinated hydrocarbons within 4 weeks of sample collection. In the absence of any previously published data on isotope stability for

a given compound, recent guidance from USEPA recommends that holding times recommended for the method used to analyze the concentration of the contaminant be used for the CSIA samples as a conservative measure (USEPA 2008a).

3.3.3 How are CSIA data interpreted?

To demonstrate how CSIA data can be used to answer the primary questions presented in Table 2.3, examples are considered at each project life cycle stage in the following sections. For the purposes of this discussion, assume CSIA data has been collected from a representative selection of wells throughout each location in the subject sites. After the discussion of how CSIA is used to answer specific questions relevant to CSIA, references for further reading are provided.

3.3.3.1 Site Characterization

B) Are contaminant-degrading microorganisms active?

A release at a site contained benzene. After this initial release, contaminant concentrations were observed to decline over time. In addition, sulfate concentrations were lower than background in the core of the plume, indicating sulfate reducing conditions. Reports suggest that benzene degrading organisms are known to exist and be active under sulfate reducing conditions (Lovley et al. 1995). Moreover, the biodegradation of benzene by sulfate reducing strains has been reported to cause isotopic fractionation of carbon in laboratory samples (Mancini et al. 2003). Therefore, to determine if benzene was being degraded (as opposed to diluted), the $\delta^{13}C$ values of benzene in groundwater were measured along a flow path downgradient of a suspected source at the site. The resulting $\delta^{13}C$ data for each well are displayed in Figure 3-10.

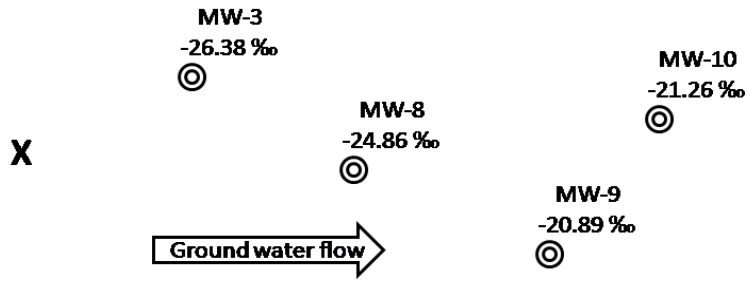

Figure 3-10. Map of an area in the site contaminated with benzene. The $\delta^{13}C$ of the benzene was measured at each of the wells shown and the results are indicated on the map. Also note that the location of the source is marked with an "X".

Source: Microseeps, Inc. Used with permission.

The combination of low sulfate concentrations, declining contaminant concentrations, and the increase in $\delta^{13}C$ presented in Figure 3-10 suggests that sulfate-reducing benzene degrading microorganisms are active in this area.

D) Is biodegradation occurring?

Benzene Biodegradation

The example for Question B, showing the analysis of $\delta^{13}C$ in benzene, answers the question of whether microorganisms are active and also provides clear evidence that biodegradation of benzene is occurring at the site. The increasing values of $\delta^{13}C$ in benzene with distance from the source are evidence for biodegradation. With knowledge of groundwater flow rates and the relevant stable isotope enrichment factor (epsilon value) for carbon during benzene biodegradation by sulfate-reducing strains (Mancini et al. 2003), estimates of biodegradation rates can be obtained using stable isotope results. See USEPA guidance (USEPA 2008a) for a detailed description of the method and constraints for determining degradation rates using CSIA results.

TCE Biodegradation

At a site, the primary contaminant is TCE, and it is unclear from the concentration and geochemistry data whether TCE is biodegrading. The TCE was released into a fractured rock aquifer where distance from the source was not necessarily proportional to the time since release. CSIA was performed to see if there was evidence that biodegradation was occurring. An example subset of the results is presented in Table 3-3. In several wells (for example, MW4) the $\delta^{13}C$ in TCE was higher, or more positive, than the highest currently published value of $\delta^{13}C$ in TCE which is -24.5 ‰ (Aelion et al. 2010). In other locations, (for example MW2) the $\delta^{13}C$ for TCE was significantly higher than similar values in the other wells (for example MW1), but not as high as in MW4. Historical records revealed all the TCE in this area was from the same source and the heavier (more positive $\delta^{13}C$) TCE was made more positive by degradation. The observation of TCE with $\delta^{13}C$ heavier than the heaviest published literature value for product and of a range of $\delta^{13}C$ values that are increasingly heavier than that found near the apparent source (well MW1 with highest residual concentrations) indicate that degradation has been active and that fractionation of $\delta^{13}C$ in TCE has occurred or is currently occurring.

These data should be combined with analysis of geochemistry, relevant intermediate products, including *cis*-DCE, VC and ethene, and appropriate microbial analyses, such as qPCR for *Dehalococcoides mccartyi (Dhc)* to provide additional supporting evidence for TCE biodegradation. The data suggest that the degradation occurring is biodegradation. This is supported by qPCR and the appearance of transformation products. Additionally, the observation of sulfide (as would be expected for respiration through sulfate reduction) and/or the presence of methane concentrations above 1,000 µg/l (as would be expected for methanogenesis) further support the conclusion of biodegradation.

Table 3-3. CSIA ($\delta^{13}C$) results for selected example wells

Well ID	Sulfide (mg/l)	Methane	TCE concentration (µg/l)	TCE $\delta^{13}C$
MW1	<2	1400	849	-28.91
MW2	2.3	750	125	-25.95
MW3	2.1	3300	21	-23.82
MW4	3.8	180	6.6	-21.04

TCE Biodegradation

At another site with TCE contamination, despite decreasing concentrations of TCE, the CSIA values were not significantly different than the heaviest of the published values. There were three potential explanations for this result:

- Degradation of TCE was proceeding, but the biodegradation mechanism active in the subject location did not have a large enough enrichment factor to observably change the isotopic ratio. Literature values of enrichment factors (Aelion et al. 2010) can typically be used to see if this is possible. For example, the biodegradation mechanism could be sMMO catalyzed co-metabolism. Under this condition, it may be more appropriate to confirm the biodegradation with EAP or qPCR.
- Degradation was proceeding, but it was masked by the introduction of undegraded TCE into the dissolved phase. This typically occurs in the presence of NAPL, and there is NAPL at this site in some of the areas where the contaminants were not observed to be heavier than the heaviest published value. In a slowly recovering aquifer, back diffusion of contaminants can continue to be an issue (Sale et al. 2008). Because degraded contaminants are continually replaced by new ones diffusing from the geological matrix, the decline in concentration is a poor measure of attenuation. In an aquifer that is otherwise "clean" the concentration of the intermediate products may be too low to measure. In this situation, CSIA provides powerful insight into an aggressive biodegradation that traditional characterization measures of contaminant concentration may miss.
- No degradation was occurring, rather only minimally fractionating attenuation mechanisms such as dilution and diffusion were responsible for the observed decline in TCE concentrations.

Based on the data, it could not be concluded that biodegradation was occurring at this site. See Aelion et al. 2010; USEPA 2008a; Gray et al. 2002; Song et al. 2002; McLoughlin et al. 2013a; Palau et al. 2010; Morrill et al. 2009.

E) Is the contaminant attenuating abiotically?

At a site, leaded fuel had been released and the fuel additive 1,2-dibromoethane (EDB) was detected significantly above the regulatory MCL of 0.05 µg/L (USEPA 2008b). The EDB concentrations were declining over time, but the mechanism of decline was unclear based on the site data. Analysis of $\delta^{13}C$ in EDB collected from several wells on-site was conducted using established methods to evaluate whether degradation could be confirmed based on stable isotopic ratios. The results from the analysis are presented in Table 3-4.

Table 3-4. EDB concentrations and $\delta^{13}C$ of EDB for selected wells (USEPA 2008b)

Well ID	Concentration (µg/l)	$\delta^{13}C$
MW5	15	-18.0
MW6	7.6	-10.5
MW7	2.7	-4.95
MW8	0.32	+11.0

Analysis of $\delta^{13}C$ in EDB revealed significant isotope fractionation as a function of distance from the spill, so it was clear that the concentration decline was due at least in part to a degradative process, rather than just dilution or dispersion. The EDB was present in groundwater that was anoxic and has a low oxidation-reduction potential, presumably due to biodegradation of the fuel hydrocarbons. However, EDB degradation has been reported to occur in anoxic environments through both biological processes (Maymo-Gatell et al. 1997) and via abiotic transformation with hydrogen sulfide (H_2S) (Schwartzenbach et al. 1985) and iron sulfide (FeS) (USEPA 2008b).

qPCR results showed no degradation capacity despite the application of a wide variety of probes, yet attenuation appeared to be occurring. CSIA results showed that the $\delta^{13}C$ of the contaminants were heavier than the heaviest published values. Combined, this strongly suggests abiotic transformation. Further, the fractionation factors (ε values) for carbon during biological and abiotic degradation as shown in Figure 3-11 are such that the very enriched values observed from the field samples strongly suggest abiotic EDB degradation (USEPA 2008b). For additional information on isotope enrichment factors, see Section 3.3.4.1.

For more information on this specific question, see USEPA 2008b; VanStone 2004; Liang et al. 2007; Jeong et al. 2011; Hofstetter et al. 2007; Elsner et al. 2007; Poulson and Naraoaka, 2002).

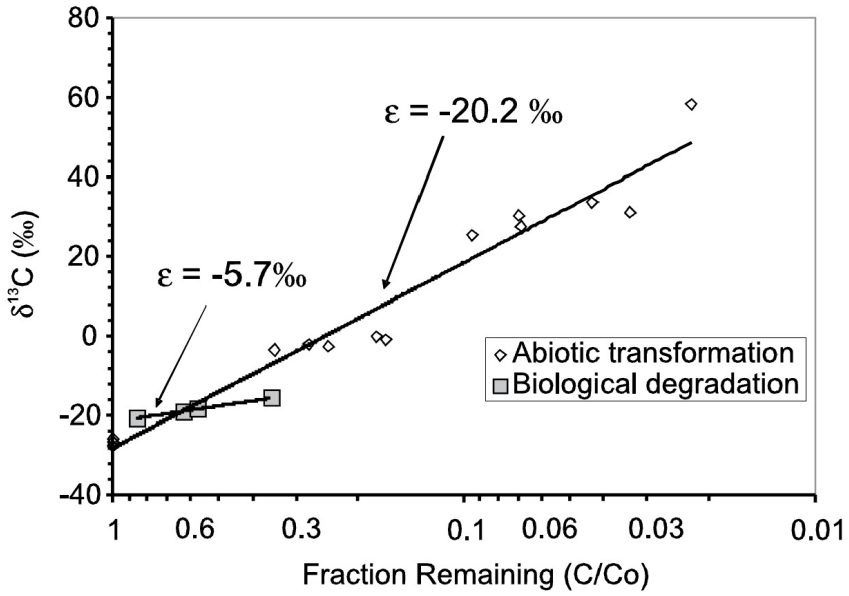

Figure 3-11. $\delta^{13}C$ of EDB versus the fraction of EDB remaining for a biological study (Henderson et al. 2008) and another study in which the EDB was transformed abiotically.

Source: USEPA 2008b.

F) Are multiple sources contributing to the contamination?

At a site perchlorate was detected in a number of monitoring wells at concentrations, ranging from a few ug/L to several mg/L. Some of the monitoring wells with high concentrations were clearly in an area of the site where propellants were discarded, and the source was easy to identify. However, several other wells with low concentrations of perchlorate were upgradient and sidegradient of this location, and did not have any other anthropogenic contaminants. Based on these data, stable isotope analysis of Cl and O in perchlorate was conducted in the primary plume location and for several of the upgradient and sidegradient wells to determine if multiple sources of perchlorate may be present at the site. The isotope data revealed that the $\delta^{18}O$, $\delta^{17}O$, and $\delta^{37}Cl$ values of perchlorate from the primary plume were consistent with values typical for synthetic perchlorate, while the same isotopic ratios for the upgradient and sidegradient wells indicated a secondary low-level source, presumably derived from the past application of natural Chilean nitrate fertilizers (later determined to contain naturally occurring perchlorate) in the region during its past history as agricultural area. An example of the differing isotopic ratios for these sources is provided in Bohlke et al. 2009, and in Case Study A.1 on this topic.

For more information on this specific question, see Bohlke et al. 2005; 2009; Sturchio et al. 2006; 2012, Jackson et al. 2010.

G) If there is a potential for multiple sources, can the sources be distinguished?

Perchlorate

For perchlorate, this question is addressed in Question F. Synthetic and natural sources of this anion can be readily distinguished by stable isotope analysis of Cl and O, although it is much more difficult to discriminate synthetic sources from each other, as values of $\delta^{17}O$ and $\delta^{37}Cl$ differ very little among synthetic sources (Sturchio et al. 2006).

TCE

For chlorinated solvents, such as TCE, a number of potential situations exist in which multiple sources may be an issue. Most commonly, multiple sources are an issue when one or more sources are contributing to a groundwater plume, or when indoor air is impacted either by vapor intrusion from a plume under the property, or by commercial products brought into a home by the occupants. Both cases were observed at the example site.

Differing $\delta^{13}C$ values have been used to discriminate sources of chlorinated ethenes (see Case Study A.3). For the purposes of this example, assume that similar analyses were conducted at one area of the site and showed multiple sources of TCE based on CSIA and supporting chemical concentration and hydrogeological information. Once multiple sources were identified, one of the important questions for wells between the sources was "how much of the contamination is from each source?" CSIA can be used to answer this question, but there are two very different applications of that question. One is for source apportionment in water, the second is for source apportionment in vapor (vapor could be ambient air or soil-gas).

Source Apportionment for a Water Sample

Source apportionment for TCE and other chlorinated solvents is most easily accomplished if biological or abiotic degradation of the parent compound has not occurred. In that case, it can be assumed that the observed $\delta^{13}C$ (and $\delta^{37}Cl$ values if available) for TCE in a well with mixed sources is just a concentration weighted average of the differing δ values of the individual sources. As an example, in one area of the site where two separate sources were identified, one off-site and one on-site, there was a downgradient well that was contaminated, but the contribution of each source to this contamination was unclear. CSIA was used in an attempt to trace the origin of the contamination and discern what percent of that impact was due to the on-site source and what percent of the impact was due to the off-site source. The site layout is presented in Figure 3-12 and the CSIA results are presented in Table 3-5.

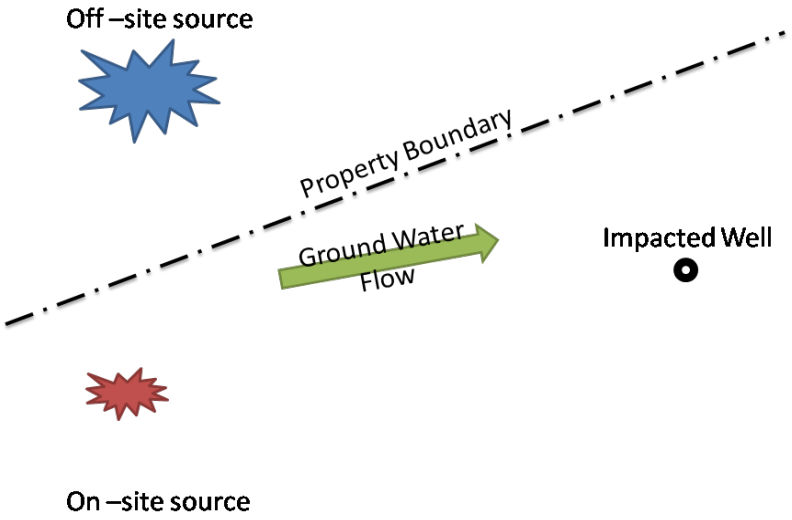

Figure 3-12. The area layout for an example site using CSIA to apportion source contributions.

Source: Microseeps, Inc. Used with permission.

In the impacted well, the $\delta^{13}C$ was between the $\delta^{13}C$ values of the two sources and the $\delta^{37}Cl$ value also was between those of the two sources. In this case, and with no evidence of biological or abiotic degradation of TCE at the site, the contribution of source X is F_x and that of source Y is F_y and the linear relationship for the two sources is as follows:

Equation 3-2:

$$F_x = \frac{\delta - \delta_y}{\delta_x - \delta_y}$$

Equation 3-3:

$$F_y = 1 - F_x$$

where δ is $\delta^{13}C$ or $\delta^{37}Cl$ for TCE from the well in question. Using the data in Table 3-5, the contribution of the off-site well is 80% for carbon. This value is corroborated by a similar calculation indicating the same contributions when chlorine is used.

Table 3-5. CSIA results for the example of apportionment in water

Sample ID	$\delta^{13}C$	$\delta^{37}Cl$
Off-site source	-30	-2
On site source	-25	+3
Impacted Well	-29	-1

Source Apportionment for a Vapor Sample

In a home situated above a plume on this site where TCE was being remediated by bio-augmentation and bio-stimulation, TCE was detected in the ambient air at concentrations above the action limit. However, the concentrations were sporadic and the plume shrinking, so it was believed that the source of the contaminant to the indoor air was not vapor intrusion. To better understand this situation, samples were collected from the air in the home and analyzed for $\delta^{13}C$ and $\delta^{37}Cl$ of the TCE. The TCE was found to be heavier than any published values and such fractionation could only come from degradation. Such degradation is clearly occurring in the treated groundwater plume based on isotope data and supporting parameters, but similar degradation is not expected for any airborne TCE brought into the home via consumer products. This result strongly implicates vapor intrusion from the groundwater plume as the cause of the indoor air concentrations. See Case Study A.2.

For more information on this specific question, see McHugh et al. 2011; Hunkeler et al. 2011; and Bouchard et al. 2008.

3.3.3.2 Remediation

K) Are intermediates being degraded?

As a chlorinated solvent is degraded, the first step is conversion to an intermediate product. For example, during reductive dechlorination, TCE typically proceeds through *cis*-DCE as an initial intermediate product (then VC and finally ethene). At a site, with analysis of $\delta^{13}C$, if the intermediate product of a reaction has the same number of carbon atoms as the starting compound (as is the case for TCE and *cis*-DCE), and the intermediate product is not degraded (e.g., during *cis*-DCE stall), the final $\delta^{13}C$ of the intermediate product when the starting compound is completely degraded will be identical to the starting $\delta^{13}C$ of the original parent compound before any degradation occurred. This relationship is shown in the top panel of Figure 3-13. If the intermediate product is also biodegrading, its $\delta^{13}C$ is not limited to that of the parent, as is shown in the bottom panel of Figure 3-13. In cases in which an undegraded sample of the starting compound is not available, the isotopic ratios can be used to infer degradation of the intermediate product (for instance, *cis*-DCE to VC).

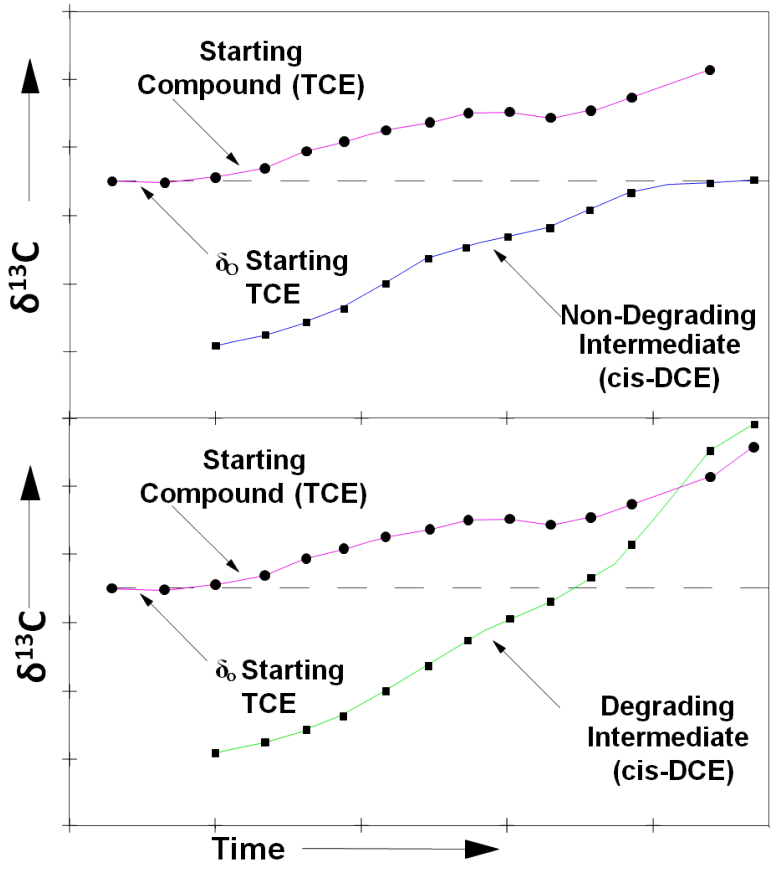

Figure 3-13. $\delta^{13}C$ of a compound and a nondegrading intermediate compound in the top panel and compound and a degrading intermediate compound in the bottom panel.

Source: Microseeps, Inc. Used with permission.

Example of Intermediates being degraded

At a site where PCE was released, the , $\delta^{13}C$ values of PCE and the intermediate products *cis*-DCE and VC, were measured where the solvent was released to groundwater. In that area, there was an application of a fermentable carbon substrate as an electron donor to promote reductive dechlorination. The appearance of the intermediates TCE and *cis*-DCE were indicative of biological reduction of PCE, but there was no evidence of VC or ethene. CSIA was used to determine if *cis*-DCE was degrading or persisting, and also to evaluate whether a *cis*-DCE accumulation (stall) was indicated. This portion of the site was small, and there were only three appropriate ground water wells to sample. The results are shown in Figure 3-14. The results were interpreted by comparing the results for each compound in each well and using the concepts discussed for Figure 3-13. In all three wells, the $\delta^{13}C$ in the *cis*-DCE was heavier (or more positive) than that of the PCE in that well, and this result indicated that the *cis*-DCE was in fact degrading.

For more information on this specific question, see McLoughlin et al. 2013a; USEPA 2008a.

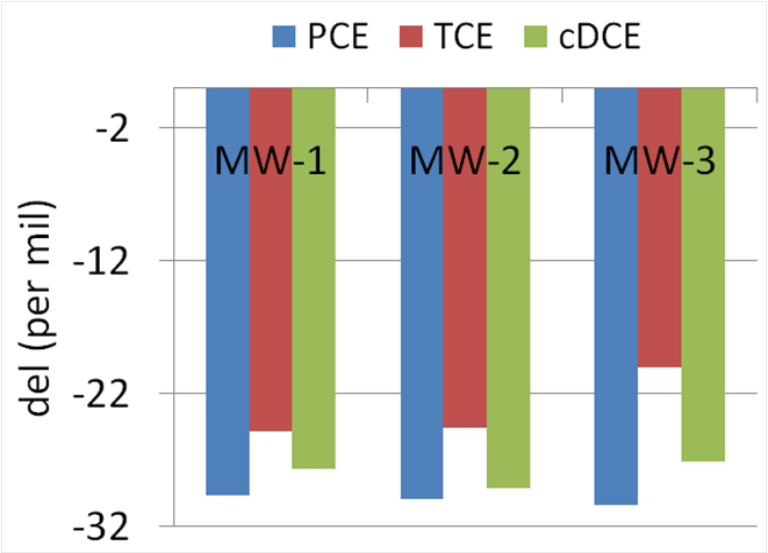

Figure 3-14. CSIA results showing the $\delta^{13}C$ of PCE, TCE and *cis*-DCE.

Source: Microseeps, 2012.Used with permission.

Example of Intermediates not being degraded

At a site where TCE had been released, the concentration of TCE declined over time and the concentration of *cis*-DCE increased. However, in this area, there were either minimal or no observations of vinyl chloride. In order to see if MNA would be an effective remedy in this area, evidence was needed that the *cis*-DCE was degrading. Samples were collected and $\delta^{13}C$ values of TCE and *cis*-DCE were measured. The data was collected and is presented in Figure 3-15

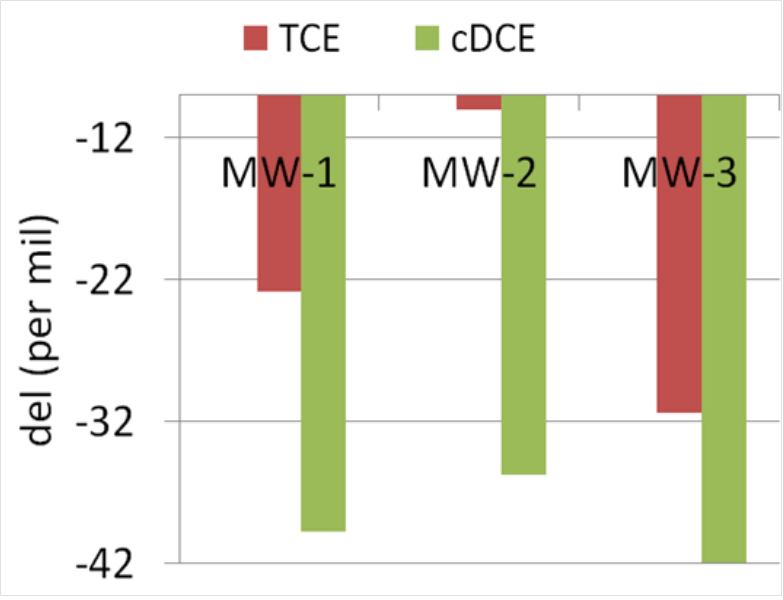

Figure 3-15. CSIA results showing the $\delta^{13}C$ of TCE and *cis*-DCE.

Source: Microseeps, 2012. Used with permission.

As shown in Figure 3-15, the *cis*-DCE in each of the wells was significantly lighter in $\delta^{13}C$ than the current TCE. However, since there were declining concentrations of TCE and since there was formation of *cis*-DCE, it can be assumed the TCE was degraded through reductive dechlorination. That degradation presumably resulted in an increase in the $\delta^{13}C$ of the TCE, so the criteria that the isotopic ratio of the *cis*-DCE must be heavier than the current value of the $\delta^{13}C$ in the dissolved TCE in each well is conservative. A sample of the undegraded parent TCE was not available to get an accurate, but less conservative measure of degradation. However, as previously noted, there have been several surveys of the $\delta^{13}C$ of manufactured TCE. The isotopic ratio of the heaviest or most positive $\delta^{13}C$ of TCE in those surveys is -24.5 ‰ (Aelion et al. 2010). It can be considered the upper limit of the $\delta^{13}C$ of undegraded TCE. None of the $\delta^{13}C$ of the *cis*-DCE in Figure 3-15 are greater than this limit. Based on this fact, it was determined that *cis*-DCE degradation was either not occurring at all or not at a rate sufficient to be protective and MNA alone would not be appropriate in this area.

L) Is there evidence of abiotic transformation?

At a site, the primary contaminant was PCE. The impacted aquifer was "aerobic" (that is, it was oxic and supported aerobic respiration) so there had been no degradation of the PCE. Since this area was to be sold within a year, it was necessary to remediate it quickly. In situ chemical oxidation (ISCO) with potassium permanganate ($KMnO_4$) was chosen as the remedial strategy.

However, the regulators at the site were concerned that the injection may simply dilute the contaminant, rather than degrade it. Analysis of $\delta^{13}C$ in PCE was conducted in the treatment area to provide evidence of PCE degradation. A previous study (Poulson and Naraoaka, 2002) has shown that an enrichment factor (ε value) for ^{13}C of -13 ‰ should be expected during PCE degradation by ISCO. Since isotopic ratios are not significantly affected by dilution, an increasing value of $\delta^{13}C$ in PCE at this site during and after treatment was considered evidence of PCE degradation. Values of $\delta^{13}C$ ranging between -4.1 ‰ and -18.7 ‰ were measured. The original $\delta^{13}C$ value for the source PCE was not available, However, since the heaviest published isotopic ratio of undegraded PCE is -23.3 ‰, the results were taken as evidence that the PCE was being degraded by the ISCO. The different ranges are shown in Figure 3-16.

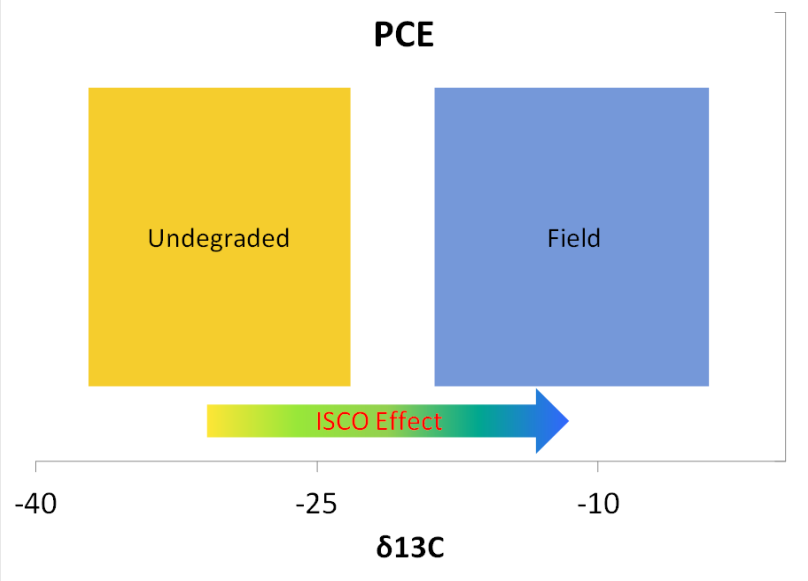

Figure 3-16. Range of undegraded product and range of field measurements of $\delta^{13}C$ of the PCE after ISCO.

Source: Microseeps, Inc. Used with permission.

M) Is biodegradation occurring?

For CSIA, the methods to answer this question are identical to those used for Question D above.

N) What is the rate of biodegradation?

At a site, MTBE had been released and the selected remedy was MNA. While the concentrations were declining, and TBA was present at the site, it was unclear if the TBA was an intermediate or if a co-contaminant that was released with the MTBE. Based on geochemical data (DO, ferrous iron, sulfate, and methane concentrations) it was determined that this location was anoxic and supported anaerobic respiration. As such, the enrichment factor for the biodegradation of MTBE under anaerobic conditions was conservatively estimated to be ε = -12 ‰. An undegraded sample of the MTBE was not available, but the heaviest (most positive) value of $\delta^{13}C$ for manufactured MTBE that is reported was used and that was -27.4 ‰. There was an obvious point of release of the MTBE, and the distance from that point to the sampled wells is given in Table 3-6, along with the well ID and the $\delta^{13}C$ of the MTBE. The groundwater seepage velocity (v) at this site is 37 meters per year. These values and the data in Table 3-6 were used to estimate the degradation rate using the formula:

Equation 3-4:

$$k = \frac{v(\delta_0 - \delta_t)}{sd}$$

Where:

k is the first order rate constant

v is the ground-water seepage velocity

δ_0 is the initial delta of the contaminant

δ_t is the delta of the contaminant at time t after the introduction of contaminant

ε is the enrichment factor for the degradation process

d is the distance from the source to the concerned well

Table 3-6. Data used for calculating MTBE biodegradation rates (adapted and simplified from USEPA 2005)

Well ID	MTBE $\delta^{13}C$ (‰)	Distance (m)	k (per year)
MW-3	+6.84	9.6	11
MW-8	+18.11	11.7	12

Under static conditions, where there is no groundwater flow, other methods exist to calculate rate constants. This is the case for microcosms, but the concentration of contaminant initially placed into the microcosm is known in microcosm studies. In those cases, CSIA is not needed to calculate rates.

For more information on this specific question, see McLoughlin et al. 2013b; Aeppli 2010b; and van Breukelen, Hunkeler, and Volkering 2005.

3.3.3.3 Monitoring

Q) Are contaminant-degrading microorganisms remaining active?

There is rarely a need to repeat CSIA once a particular question is answered. However, site managers may repeat analyses to see what changes have occurred since a previously established baseline, or as part of a monitoring program designed to use CSIA to establish more definitive answers with less frequent sampling, or to rule out contributions from new sources. An example of the value of repeat analyses is provided in Figure 3-17. MNA was the selected remedy at a site impacted with MTBE, and annual CSIA sampling was used to ensure that remedy was still appropriate. In Figure 3-17, the $\delta^{13}C$ of MTBE is plotted against the natural logarithm of the concentration. The dotted lines represent the range of expected $\delta^{13}C$ for MTBE in gasoline. In a sealed microcosm, Rayleigh's law predicts a linear relationship, but at field scale this is not expected because of contaminant flow. Nonetheless, the relationship has been observed multiple times and can be used to assess data from multiple sampling events to confirm that degradation continues, that dilution, dispersion, sorption and volatilization are negligible, and that there are no additional sources contributing to the contaminant mass. Even in cases like the one in Figure 3-17, other than to confirm degradation during active remediation, CSIA is rarely done more often than yearly.

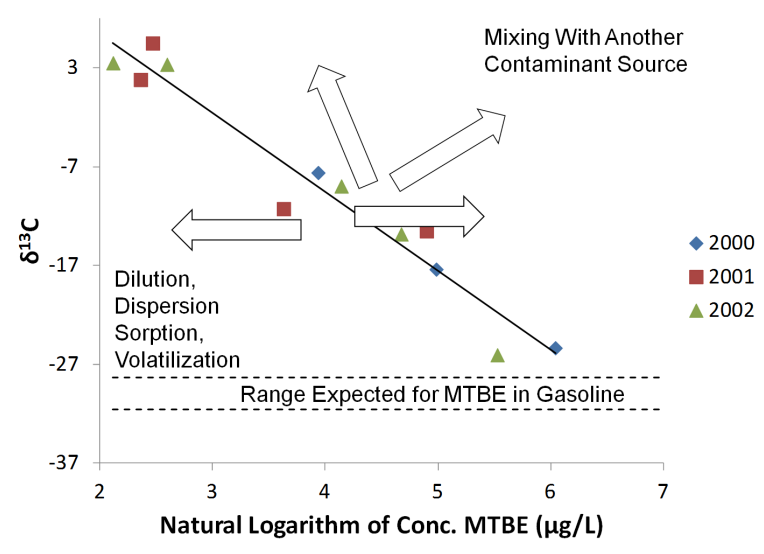

Figure 3-17. Plot of the $\delta^{13}C$ of MTBE vs. the natural logarithm of the concentration. Data from Table 1 of Kolhatkar et al. (2002).

Source: Adapted from USEPA 2008a.

Additionally, consider the methods to answer this question discussed in Question B above.

S) Are intermediates being degraded?

For CSIA, the methods to answer this question are identical to those used in Question K above.

T) Is there evidence of abiotic transformation?

For CSIA, the methods to answer this question are identical to those used in Question L and Question E above.

U) Is biodegradation occurring?

For CSIA, the methods to answer this question are identical to those used for Question D above.

V) What is the rate of biodegradation?

For CSIA, the methods to answer this question are identical to those used for Question N above.

3.3.3.4 Closure

Some variability of closure requirements exists among states and programs. However, in many situations, EMD data could serve as an additional line of evidence for understanding what processes are important in reducing concentrations and reaching the applicable closure levels. The evidence provided by EMD data would reveal whether biodegradation processes are occurring, have sufficiently proceeded, or are likely to continue.

CSIA provides information on the degradation of a contaminant either since the last time CSIA was used or back to the manufacturing of that contaminant if CSIA was not previously performed at a site. As such, without baseline CSIA data, it is impossible to determine the timeframe over which degradation has occurred or is occurring (i.e., as evidenced by δ values for one of more elements in a contaminant). If CSIA is used to support site closure, particularly as evidence of continuing degradation of a contaminant, measurements must be taken over time. CSIA either needs a baseline or to be complemented with concentrations of short lived terminal electron acceptors (see Appendix D, Question 15) such as ferrous iron or sulfide.

W) Is contaminant degradation likely to continue?

CSIA measures and defines processes that have occurred in the past or are occurring presently. Like most techniques, CSIA is not a predictive tool. However, CSIA can be used to estimate the degradation rate of a compound as discussed in Question K above, and to provide a line of evidence completely independent of the more traditional concentration measurements that biodegradation is occurring at a site. Using multiple CSIA measurements in the same area to establish timeframe, combined with other EMD tools to evaluate microbial populations, this technique can provide useful information for decision making concerning site closure. At a site, rates were measured for TCE biodegradation using CSIA values on multiple occasions and they were similar over time, and compared favorably with available literature rates. In addition, $\delta^{13}C$ values were measured for *cis*-DCE to ensure that this intermediate was continuing to degrade at the site. This result was interpreted as an indication that contaminant degradation was ongoing and the rates were consistent over time.

For more information on this specific question, see USEPA 2005; McLoughlin et al. 2013b; Aeppli 2010b; van Breukelen, Hunkeler, and Volkering 2005.

X) Are intermediates being degraded?

For CSIA, the methods to answer this question are identical to those discussed in Question K above. For closure, review the stable isotopic ratios of intermediate products (such as *cis*-DCE at TCE or PCE sites) even when traditional concentration measurements cannot detect downstream intermediates such as vinyl chloride.

Y) Is biodegradation occurring?

For CSIA, the methods to answer this question are identical to those discussed in Question D above.

Z) What is the rate of biodegradation?

For CSIA, the methods to answer this question are identical to those discussed Question N above.

3.3.4 Practical Considerations

CSIA is a powerful tool that allows site managers to evaluate contaminant fate and transport

independently of traditional concentration measurements. This tool also can provide valuable forensic information concerning the original source of contaminants in groundwater, soils, or air. However, as with any technique, be aware of both the advantages and the limitations of this technique.

3.3.4.1 Using Enrichment Factors

Because of slight differences in bond energy, biodegradation and chemical degradation occur slightly more rapidly for molecules containing only elements with light isotopes compared to those with both light and heavy isotopes. This difference leads to an isotopic enrichment in the parent molecule, and the strength of this enrichment is termed the enrichment factor (ε), as previously described in Figure 3-13 and accompanying text. Both in concept and in practice, enrichment factors are useful and accurate. The following lists points out important issues that should be considered when using enrichment factors.

- Assess redox conditions. A contaminant can be degraded by different processes under different conditions. A classic example is MTBE (Rosell et al. 2012). By knowing the redox conditions, the range of applicable enrichment factors can often be narrowed.
- Consider if the observed attenuation could be described by a single mechanism. Reductive dechlorination is generally considered the dominant degradation mechanism of chlorinated ethenes and intermediate products. However, part of the degradation may also be a result of oxidation (Bradley 2011; Gossett 2010; Bradley and Chapelle 2011), co-metabolism (Barth et al. 2002; Chu et al. 2004; Wymore et al. 2007) and/or abiotic degradation (Brown et al. 2009; Hofstetter et al. 2007; Liang et al. 2007).
- Know the range of reported enrichment factors for a particular mechanism under a given set of redox conditions. This knowledge helps to evaluate the plausibility of a particular interpretation. For example, the reductive dechlorination of chlorinated ethenes has considerable variation among the reported enrichment factors. For example, compare those recommended by Slater et al. 2001 with those found by Cichocka et al. 2008. Despite the potential variation in ε values among studies, it is possible to use these values to interpret field data and constrain degradation rates if conservative choices are made (McLoughlin et al. 2013a and McLoughlin et al. 2013b).
- Consider gathering supporting evidence for the degradation mechanism. Biodegradation processes, even with the same initial reaction, can sometimes be carried out by different enzymes. Since the enrichment factors for different enzyme-catalyzed reactions can vary widely, it may be necessary to refine the mechanism specifics with tools such as qPCR. A classic example is the co-metabolic biodegradation of TCE. One group of bacteria carries that process out using the sMMO enzyme (Chu et al. 2004; Wymore et al. 2007) and another group of bacteria using a TMO enzyme (Barth et al. 2002).
- Monitor changes in redox conditions as well as CSIA. Enrichment factors are specific to a particular strain, and as redox conditions change one strain may out-compete another. This situation may not change the biodegradation mechanism, but can dramatically change enrichment factors. This is the case for two bacteria that each aerobically oxidize MTBE: PM1 and

L108. It is possible to have those two bacterial strains simultaneously present at a site. However, under oxygen limited conditions L108 could out-compete PM1, thus changing the enrichment factor for ^{13}C during biodegradation. Understanding the site microbiology can be important in this case, because recent studies suggest that both the hydrogen and carbon enrichment factors for strain L108 are near zero (Rosell et al. 2010).

Despite some limitations, enrichment factors can provide important information concerning the fate of an environmental contaminant. They can be used to discriminate degradation mechanisms and aid in monitoring the effectiveness of a remediation approach. Moreover, enrichment factors can be used with site specific CSIA data to calculate degradation rates for simple mechanisms (USEPA 2008b) and can help constrain the rates in complex mechanisms such as those involving intermediate products (van Breukelen, Hunkeler, and Volkering 2005) or even be combined with other techniques allowing for determination of location-specific rate constants (Aeppli et al. 2010b).

3.3.4.2 Non-dissolved Contaminant

CSIA examines one environmental medium at a time, but the isotopic ratios of contaminant mass are controlled by what occurs in the dissolved phase as well as the non-aqueous phase and the vapor phase. (Morrill et al. 2009; ISCO site; NAS Pensacola site).

3.3.4.3 Isotopic Effects Remain Over Time

While continual processes build upon previous isotopic effects, once an isotopic effect occurs, it remains. As such, CSIA does not necessarily represent recent history unless certain precautions are taken. It may be desirable to couple CSIA measurements with a time sensitive indicator such as dissolved hydrogen or to use multiple sampling events to evaluate temporal evolution (McLoughlin et al. 2013a).

3.3.4.4 Heterogeneity

Heterogeneity creates substantial variation across a site. Degradation occurring in one area may not be occurring in another. The primary source influencing one portion of a plume may be different than the primary source influencing another portion of the plume. Because CSIA is relatively affordable, it can and should be done at multiple points across a site to account for variability (Courbet et al. 2011; Gaganis, 2005; Song et al. 2002), since the observations at one location may not reflect the rest of the site.

3.3.4.5 Environmental Forensic Considerations

While CSIA data provide useful forensic evidence, several issues must be considered before using the method in forensic investigations:

- Distinct sources with similar isotopic composition may make it impossible to use CSIA to determine the impact of a particular source on a particular well.

- Alteration of the original isotopic composition begins once contaminants are released to the environment. This alteration is mainly due to biodegradation, although other weathering processes may also affect the isotopic composition to a more limited degree. Thus the forensic information in CSIA can, in some instances, be hidden by biodegradation. In other cases, the original source of a partially biodegraded compound can be readily distinguished from an alternate source, particularly when multiple isotopes are measured (e.g., perchlorate; Sturchio et al. 2007). In either case, it is important to evaluate the potential for biodegradation of the target contaminant at the site under evaluation.
- The introduction of even minute quantities of an isotopically labeled compound in a well alters the fundamental assumptions for the evaluation of CSIA data, which reflect "natural abundances" of isotopes. Because of this, any well used for SIP cannot subsequently be used for CSIA for a prolonged period. For this reason, CSIA samples should be collected before SIP is performed if both techniques are desired at a site.

3.4 Additional Information

Further reading specific to CSIA is provided in Appendix F.

4.0 QUANTITATIVE POLYMERASE CHAIN REACTION

4.1 Summary of qPCR

The quantitative polymerase chain reaction (qPCR), also called quantitative real-time polymerase chain reaction, and reverse transcriptase-qPCR (RT-qPCR) methods are typically used to quantify the abundance and activity of target microorganisms capable of contaminant biodegradation or of genes (DNA by PCR and qPCR) or transcripts (RNA by RT-qPCR) in biodegradation pathways. These methods can provide a direct line of evidence for evaluating the feasibility and performance of the biodegradation processes underlying monitored natural attenuation (MNA) and bioremediation strategies. Traditionally, cultivation-based methods that rely on growth of the target microorganisms in the laboratory (such as microcosms, plate counts, or most probable number (MPN) analyses) have been used to estimate contaminant-degrading microbial populations. However, cultivation dependent techniques are laborious, time-consuming, and most importantly biased, because over 99% of microorganisms present in the environment cannot be grown under standard laboratory conditions (Amann et al. 1995).

Additional introductory information regarding PCR, qPCR, and RT-qPCR methods is available in the PCR Fact Sheet and the qPCR Fact Sheet. In addition, the qPCR Fact Sheet includes a list of current qPCR targets.

Conversely, qPCR and RT-qPCR can be applied to environmental samples independent of cultivation in the laboratory. Nucleic acids (DNA and RNA) are extracted directly from the microorganisms associated with a soil, sediment, or water sample. Thus, qPCR avoids the complications associated with cultivation and provides a direct, sensitive, and accurate method to quantify specific target genes indicative of specific microorganisms or biological processes. In remediation, qPCR has been used to quantify microorganisms capable of a variety of environmental processes including biodegradation of chlorinated solvents, petroleum hydrocarbons, and fuel oxygenates. Within the environmental industry, qPCR analysis has been offered on a commercial basis since 2002. qPCR is a reliable and frequently used method for rapid and accurate enumeration of gene targets in clinical, pharmaceutical, agricultural, and environmental applications and in academic research. Figure 4-1 includes the steps involved in qPCR.

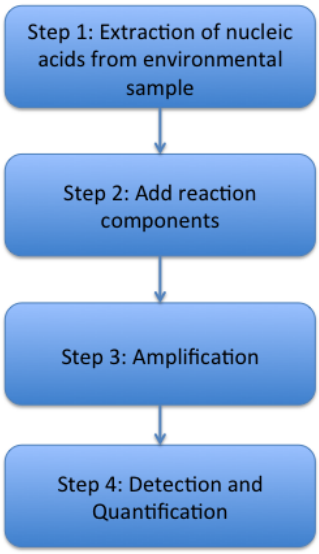

Figure 4-1. qPCR Flow Diagram.

4.2 Applications

Site characterization, remediation, monitoring, and site closure require thorough examination of available chemical, geochemical, and microbiological data. In addition to identifying contaminants and delineating the spatial extent of contamination, site characterization includes defining baseline conditions for a preliminary evaluation of potential remedies (including MNA). The purpose of performing qPCR and RT-qPCR analyses is to provide the microbiological lines of evidence: quantification of the abundance and activity of microorganisms capable of biodegradation of the relevant contaminants at a site. Examples of sites where qPCR and RT-qPCR have been successfully used are presented in Table 4-1.

Table 4-1. Example applications of qPCR

Title and location	General information	Contaminants	EMDs used	Project life cycle stage
Dry Cleaners, Eastern PA (see description below)	Evaluate feasibility of MNA vs. enhanced bioremediation strategies	PCE, TCE	qPCR	Site characterization
New York Site (see description below)	Confirm biodegradation after ISCO	Benzene	RT-qPCR	Remediation

Table 4-1. Example applications of qPCR (continued)

Title and location	General information	Contaminants	EMDs used	Project life cycle stage
Naval Weapons Station, SC (see description below)	Evaluate performance of electron donor addition and pH modification	TCE, *cis*-DCE	qPCR	Remediation
qPCR Case Study, NY (Appendix A.4)	Select electron donor and evaluate performance	PCE, TCE, *cis*-DCE, VC	qPCR	Site characterization, remediation, and monitoring
RT-qPCR Case Study, Northern California (Appendix A.6)	Evaluate oxygen injection at a gasoline impacted site	BTEX, MTBE	RT-qPCR	Remediation and monitoring
qPCR Case Study, CA (Appendix A.5)	Monitor biodegradation at Seal Beach Naval Weapons Station	PCE, TCE, *cis*-DCE and VC	qPCR, CSIA	Monitoring
EAP Case Study - Gaseous Diffusion Plant, KY (Appendix A.7)	Evaluated aerobic co-metabolism targeting aerobic oxygenases	TCE	EAP, CSIA, qPCR	Site characterization and remediation
SIP Case Study - Air Force Plant 44, AZ (Appendix A.8)	Evaluated aerobic co-metabolism targeting aerobic oxygenases	TCE; 1,4-dioxane	PLFA-SIP, qPCR, EAP	Remediation Selection (Natural attenuation)
SIP Case Study – Fuel Compounds, NJ (Appendix A.9)	Evaluated presence and activity of naphthalene-degrading bacteria	Naphthalene	PLFA-SIP, RT-qPCR, qPCR	Remediation

Dry Cleaners, Eastern PA

The historical groundwater monitoring data for this TCE-impacted site revealed predominantly anoxic conditions and formation of vinyl chloride and ethene—but at low concentrations. During site characterization, qPCR was performed to quantify *Dehalococcoides mccartyi* (*Dhc*). *Dhc* and vinyl chloride reductase genes were detected, but at low abundances. This result indicated that bioaugmentation would not be necessary but also suggested that MNA would not be an appropriate site management strategy. Site managers elected to pursue enhanced bioremediation and biostimulation was implemented. For a more complete example detailing how qPCR can aid in site characterization, remedy selection, and performance monitoring, see Case Study A.4.

New York Site

At a petroleum-impacted site in New York, the corrective action plan employed in situ chemical oxidation (ISCO) with a calcium peroxide activated sodium persulfate product to destroy contaminant mass, followed by bioremediation as a polishing step. Since reaching the performance objectives often relies upon biodegradation of residual contaminants following the direct ISCO phase, RT-qPCR quantification of naphthalene dioxygenase (NAH) and toluene monooxygenase (TMO) gene expression was performed to monitor the activity of aerobic BTEX and PAH degrading bacteria throughout the project. Dissolved benzene concentrations decreased rapidly during the

active ISCO phase. Even after depletion of the chemical oxidizing agent, concentrations of petroleum hydrocarbons including benzene continued to decrease albeit at a slower rate. While dissolved oxygen was produced by decay of the calcium peroxide, the observed increase in groundwater pH accompanying ISCO led stakeholders to doubt that the continued decrease in contaminant concentrations was due to biodegradation. However, RT-qPCR results revealed expression of NAH and TMO genes during these sampling events, demonstrated the activity of aerobic BTEX and PAH utilizing bacteria, and indicated that biodegradation was indeed the treatment mechanism despite elevated groundwater pH.

Naval Weapons Station, Charleston, SC

At this site injection of a pH-buffered emulsified vegetable oil was conducted (Vroblesky et al. 2010). qPCR demonstrated that electron donor addition and pH modification resulted in substantial increases in the abundance of *Dhc* and TCE reductive dehalogenase genes, which corresponded to dechlorination of TCE and *cis*-DCE.

When site characterization results and closure objectives dictate that MNA is not an appropriate site management strategy, engineered remediation options must be considered. Often, qPCR results obtained during the site characterization stage demonstrate that the contaminant degrading microorganisms are present at low abundances under the existing site conditions. In such cases, enhanced bioremediation through biostimulation (such as the addition of an electron donor, electron acceptor, or nutrient) is commonly performed to promote growth and activity of contaminant degrading microorganisms. One of the most common applications of qPCR and RT-qPCR is to document that biostimulation did indeed result in the desired increase in the abundance and activity of specific contaminant degrading microorganisms. Case Study A.4 and Case Study A.5 provide detailed examples of how qPCR is used to determine the effectiveness of biostimulation by electron donor addition at sites impacted by chlorinated ethenes. Likewise, Case Study A.6 shows how RT-qPCR quantification of toluene dioxygenase (TOD), phenol hydroxylase (PHE), as well as the measurement of *Methylibium petroleiphilum* PM1 16S rRNA, were used to demonstrate that oxygen addition promoted the activity of aerobic BTEX- and MTBE-degrading microorganisms at a gasoline impacted site.

While perhaps not as common, qPCR and RT-qPCR analyses are also used to assess the impact of physical and chemical treatment approaches on contaminant-degrading microorganisms to evaluate the potential for subsequent biodegradation to achieve site closure.

4.3 Data Interpretation

The application, analysis, and interpretation of microbiology-based EMD methods differ from typical soil and groundwater geochemical measurement in a number of ways. For example, microbial biomarkers cannot easily be preserved, and sample handling and processing requires special care. The analysis of microbial parameters requires specific data quality considerations for sampling plans, sample collection and handling, quality control and laboratory procedures, and these are discussed in Section 10.0. Included below is a brief introduction to how qPCR data are typically reported and some specific examples of how the data would be interpreted in answering the questions presented in Table 2-3.

4.3.1 How are the data typically reported?

qPCR results are generally presented as gene copies per milliliter (mL) or per liter (L) of water or per gram (g) of solids. If a cell contains only one copy of the target gene, the number of gene copies equals the number of cells. When a cell contains multiple copies of the target gene, the reported number can be converted based on knowledge of the number of target gene copies per genome. RT-qPCR results are reported as transcript copies per mL of water or per g solids. In many cases, genes of active degradation pathways are transcribed (messenger RNA [mRNA] is produced) and transcripts can be quantified by RT-qPCR. Since a microbial cell can contain a few to many transcript of a target gene, meaningful comparisons between samples can be obtained by normalizing the numbers of transcript abundance per cell (determined by qPCR).

Table 4-2 describes information that should be provided in laboratory reports of qPCR EMD data, including common laboratory report information, recommended information about the qPCR method, and desirable information about the qPCR method and results. The analytical laboratory itself should follow the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for qPCR (Bustin et al. 2009).

Table 4-2. Recommended and desirable information for qPCR laboratory report

Report information	Typical information or acceptable ranges
Common Laboratory Report Information	
Site Identifier	Location, name, monitoring well
Sample type or matrix	Water, sediment, soil, Bio-Trap®
Specific qPCR Information	
Recommended	
Results	Number of gene copies present in the environmental sample. In some cases, number of organisms if they contain one copy of the 16s rRNA gene, such as *Dhc*.
Reporting Units	Gene copies per volume of water or mass of soil
Typical Reporting Limits	100 gene copies per sample volume or mass
Limit of Detection	Varies, should be adequate for the application.
Sample storage/transportation	
Gene Target, Specificity	VC reductase
Primer name or sequence	
DNA extraction method	
Detection Chemistry	TaqMan® or SYBR® Green
Extraction blank	
Laboratory Control Sample	
No Template Controls (NTC)	
Inhibition testing	Dilutions, Spiked samples
Desirable	
Analysis Method	
Volume Extracted	0.5 to 2 L (see Note)

Table 4-2. Recommended and desirable information for qPCR laboratory report

Report information	Typical information or acceptable ranges
Processing time after sampling	Preferably within 24 hours
Note: The reports should clearly state the exact volume originally sampled, which can be particularly important if filtration was used for sampling.	

4.3.2 How are qPCR data interpreted?

Interpretation of qPCR and RT-qPCR results depends on the contaminant, the biodegradation pathway, and site-specific factors including geochemical conditions and stage in the overall project life cycle. To illustrate interpretation of qPCR and RT-qPCR results, each of the questions relevant to qPCR and RT-qPCR in Table 2-3 is discussed below.

4.3.2.1 Site Characterization

A) Are contaminant-degrading microorganisms present?

As a part of site characterization and to aid in remedy selection, qPCR and RT-qPCR can be used to detect and quantify biomarkers of contaminant-degrading microorganisms. The presence/absence of keystone bacteria responsible for the degradation of specific contaminants (i.e., *Dhc* for chlorinated ethene detoxification) provides relevant information about the potential success of MNA as a viable site management approach. On the other hand, qPCR results revealing low abundances or non-uniform distribution of contaminant-degrading microorganisms indicate that bioremediation options (that is, bioaugmentation and/or biostimulation) may be needed. As discussed in the qPCR Fact Sheet, qPCR analyses have been developed for a broad spectrum of genes implicated in biodegradation pathways. Thus, selection of an appropriate qPCR analysis depends upon the contaminants and geochemical conditions (for example, oxic or anoxic) as illustrated in the following example.

Consider a site that is impacted by the chlorinated solvents PCE and TCE. Under anoxic conditions, PCE and TCE can undergo sequential reductive dechlorination through the intermediate products *cis*-dichloroethene (DCE) and vinyl chloride (VC) to ethene (DiStefano et al. 1991; Freedman and Gossett, 1989). To date, *Dhc* is the only bacteria capable of complete reductive dechlorination of PCE to ethene (Maymó-Gatell et al. 1997). In addition, some of the VC reductase genes that encode the enzyme responsible for dechlorination of VC to produce ethene have been identified (Müller et al. 2004, Krajmalik-Brown et al. 2004). Performing qPCR quantification of *Dhc* 16S rRNA genes and VC reductase genes provides a direct line of evidence to evaluate the feasibility and the long-term performance of MNA at PCE and TCE impacted sites. If *Dhc* biomarker genes are not detected in samples obtained from the impacted zone, complete reductive dechlorination to ethene is unlikely and other site management strategies should be considered. Conversely, the detection of *Dhc* biomarkers indicate at least the potential for complete reductive dechlorination.

To evaluate the biodegradation component of MNA feasibility and performance, the abundance of these key dechlorinating bacteria must also be considered. Lu et al. (2006) proposed a screening

criterion of 10^7 *Dhc* cells per L to identify sites where MNA may be effective. Further research has indicated that ethene formation coincides with *Dhc* cell titers of >2 x 10^6 per L. High numbers (> 10^5) of the VC reductive dehalogenase genes *vcrA* and/or *bvcA* are strong indicators for complete dechlorination to ethene, however, VC reductase genes that are not yet identified may exist and may contribute to ethene formation (see P.K. Lee et al. 2008).

B) Are contaminant-degrading microorganisms active?

The presence of biodegrading bacteria may not ensure efficient contaminant removal, so activity must sometimes be measured as well. RNA is generally a short-lived molecule that is central to the production of proteins, including the enzymes responsible for contaminant biodegradation (see Appendix D, Microbiology FAQ). RT-qPCR measures RNA rather than DNA, and thus quantifies target gene activity (i.e., transcription) as a measure of contaminant degradation, further information about RT-qPCR can be found in Section 4.3.3. Logically, contaminant-degrading microorganisms must not only be present in sufficient abundance but also active under existing site conditions for a successful remedy. Conversely, qPCR results may show that contaminant-degrading microorganisms are present but RT-qPCR could reveal that these microorganisms or biodegradation pathways are not active. In such a case, bioremediation options such as biostimulation (the addition of an electron acceptor such as oxygen for the remediation of petroleum compounds) should be considered.

As an example, consider a site that is impacted by petroleum hydrocarbons, where benzene, toluene, ethylbenzene, and xylenes (BTEX) are the primary contaminants. BTEX compounds are susceptible to biodegradation by different pathways under oxic and anoxic conditions. RT-qPCR analyses are performed to quantify activity of specific genes to determine whether or not BTEX biodegradation pathways are active under existing site conditions. More specifically, RT-qPCR quantification of benzylsuccinate synthase (*bssA*) genes is employed to determine whether anaerobic pathways for the biodegradation of toluene, ethylbenzene, and xylenes are active. To evaluate aerobic BTEX biodegradation activity, a site manager would submit samples for RT-qPCR analyses targeting aromatic oxygenase genes encoding toluene/benzene dioxygenases. There may be other pathways and enzymes that contribute that would not be captured in the specified analyses.

C) Are the microorganisms capable of complete degradation?

Partial biodegradation of some contaminants will result in the accumulation of intermedate products, which can pose a greater threat to human health and the environment. For example, partial reductive dechlorination of PCE and TCE yields *cis*-DCE and VC, both of which are more mobile and toxic than the parent compounds. While a number of bacteria have been identified that are capable of reductive dechlorination of TCE to *cis*-DCE (members of the genera *Dehalobacter*, *Desulfuromonas*) to date, *Dhc* is the only bacteria known to be capable of complete reductive dechlorination of chlorinated ethenes such as TCE to ethene. qPCR analyses are available to quantify the genes encoding the enzymes responsible for the dechlorination of chlorinated ethenes. A transient increase in intermediate product concentrations during anaerobic treatment of PCE/TCE would be expected, DCEs and VC can persist if *Dhc* strains capable of efficiently

degrading the lower chlorinated ethenes are present in low abundance or absent. In this case, a site manager should consider qPCR quantification of *Dhc* 16S rRNA gene and VC reductase gene copies to determine whether *Dhc* strains capable of complete reductive dechlorination to ethene are present and abundant.

4.3.2.2 Remediation

The following questions address typical issues that may arise at many sites and focus on different areas where qPCR may be useful.

H) Are numbers of contaminant-degrading microorganisms and/or genes changing?

Ultimately, MNA can be an effective site management strategy when target microorganisms capable of biodegrading the contaminants are present and active under existing environmental conditions. On the other hand, qPCR results revealing a low abundance of target microorganisms with non-uniform distribution within the aquifer would indicate that biostimulation or bioaugmentation options may be needed.

For example, consider a site impacted by PCE and TCE. In addition to the qPCR detection of VC reductase genes, Lu et al. (2006) proposed that a *Dhc* abundance of 10^7 cells/L be used as a screening criterion for sites at which MNA would provide a generally acceptable rate of reductive dechlorination. In other words, qPCR results can be a powerful supplemental line of evidence along with traditional chemical and geochemical analyses that could be used as a remedy screening and performance-monitoring tool.

For other common contaminants, most notably petroleum hydrocarbons, RT-qPCR analyses may be more appropriate. For a site where MNA is being considered for a dissolved BTEX plume, BTEX concentrations may appear to be decreasing but could be the result of physical processes rather than biodegradation. RT-qPCR quantification can determine whether known pathways for aerobic and anaerobic BTEX biodegradation are active under existing site conditions. When viewed along with the trends in contaminant concentrations and geochemical parameters, the lack of or decrease in the activity of these degradation pathways as determined by RT-qPCR would indicate that MNA may not be appropriate and that bioremediation (for example electron acceptor addition) should be considered.

I) Is the remediation strategy affecting the numbers or types of contaminant-degrading microorganisms?

The purpose of any biostimulation strategy is to add an amendment such as an electron donor (e.g., lactate, emulsified vegetable oil) or acceptor (e.g., oxygen) that will stimulate growth and activity of contaminant-degrading microorganisms. Thus, qPCR is a direct route to assess the feasibility, evaluate the effectiveness, and monitor the progress of biostimulation as a treatment strategy. Whether during feasibility studies or in full-scale implementation, qPCR or RT-qPCR results should reveal an increase in the abundance and activity of contaminant degrading microorganisms in response to the amendment. When such increases are not initially evident, qPCR should be considered to evaluate the possibility that othermicrobial groups (for example methanogens or sulfate reducers) are competing with the biodegrading microorganisms for the added electron donor.

As an example, consider a site in which qPCR revealed *Dhc* abundance on the order of 10^3 to 10^4 cells per liter (cells/L) and VC reductase genes also have been detected in pretreatment

groundwater samples obtained along the dissolved plume. Based on the observed *Dhc* abundance and the screening criterion of 10^7 *Dhc* cells/L proposed by Lu et al. (2006), complete reductive dechlorination of TCE may be possible under existing site conditions but not necessarily at an acceptable rate. Sites impacted by chlorinated ethenes, where *Dhc* 16S rRNA genes and VC reductase genes are detected but in low abundance, may require the addition of an electron donor to stimulate the growth of these key dechlorinating bacteria and promote reductive dechlorination. In such cases, qPCR can be useful to measure the increases in the numbers of *Dhc* bacteria and VC reductase genes to ensure that biostimulation achieves detoxification.

In some cases, qPCR analysis may indicate that contaminant-degrading microorganisms are not present or are present in such low abundance that initiating degradation activity requires bioaugmentation. Similar to the discussion for biostimulation, qPCR analysis is used to document the in situ maintenance of key strains of the bioaugmentation culture. qPCR can also reveal decreases in target gene abundances, suggesting that the biodegrading bacteria experience a limitation, and another injection of electron donor should be considered to sustain biodegradation.

J) Is there a biological basis for intermediates accumulating?

Partial biodegradation of some contaminants like PCE and TCE can result in the accumulation of toxic intermediate products. To examine the potential for accumulation of intermediates, qPCR can be used to monitor specific microorganisms and functional genes involved in the degradation of the intermediate compounds. As an example, at a site impacted by TCE, groundwater monitoring results may indicate anoxic conditions but suggest that DCE is accumulating with little to no production of VC.

4.3.2.3 Monitoring

O) Does microbial community composition support the remediation strategy?

Monitoring a single group of contaminant-degrading microorganisms or one particular functional gene may not provide all data needed to assess and monitor a remediation strategy. At many sites, particularly those impacted by contaminant mixtures, site managers should consider qPCR quantification of multiple gene targets to obtain more comprehensive information. Since DNA is already extracted from the sample for the first target gene, with marginal additional effort qPCR analysis of additional target genes can provide more complete information.

For instance, a site is impacted not only by the chlorinated ethenes PCE and TCE but also chlorinated ethanes (such as 1,1,1-trichloroethane) and chlorinated methanes (chloroform). Electron donor addition has been performed to stimulate reductive dechlorination. With the mixture of contaminants present, a site manager may wish to perform qPCR quantification of other relevant dechlorinators that may contribute to PCE/TCE, 1,1,1-trichloroethane, and chloroform biodegradation (for example, *Dehalobacter restrictus*, *Geobacter lovleyi*, *Desulfuromonas spp*., and *Desulfitobacterium spp*.) in addition to *Dhc*. Furthermore, electron donor addition stimulates growth of microorganisms such as sulfate reducing bacteria (SRB) and methanogens, which are

competing with *Dhc* for hydrogen. In fact, initial stimulation of SRBs and methanogens following electron donor addition is frequently observed.

P) Do contaminant-degrading microorganisms continue to be sufficiently abundant?

Ultimately, the success of any biodegradation remediation strategy depends upon maintaining conditions that sustain the activity of a sufficient population of contaminant-degrading microorganisms. qPCR results can help to ensure that target microbial populations are maintained and biodegradation activity is sustained. Substantial decreases in the abundance of key biomarker genes or transcripts provide direct evidence that the conditions are not favorable for sustained contaminant degradation, and that amendment addition may be required. This relevant information may not be readily available from contaminant and geochemical monitoring data and demonstrates the value of qPCR.

The most common use of qPCR as a performance-monitoring tool is tracking the abundance of *Dhc* and associated reductive dehalogenase genes to evaluate biostimulation as a remediation strategy for chlorinated ethenes. If qPCR results indicate a stable *Dhc* population size of 10^6-10^7 cells/L, complete reductive dechlorination to ethene is likely to continue. Conversely, consistent decreases in the *Dhc* abundance should trigger re-evaluation and potentially additional corrective actions to stimulate and maintain these contaminant-degrading bacteria.

While *Dhc* is the most common example, with a broad spectrum of analyses available, qPCR can be used to monitor populations of a variety of contaminant-degrading bacteria (such as for a site impacted by perchlorate and ammonium perchlorate). Biostimulation through the addition of an electron donor is also a common remediation approach at sites impacted by perchlorate. qPCR detection and quantification of perchlorate reductase gene (*pcr*) and chlorite dismutase gene (*cld*) gene encoding the enzymes responsible for the biodegradation of perchlorate in environmental samples indicates biodegradation potential (Lieberman and Borden 2008). Absence of both of these genes indicates that biodegradation is unlikely, as all known perchlorate reducers use the enzymes encoded by *pcr* and *cld*. Thus, qPCR monitoring of the abundance these genes can provide direct feedback on the performance of the bioremediation strategy.

Q) Are contaminant-degrading microorganisms remaining active?

As discussed in Section 4.3.3.2, for some classes of contaminants like petroleum hydrocarbons, contaminant-degrading microorganism may be abundant but not active under the existing site conditions. For example, consider a gasoline-impacted site where geochemical parameters indicate oxic conditions but the trends in contaminant concentrations suggest that MNA will not meet site remediation goals in a reasonable timeframe. To assess whether oxygen addition can enhance BTEX biodegradation, RT-qPCR could be used to determine whether the transcription of aromatic oxygenase genes increases following oxygen biostimulation, which is generally followed by decreases in parent compound concentrations. RT-qPCR quantification of aromatic oxygenase gene transcription could then be used as a performance monitoring tool.

R) Is there a biological basis for intermediates accumulating?

Partial biodegradation of some contaminants like PCE and TCE can result in the accumulation of intermediates (such as *cis*-DCE and VC). To examine the potential for accumulation of intermediates, qPCR can be used to monitor specific microorganisms and functional genes involved in the degradation of the intermediate compounds. As an example, consider a site impacted by TCE where groundwater monitoring results indicate anoxic conditions but also suggest that *cis*-DCE is accumulating with little to no production of VC. While a number of bacteria have been identified that are capable reductive dechlorination of TCE to *cis*-DCE (e.g. *Dehalobacter restrictus*, *Geobacter lovleyi*, *Desulfuromonas spp.*), *Dhc* is the only bacteria currently known to be capable of complete reductive dechlorination of TCE to ethene. Moreover, qPCR analyses can quantify the genes encoding the enzymes responsible for the dechlorination of DCEs and VC. While a transient increase in intermediate product concentrations would be expected, qPCR results indicating that *Dhc* 16S rRNA gene and VC reductase gene copies are low would suggest that intermediate products would continue to accumulate.

At many sites where PCE/TCE contamination persists, the addition of electron donor can initiate reductive dechlorination activity. Several different PCE- and TCE-dechlorinating bacteria have been identified, and such microbes are commonly present in aquifers. Common PCE/TCE-dechlorinating bacteria generate *cis*-DCE as dechlorination end product. To achieve further degradation and detoxification (i.e., ethene formation), *Dhc* strains harboring DCE and VC reductive dehalogenase genes such as *vcrA* and *bvcA* are required. qPCR is an ideal method to determine if *Dhc* strains with *vcrA* or *bvcA* genes are present and this information can be used to judge the feasibility of complete reductive dechlorination to non-toxic ethene. An increase of VC reductive dehalogenase gene copies following extensive PCE/TCE reductive dechlorination to *cis*-DCE indicates that *Dhc* capable of ethene formation are active and grow at the expense of DCE and VC reductive dechlorination.

4.3.2.4 Closure

Closure requirements vary among states and programs. However, in many situations, EMD data can provide additional lines of evidence for understanding the processes that will sustain reduction of contaminant concentrations and reach the applicable closure levels. EMD data can provide evidence that shows whether or not biodegradation processes are occurring or are likely to continue.

The following question is a typical one that may arise at many sites and focuses on where qPCR may be useful.

W) Is contaminant degradation likely to continue?

Some sites may be granted no further action status under risk-based closure procedures with contaminant concentrations exceeding groundwater MCLs. In such cases, some assessment of whether biodegradation of residual contaminants will continue is warranted. If qPCR or RT-qPCR demonstrate the contaminant-degrading bacteria are present in sufficient abundance and are active, biodegradation is likely to continue. Physical processes (such as sorption) may also contribute to

contaminant concentration reductions; however, nondestructive processes do not result in true contaminant removal. Risk-based closure may not be appropriate if the contaminant is not truly removed.

As an example, consider a site impacted by petroleum hydrocarbons. Based on historical groundwater monitoring data, contaminant concentrations are decreasing, the plume is shrinking, and residual contamination poses no imminent threat to sensitive receptors. Geochemical data indicate that electron acceptors (such as sulfate) are still present within the dissolved contaminant plume. qPCR quantification of benzylsuccinate synthase (*bssA*) genes or RT-qPCR quantification of *bssA* gene activity would support the conclusion that biodegradation of benzene, toluene, ethylbenzene, and xylenes is likely to continue.

4.3.3 Practical considerations

The perceived limitation of DNA-based technologies, including qPCR, is that the detection of a target gene as an indicator of a specific microorganism or biodegradation pathway does not necessarily indicate corresponding activity. As discussed below, RT-qPCR is based on RNA rather than DNA, and is commercially available. RT-qPCR has been used to quantify gene transcription and microbial activity in environmental samples (Baldwin et al. 2010). Site managers can thus use qPCR as an indicator of biodegradation processes and RT-qPCR for confirmation of these processes.

4.3.3.1 qPCR

An important potential limitation of all DNA-based technologies, including qPCR, is that the detection of a target microorganism or functional gene is not necessarily indicative of the corresponding activity in the subsurface. As discussed below, analyzing for mRNA can be a more direct method to measure activity, and that can be done by using the RT-qPCR method (Baldwin et al. 2010). However, for many contaminants and target microorganisms, qPCR combined with a carefully considered sampling plan can provide the actionable data needed for site management. The qPCR analysis of *Dhc* biomarkers is an example how qPCR data can link the presence of a specific bacterial population with a particular process, in this case reductive dechlorination. In the case of *Dhc*, qPCR data derived from groundwater samples can indicate if "generally useful rates" of dechlorination can be achieved. For example, a *Dhc* population size exceeding 10^7 cells/L at sites contaminated with chlorinated ethenes is generally associated with ethene formation and this value has been proposed as a screening criterion for the feasibility of MNA at chlorinated ethene sites (Lu et al. 2006).

For other contaminants, qPCR data have proven helpful in making management decisions, along with chemical and geochemical data, even though a clear relationship between population size and activity has not been established. Comparisons of DNA abundance by qPCR to background (unimpacted, upgradient) or baseline (prior to treatment) samples can indicate growth of target microorganisms, particularly if the increases are substantial (several orders of magnitude). In this regard, it

is helpful that the background populations of microorganisms capable of biodegrading many of the common groundwater contaminants are typically very low, and often are not detectable.

The successful application of qPCR requires that biomarker genes for the process of interest are available. Specifically, genes of the biodegradation pathways must be known, and sequence data for the specific target gene(s) of interest are available. Such information is not currently available for all contaminants of interest, but with ongoing research, additional qPCR targets will be identified, and should expand the applicability of the technique to other contaminants and newly identified biodegradation pathways.

PCR inhibitors including certain metals and humic acids can affect target gene amplification and bias qPCR results, particularly when the template was obtained from environmental samples. Although inhibitors are commonly present in environmental samples, PCR inhibition is readily identified with basic QA/QC procedures, and qPCR data affected by inhibition can be identified and eliminated. Additionally, nucleic acid extraction procedures are available that eliminate potential PCR inhibitors, and may be required to prepare samples with high humic acid content (Bustin et al. 2009).

Other potential limitations include for example lack of primer specificity and DNA or RNA extraction efficiency.

Finally, the use of qPCR has also been somewhat limited in the past by a lack of standardized protocols for sample collection, storage, DNA extraction, and qPCR analysis itself. However, efforts to generate standard operating procedures are currently under way (Lebrón et al. 2008) and should lead to greater consistency and confidence in the results.

4.3.3.2 RT-qPCR

RT-qPCR quantifies transcriptional activity of target functional genes in environmental samples. For example, consider evaluating aerobic biodegradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) at a gasoline-impacted site. A number of analyses have been developed to quantify genes encoding aromatic oxygenases responsible for the first step in aerobic BTEX biodegradation (see Table 1 in the qPCR Fact Sheet). Microorganisms containing these functional genes are commonly present in the environment but, due to subsurface conditions such as low oxygen availability, they may not be active. With ubiquitous distribution of aerobic BTEX degraders, remedial actions such as injection of oxygen-releasing materials may result in an increase in oxygenase gene transcription and ultimately in an increase of contaminant biodegradation (activity). For such cases, RT-qPCR is an appropriate tool to evaluate the feasibility and performance of bioremediation alternatives (Baldwin et al. 2010).

To date, RT-qPCR has seen very limited application at field sites and it is uncertain if the analysis of *Dhc* biomarker gene transcripts will emerge as a productive approach to assess reductive dechlorination activity. One potentially important limitation of the use of RT-qPCR is that extracting high quality mRNA from environmental samples is very challenging, since RNA and mRNA in par-

ticular are generally short-lived molecules. Samples also must be stored immediately at -80°C or treated with an RNA stabilizer in the field to prevent RNA degradation prior to lab analyses.

4.4 Additional Information

Further reading specific to qPCR methods is provided in Appendix F.

5.0 MICROBIAL FINGERPRINTING METHODS

5.1 Summary of Microbial Fingerprinting

Microbial fingerprinting methods can provide a comprehensive assessment of the microbial community. Fingerprinting methods require little prior knowledge about which microorganisms are of interest and the genetic fingerprinting methods allow identification of dominant members of the microbial community to the family or even genus level. Microbial fingerprinting methods differentiate microorganisms or groups of microorganisms based on unique characteristics of a universal component or section of a biomolecule (such as phospholipids, DNA, or RNA). Microbial fingerprinting methods provide an overall profile of the microbial community, indications of microbial diversity, insights into the types of metabolic processes occurring, and in some cases can be used to identify subsets of the microorganisms present. For more information on the basic biology behind each of the methods, please refer to the Microbial Fingerprinting Fact Sheet.

Three microbial fingerprinting methods are described in this guidance:

- PLFA analysis provides a measure of total viable biomass and a broad-based profile of the microbial community composition grouped into general categories. The general process is illustrated in Figure 5-1. Other than in combination with stable isotope probing (SIP), PLFA analysis is best suited for general questions such as whether a treatment increased (or decreased) total biomass or substantially altered redox conditions. It should be noted that PLFA can not analyze for Archaea such as methanogens.

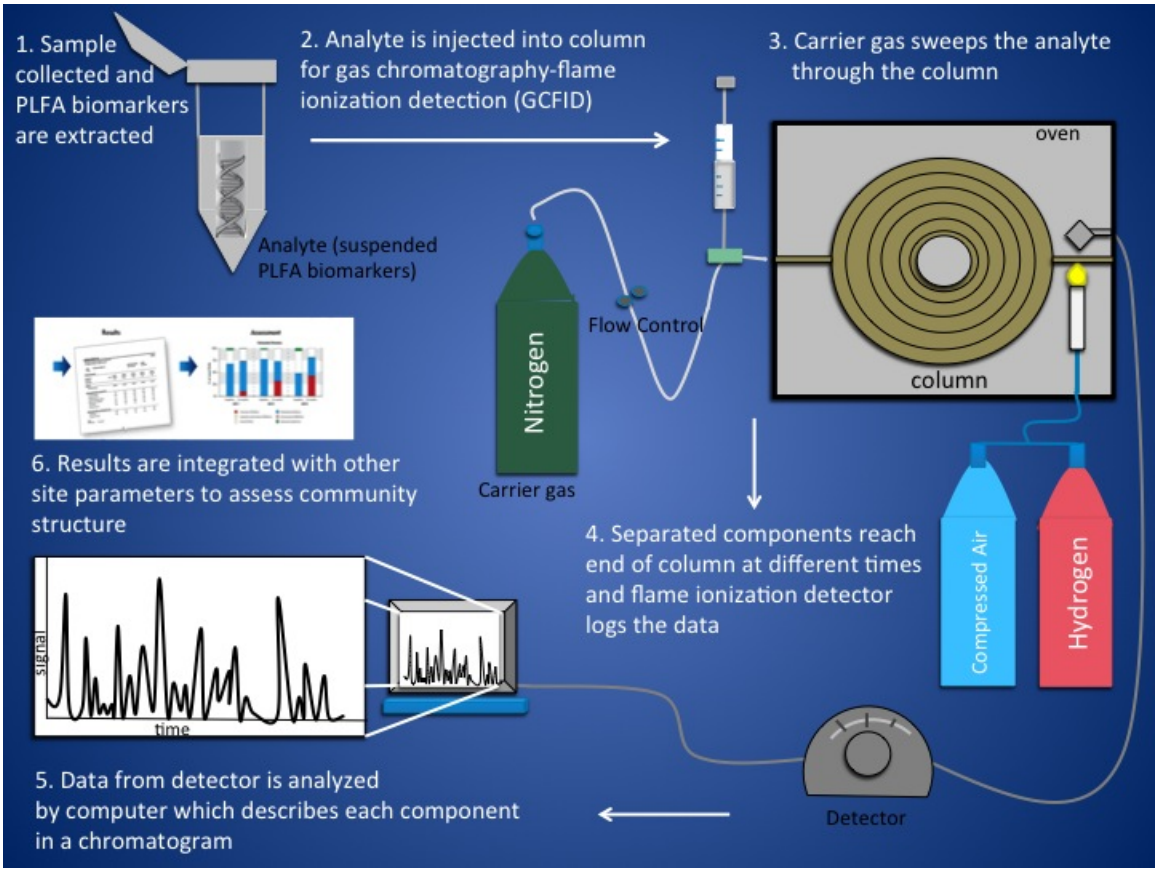

Figure 5-1. PLFA process.

Source: Microbial Insights, Inc, 2010. Used with permission.

- DGGE and sequence analysis provides a DNA- based profile of the microbial community and allows identification of the predominant organisms generally to the family or genus level. DGGE analysis cannot, however, quantify specific organisms or microbial functions present within a sample. The DGGE process is illustrated in Figure 5-2. DGGE profiles are used to visually display differences or shifts in microbial community composition over time or in response to treatment. Sequence analysis following DGGE is somewhat exploratory, seeking to answer the question "Who is there?" In addition, DGGE can also be used to identify and compare the presence/absence of specific organisms among samples. Most often, DGGE analysis followed by sequencing is performed when identification of the predominant organisms is required but little is known about the microbial community of the sample prior to the analysis.

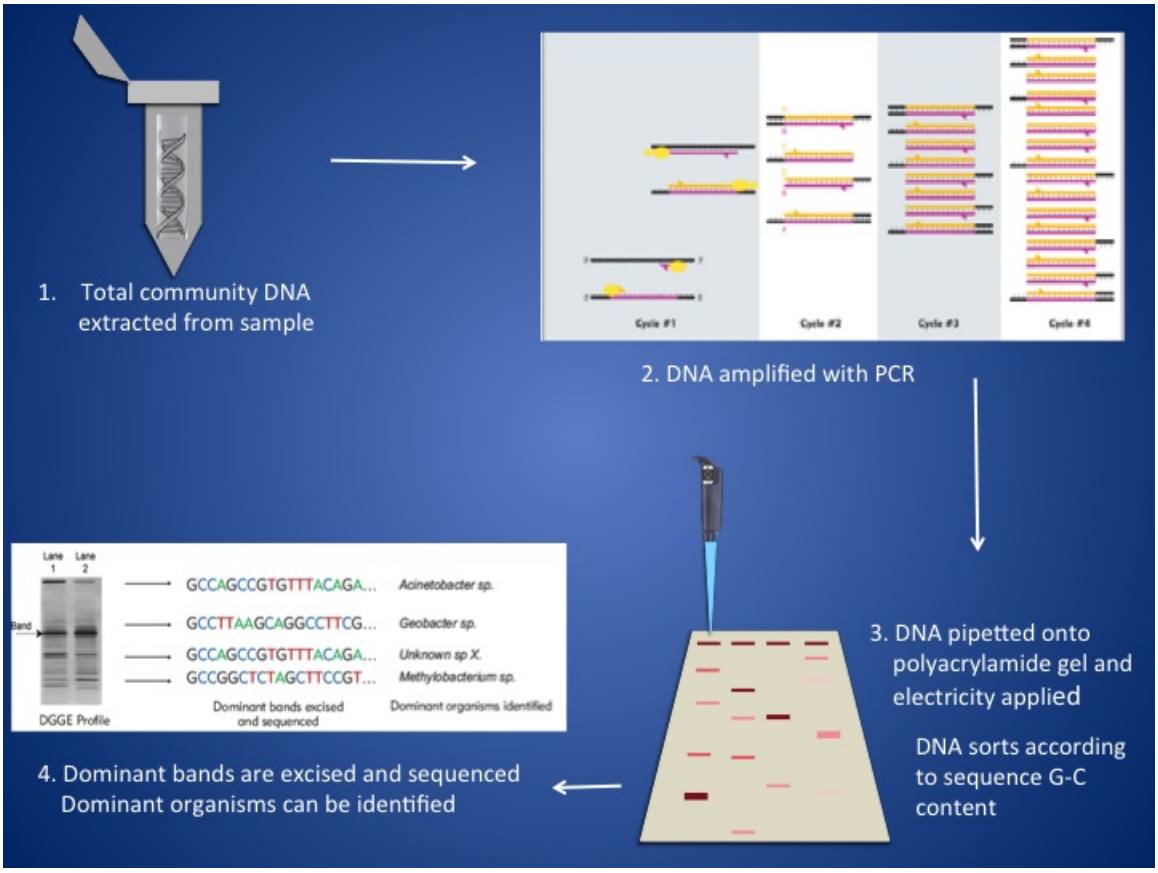

Figure 5-2. DGGE process.

Source: Microbial Insights, Inc, 2010. Used with permission.

- T-RFLP involves digestion of genetic material (DNA) with restriction enzymes that cleave DNA at specific sites. These sites differ between microorganisms, resulting in different DNA fragment lengths (and sequences), which can be used to identify some members of a microbial community and provide a fingerprint of its composition. The T-RFLP process is illustrated in Figure 5-3. As with DGGE, T-RFLP information is nonquantitative or at best semi-quantitative.

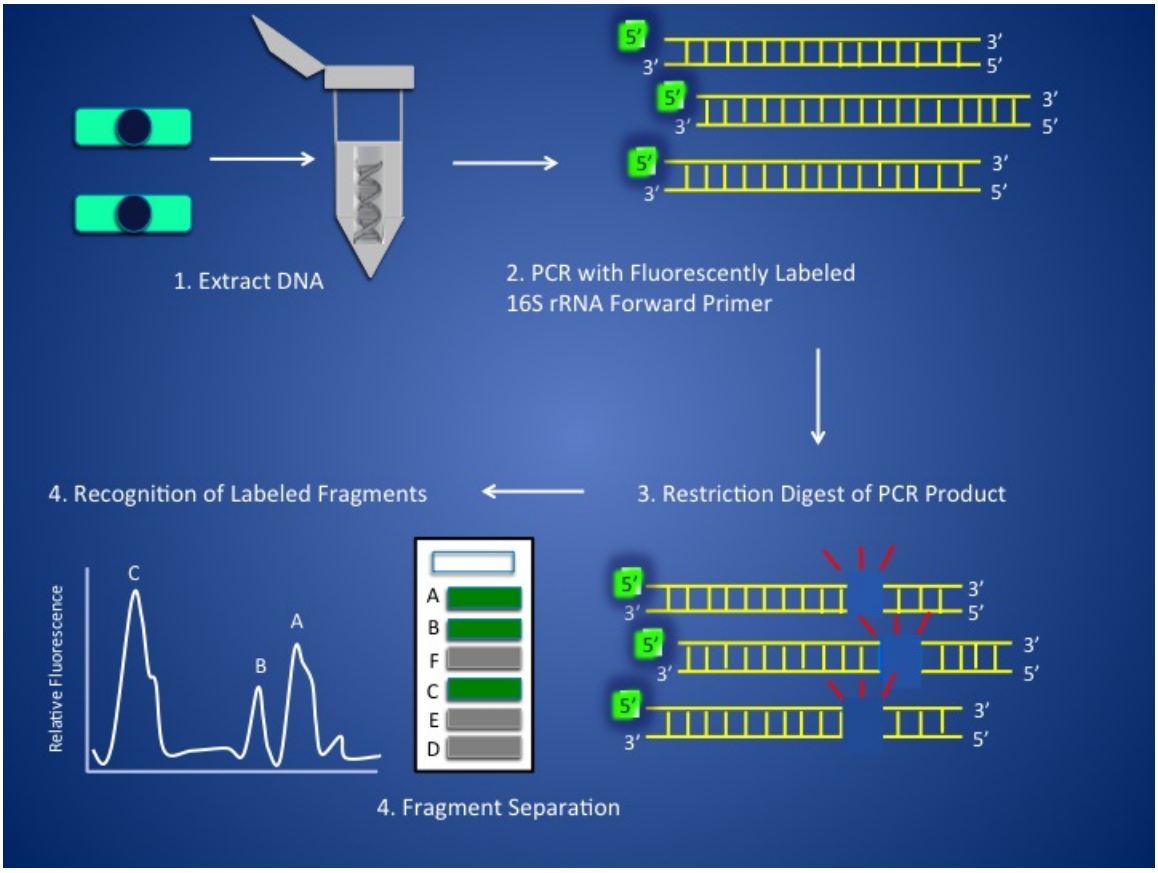

Figure 5-3. T-RFLP process.

Source: Microbial Insights, Inc. 2011. Used with permission.

Microbial fingerprinting methods have been used to investigate microbial populations at many different environmental remediation sites ranging from metal contaminated sites (USEPA 2009), to retail gasoline stations (Nales 1998), to Superfund sites (USEPA 2006). Microbial fingerprinting techniques can be used with other EMDs (see Section 7.0 and Table 2.2). For example, PLFA analysis is used in conjunction with SIP to document that biodegradation is occurring. Although often requiring greater quantities of the isotopically labeled contaminant , the genetic fingerprinting techniques can also be used with SIP to not only demonstrate contaminant biodegradation but also to potentially identify microorganisms responsible.

5.2 Applications

Site characterization, remedy selection, and monitoring require thorough examination of available chemical, geochemical, and microbiological data. Using microbial fingerprinting techniques such as PLFA and DGGE can provide evidence of the functional groups present and the diversity of the microbial community of a site. These methods can elucidate the initial community structure of a site as well as show the changes in microbial activity following treatment. Examples of sites where fingerprinting methods have been successfully used are presented in Table 5-1.

Table 5-1. Example applications of fingerprinting methods

Title	General information	Contaminants	EMDs	Project life cycle stage
Fort Lupton, CO (see description below)	Understand the role of the vadose zone in bio-degradation of hydro-carbons	BTEX	PLFA	Site Char-acterization
Tallgrass Prairie Reserve, OK (see description below)	Understand crude oil impacts on soil ecology	Crude Oil	PLFA	Monitoring and Closure
Microbial Fingerprinting Case Study – PCB Dechlorination, Washington DC (see description below)	PCR-DGGE was used to evaluate changes in dechlorinating bacteria	PCBs	DGG-E	Remediation
Microbial Fingerprinting Case Study – BTEX Degradation, WA (see description below)	Confirm biodegradation with sulfate amendment	BTEX (Benzene, Toluene, Xylene, Ethylbenzene)	PLF-A, qPC-R	Monitoring

Microbial fingerprinting techniques are often employed at the site characterization stage to investigate the microbial community structure and composition, to evaluate the types of microbial processes that may be occurring, and to aid in development of a conceptual model.

Fort Lupton, CO site

This is a site at which groundwater was impacted with gas condensate hydrocarbons from a leaking underground sump used to store produced water. Long-term monitoring had demonstrated contaminant biodegradation and generation of anaerobic conditions in the saturated zone. In this case, the goal was to examine the microbial community and assess the microbial processes occurring in the vadose zone above the dissolved plume in order to evaluate the role of the unsaturated zone in natural attenuation. PLFA and DGGE analyses along with traditional chemical measurements were performed on soil cores obtained above the dissolved plume and in background areas. At increasing depths below ground surface, PLFA analysis revealed decreasing total biomass and DGGE profiles showed decreasing bacterial diversity. At specific depths in contaminated samples that corresponded to higher levels of total petroleum hydrocarbons, CO_2, and methane in soil gas however, total PLFA increased (although methanogens will not be detected by this technique). This result demonstrated an increase in bacterial biomass while DGGE revealed the selection of specific members of the microbial community, suggesting a microbial community response to the increased carbon pool (gas condensates and biogenic methane) above the water table. Moreover, PLFA and DGGE results provided the following insights into that response:

- Significantly lower proportions of anaerobic PLFA biomarkers at depth (60 – 144 cm) in the impacted zone than at the same depths outside the impacted area suggested a predominantly aerobic microbial community in the vadose zone.
- Decreasing ratios of specific PLFAs indicated increasing growth rates with depth possibly due to increased availability of gas condensates and biogenic methane from the dissolved plume.

- Identification of methanotrophic 16S rRNA sequences in the DGGE profiles from core samples obtained from this depth above the groundwater plume.

Overall, the microbial fingerprinting results suggested an active, predominantly aerobic microbial community. This community was enriched in methane oxidizing bacteria, which were most likely supported by condensate hydrocarbons and biogenic methane (produced by anaerobes in the saturated zone) transported into the vadose zone by evaporation at the water table (Sublette et al. 2002).

Microbial fingerprinting techniques can provide valuable insight during the remediation phase and can be critical during the monitoring phase and ultimate site closure.

Tallgrass Prairie Reserve, OK

A pipeline break resulted in the release of an unknown quantity of dewatered crude oil at the Tallgrass Prairie Reserve in Oklahoma (Sublette et al. 2007). The heavily impacted portion of the area was tilled and fertilized to promote bioremediation. Approximately six years after the spill and subsequent treatment, TPH levels were near detection limits and microbial fingerprinting methods were used as a component of a study to evaluate the degree of restoration of the soil ecosystem. PLFA indicated the following at this site:

- Biomass as indicated by total PLFA increased with distance from the pipeline presumably due to lower exposure to hydrocarbons at increasing distances from the release.
- Ratios of specific monoenoic PLFAs suggested decreased growth rates and membrane permeability, an adaptation to unfavorable environments by some *Proteobacteria*, closer to the pipeline despite low residual TPH levels.
- Overall, PLFA analysis suggested that the spill continued to have a measurable effect on the microbial component of the impacted soil ecosystem.

PCB Dechlorination

This study investigated the enhanced microbial transformation of low concentration PCBs after biostimulation and bioaugmentation. Microcosms were prepared with sediment samples from the Anacostia River. The sediments were contaminated with a weathered mixture of urban and industrial sources containing PCBs, polycyclic aromatic hydrocarbons, chlorinated pesticides, and heavy metals (Reible et al. 2006; Horne Engineering Services 2003). PCR-DGGE was used as a fingerprinting technique to detect changes in the dechlorinating bacteria population, specifically *Dehalococcoides mccartyi* (*Dhc*), in the microcosms.

Dechlorinating bacteria were detected in several of the various microcosm studies, augmented with alternate electron acceptors and/or directly bio-augmented with *Dhc*. The PCR-DGGE analysis bands were excised and sequenced to identify the microorganisms present. Identifying the microorganism that can dechlorinate low concentration PCBs has significant benefits for site remediation. If the microbial dechlorination of PCB could be implemented and monitored in the field, as observed in the microcosm studies, then low concentration PCB sites would not require the more

expensive remediation methods currently used like dredging and ex situ treatment. (Krumins et al. 2009).

BTEX Degradation

At a gasoline-contaminated groundwater site, bioremediation was tested using sulfate injections. EMDs, PLFA and real-time PCR for benzylsuccinate synthase (*bssA*), were used to monitor the conditions in the aquifer to determine if anaerobic bacteria were stimulated and if the biodegradation rates for dissolved-phase gasoline and BTEX were increased. Both groundwater samples and Bio-Trap® sampling devices were used for microbial analyses. The site chemistry and geochemistry were also monitored. The PLFA profiles indicated an increased percentage of cyclopropyl fatty acids in biofilms as a result of the sulfate injections. Cyclopropyl fatty acids are found in anaerobic bacteria and are components of *Desulfobacter* species. The evaluation of the chemical, geo-chemical and microbial analyses was used to understand the success of the bioremediation (Sublette et al. 2006).

5.3 Data Interpretation

The application, analysis, and interpretation of microbiology-based EMD methods differ from typical soil and groundwater geochemical measurement in a number of ways. For example, microbial biomarkers cannot easily be preserved, and sample handling and processing requires special care. The analysis of microbial parameters requires specific data quality considerations for sampling plans, sample collection and handling, quality control and laboratory procedures, and these are discussed in Section 10.0. Included below is a brief introduction to how fingerprinting data are typically reported and some specific examples of how the data would be interpreted in answering the questions presented in Table 2-3.

5.3.1 How are the data typically reported?

For PLFA, the total biomass in the sample is presented as the total number of cells per milliliter (ml) of water or per gram (g) of solid matrix (usually soil). Community structure is presented as the percentage of the different functional groups (such as iron reducers, sulfate reducers, or fermenters). The physiological responses of *Proteobacteria* to different environmental stresses are reported as decreased membrane permeability and slowed growth ratios. These ratios are best used in long-term monitoring projects where multiple measurements are taken over time (Hedrick 2000; MacNaughton et al. 1999; Frostegård, Tunlid, and Baath 1996; Frostgård, Tunlid, and Baath 2011; Fischer, Schauer, and Heipieper 2010).

For DGGE and T-RFLP, the identities of the dominant genera within the microbial community are presented. A DGGE report typically includes a photo of the gel (see Figure 5.2), the family or genus of the microorganisms identified, and the similarity index to gauge how well the DNA sequence recovered from the sample matched the sequence found in public databases (Muyzer, de Wall, and Uitterlinden 1993). However, since individual “bands” are excised from the gel for sequencing, typically only 3 to 10 microorganisms are identified by DGGE analysis. The number

of microorganisms that can be identified by T-RFLP (Osborn 2001) can be ten times greater, thus providing more comprehensive examination of the microbial community composition.

Table 5-2 includes information that should be provided in laboratory reports of fingerprinting EMD data including common laboratory report information, recommended information about the fingerprinting methods, and desirable information about the fingerprinting method and results.

Table 5-2. Recommended and desirable information for fingerprinting laboratory report

Report information	Typical information or acceptable ranges
Common Laboratory Report Information	
Site Identifier	Location, name, monitoring well
Sample type or matrix	Water, sediment, soil, Bio-Trap®
Specific qPCR Information	
Recommended	
Results	• PLFA: Total biomass, community structure, physiological response • DGGE and T-RFLP: Comparison of community profiles (spatially or temporaly), Identity of dominant taxa within the community (if paired with sequencing; methods of ID should be reported with sequencing data)
Reporting Units	• PLFA: Viable cells per volume of water or mass of soil; relative percentage of different populations; ratio of various fatty acids • DGGE: Family or genus of microorganisms identified; similarity to documented organisms • T-RFLP: Family or genus of microorganisms identified (if paired with sequencing); relative abundance of each T-RF
Typical Reporting Limits	• PLFA:Greater than 10,000 cells per sample • DGGE: 1000 gene copies per sample volume or mass.
Limit of Detection	Varies, should be adequate for the application
Sample storage/transportation	
Gene Target, Specificity	
Extraction blank	
Laboratory Control Sample	
No Template Controls (NTC)	
Inhibition testing	Dilutions, Spiked samples
phospholipids extraction method	
DNA extraction method	
restriction enzymes	
primers	
Desirable	
Analysis Method	
Volume Extracted	1 to 2 L
Processing time after sampling	Preferably within 24 hours

5.3.2 How are the data interpreted?

Interpretation of results depends on the specific microbial fingerprinting method, the questions being addressed, and stage in the overall project life cycle. To illustrate interpretation of PLFA, DGGE, and TRFLP results, each question posed in Table 2.3 is discussed below.

5.3.2.1 Site Characterization

A) Are contaminant-degrading microorganisms present?

Although other EMDs (such as qPCR) should be used to detect and quantify target microorganisms, fingerprinting techniques can provide valuable insight when specific target microorganisms and biochemical pathways are unknown. In general terms, evaluation of microbial community profiles and composition should focus on comparisons between background (non-impacted) and impacted samples. For example, at a PCE contaminated site, if total PLFA bacterial biomass is substantially lower in samples from the impacted zone than in background areas, microbial growth may be inhibited and MNA strategies that rely primarily on biodegradation may not be feasible. Alternatively, biostimulation may need to be considered. In addition, certain classes of PLFA biomarkers can supplement traditional geochemical analyses to assess the dominant redox conditions (such as aerobic or anaerobic) within and outside the contaminated zone. Finally, PLFA analysis is often performed as a component of an SIP study to evaluate the feasibility and performance of MNA by conclusively determining whether biodegradation of specific a contaminant is occurring under existing site conditions.

The nucleic acid-based fingerprinting methods (DGGE, T-RFLP) are used to identify the predominant microorganisms present in a sample when used in conjunction with sequencing information. Theoretically, the presence of a contaminant exerts a selective pressure on the microbial community, promoting growth of microorganisms capable of using the contaminant under the given subsurface conditions. For example, consider a site impacted by an emerging contaminant—a biodegradation pathway is not yet known and contaminant-degrading microorganisms have not been identified. As an exploratory tool, this comparison of microbial community composition in background versus impacted samples could provide an initial indication of what microorganisms may be important specific to biodegrading the emerging contaminants.

5.3.2.2 Remediation

H) Are numbers of contaminant-degrading microorganisms and/or genes changing?

As mentioned previously, other EMDs should be used to detect and quantify contaminant-degrading microorganisms or functional genes when degraders are known and pathways have been identified. However, fingerprinting techniques can provide valuable insight when specific target microorganisms and biochemical pathways are unknown. Moreover, fingerprinting techniques can answer general questions relating to the viability, health, and diversity of the microbial community.

Many remediation plans include a tiered approach (a treatment train) where physical (air sparging, soil vapor extraction, or multi-phase extraction) or chemical (in situ chemical oxidation using Fenton's reagent, permanganate, or persulfate) alternatives are employed to address contaminant mass in the source area, followed by MNA or bioremediation, to meet site closure requirements. Fingerprinting can be used to understand how the microbial community responds to the physical or chemical remediation and then recover for ongoing MNA or bioremediation. Specifically, subsurface conditions resulting from physical or chemical treatment processes can initially decrease the biomass and diversity of the microbial community composition. Fingerprinting methods can also be used to gain additional insight into the predominant terminal electron accepting processes following the physical or chemical treatment.

For a site with BTEX and petroleum hydrocarbon contamination, fingerprinting methods can reveal microbial responses during various phases of the remediation. A tiered site remediation plan at the site called for in situ chemical oxidation treatment using active persulfate to reduce contaminant mass in the source area followed by MNA or a bioremediation strategy to meet site closure requirements. The choice to use either MNA or bioremediation was made after fingerprinting was conducted again to see how the microbial community responded and recovered after the chemical oxidant completed its reaction. Initially, decreases in total PLFA (bacterial biomass) along with decreases in the ratios of specific PLFAs directly indicated an immediate adverse impact to the microbial community following the chemical oxidant addition. However, after the chemical oxidant reacted, residual sulfate from the persulfate was suspected to be present and may have served as an alternative electron acceptor to subsequently stimulate the biodegradation of the residual petroleum through sulfate reduction. Therefore, in addition to sampling for residual sulfate in the subsurface, microbial fingerprinting techniques can also be included in the remedy performance sampling plan to provide indications whether and when sulfate reducers may become stimulated to address the remaining petroleum contaminants.

I) Is the remediation strategy affecting the numbers or types of contaminant-degrading microorganisms?

Again, microbial fingerprinting methods cannot be used to quantify specific contaminant-degrading microorganisms or functional genes involved in contaminant biodegradation. However, DGGE and T-RFLP can indicate whether or not there are shifts in the total community structure over time, which might be used to inform the relative abundance of contaminant degraders depending on the type and extent of information available (that is, if a specific restriction fragment or DGGE band has been linked to a degrader). Microbial fingerprinting techniques are best suited for general questions relating to either identifying the predominant microorganisms present in the sample or the viability, health, and diversity of the microbial community.

One example of the use of fingerprinting techniques during remediation is the evaluation of the secondary impacts of a treatment technology such as in situ chemical oxidation. PLFA analysis can be used to determine whether chemical oxidation has adversely impacted the microbial community or to monitor recovery of the microbial community following ISCO and assess the feasibility of subsequent biodegradation. While other EMDs are more appropriate for tracking known contaminant

degraders, microbial fingerprinting techniques can be employed to investigate the overall impacts of a remediation strategy. For example, PLFA results showing an increase in total biomass relative to baseline levels indicate microbial growth in response to the treatment approach. Likewise, comparison of baseline DGGE to post-treatment DGGE profiles can reveal which microorganisms or types of microorganisms were enriched by the treatment approach as well as those which may no longer be dominant following the change in subsurface conditions. In other words, the comparison is used to determine the impact of the treatment approach on the overall microbial community composition.

O) Does the microbial community composition support the remediation strategy?

Monitoring specific groups of contaminant-degrading microorganisms or one particular functional gene does not always provide the complete picture needed to assess a remediation strategy. For example, although PLFA is not capable of directly measuring the effect of amendments on the specific organisms involved in biodegradation, it can be used to indirectly assess the impact by measuring overall changes in biomass, microbial composition of the site, and redox state. Thus, if an electron donor was used as an amendment, an increase in the proportion of the anaerobic PLFA biomarkers using that amendment should occur, whereas if an electron acceptor such as oxygen was used, a decrease in the proportion of the same biomarkers should be noted.

Moreover, biostimulation can depend upon interactions between different microorganisms and be hindered by other microbial interactions. At a site which was impacted by a mixture chlorinated solvents undergoing biostimulation, the injected electron donor was fermented by a diverse group of microorganisms producing hydrogen, which in turn was used by many of the known dechlorinating bacteria and competing microorganisms. While qPCR would be more appropriate to track stimulation of known contaminant-degrading microorganisms (such as *Dhc*) and competitors (sulfate reducing bacteria), nucleic acid-based fingerprinting techniques or microarrays could be used for more comprehensive evaluation of the overall microbial community. For example, a T-RFLP profile may reveal an increase in the proportions of potential dechlorinating microorganisms (such as *Chloroflexi*) and supporting microbial populations involved in fermentation (*Firmicutes*).

P) Do contaminant-degrading microorganisms continue to be sufficiently abundant?

See Question H.

5.3.2.3 Closure

Some variability of closure requirements exists among states and programs. However, in many situations, EMD data can serve as an additional line of evidence for understanding what processes are important in reducing contaminant mass and concentrations and reaching the applicable closure levels. The evidence provided by EMD data can reveal whether biodegradation processes are occurring, have sufficiently proceeded, or are likely to continue (See Sublette et al. 2007).

W) Is contaminant degradation likely to continue?

Fingerprinting techniques do not provide direct evidence regarding degradation, but fingerprinting can provide indirect evidence that the contaminant degradation is occurring during the monitoring phase. Once it is established, continued degradation is possible as long as no substantial changes occur in subsurface geochemical conditions and or in microbial community composition thereafter. For example, consider a site which has had PCE contamination in which PLFA analysis was used to monitor viable biomass and community structure after the completion of an in situ oxidation. Changes in total PLFA (biomass), anaerobic PLFA biomarkers, or alterations in the ratios of specific PLFAs (for example relevant to sulfate reducers) could signal changes in the health and viability of the subsurface microbial community which may impact further contaminant biodegradation. Furthermore, a stable microbial population and community composition would suggest that degradation is likely to continue.

AA) Does the microbial community composition suggest that sufficient contaminant degradation has occurred?

Throughout the project life cycle, from release of the contaminants through remediation to closure, site management activities will impact the subsurface microbial community. For example, bioremediation will stimulate a relatively small number of microorganisms, and over time the diversity should increase as the contaminants are removed. Fingerprinting techniques can be useful for tracking such changes in community composition, but only if there are relevant baseline or background samples for comparison and several samples taken over time to monitor the changes over time. While a complete return to baseline conditions may not be feasible, a diverse microbial community with viable biomass levels comparable to background would provide an indication of recovery. For example, comparison of DGGE profiles from samples obtained in impacted areas after treatment with those of baseline or background samples should reveal microbial community structures of similar, but likely not identical, diversity.

5.3.3 Practical considerations

Interpretation of most site monitoring data, including microbial fingerprinting, is most conclusive when based on comparisons to corresponding results for background (non-impacted, upgradient) or baseline (prior to treatment) samples. For example, PLFA results showing an increase in total biomass relative to baseline levels indicate microbial growth in response to the treatment approach. Likewise, comparison of baseline DGGE to post-treatment DGGE profiles can reveal which microorganisms or types of microorganisms were enriched by the treatment approach as well as those which may no longer be dominant following the shift in subsurface conditions. In other words, the comparison is used to determine the impact of the treatment approach on the overall microbial community composition. Other EMDs such as qPCR (if available) may be more appropriate for tracking numerical and functional changes in specific microbial populations in response to treatment. Overall, the central lesson learned in microbial fingerprinting for environmental restoration applications is that analysis of background or baseline samples is invaluable for productive interpretation of results. Additional information about fingerprinting methods is included in the

fingerprinting fact sheet.

5.3.3.1 Choosing between PLFA, DGGE, T-RFLP, and other EMDs

The primary difference in the results provided by each technique is in the degree of resolution or specificity. Choosing between these techniques therefore depends primarily upon the specificity of the questions that need to be addressed and the current state of knowledge regarding the microbial process in question.

Fingerprinting methods are used to provide an overall view of the microbial community, indications of microbial diversity, insight into the types of metabolic processes occurring in the sample (notably the terminal electron accepting processes, such as sulfate reduction), and some can be used to identify a subset of the microorganisms present in the sample. This information is relevant because biodegradation inherently depends upon the types and abundance of microorganisms present in the subsurface. For example, microbial fingerprinting methods can identify when adverse conditions (such as low pH), either natural or following a remedy such as chemical oxidation, result in low microbial biomass and microbial diversity rendering biodegradation unlikely under existing conditions. Similarly, microbial fingerprinting methods can be used to determine if the overall microbial community has recovered or responded to remedial actions. While other EMDs are more appropriate to detect and quantify known contaminant degrading microorganisms, several microbial fingerprinting techniques can be used to identify the predominant microorganisms present in the sample.

Genetic fingerprinting methods are not quantitative, and many important microbial processes are conducted by a numerically small portion of the total population (<1%) that may not be detected by fingerprinting techniques. T-RFLP may be more sensitive than DGGE for detecting less abundant microorganisms, but it is still limited for such uses as compared to more specific methods such as PCR. Interpretation of microbial community fingerprints is somewhat subjective and less straightforward than for other EMDs and identification is limited to known microorganisms and available DNA sequences.

PLFA analysis provides a measure of total viable bacterial biomass and a broad-based profile of the microbial community composition grouped into general categories. PLFA analyses can provide information on the changes in the community without determining the exact species composition, since a change in the PLFA pattern should indicate an altered community. PLFA analysis can address general questions such as whether a treatment increased or decreased total bacterial biomass or substantially altered redox conditions.

DGGE and sequence analysis provides a DNA-based profile of the microbial community and allows identification of the predominant organisms generally to the family or genus level but cannot quantify specific organisms or microbial functions. DGGE profiles are used to visually display differences or shifts in microbial community composition over time or in response to treatment. Subsequent sequence analysis is somewhat exploratory, seeking to identify specific types of organisms. Most often, DGGE analysis is performed when identification of the predominant organisms is

required but little is known about the microbial community of the sample prior to analysis. Although DGGE can be used to identify microorganisms (unlike PLFA, for example), the number of microorganisms that can be identified depends on the complexity of the microbial population. Typically, only three to ten microorganisms can be identified per sample.

While the DNA-based microbial fingerprinting methods (DGGE, T-RFLP) are used to identify microorganisms present in a sample, other EMDs provide more specific results and may be more appropriate for evaluating contaminant biodegradation. For example, qPCR provides very specific results—quantification of a specific microorganism (for example, *Dhc*) or genes encoding a specific function (reductive dechlorination of vinyl chloride) responsible for biodegradation of common groundwater contaminants. In these cases where site management questions focus on evaluating biodegradation of a specific contaminant or group of compounds, other EMDs such as qPCR are often more applicable.

5.3.3.2 Pyrosequencing

Despite their relative ease of performance, some common DNA-based fingerprinting techniques such as T-RFLP and DGGE have biases that can oversimplify the diversity in complex microbial communities. For example, T-RFLP analyses cannot differentiate terminal fragments (and consequently microbes) that share the same restriction site. Similarly, amplified 16S rRNA gene fragments that contain more than one base difference sometimes cannot be effectively separated by the denaturing conditions used in conventional DGGE analyses.

Like many other molecular approaches, DNA-based fingerprinting methods are likely to be replaced by approaches that exploit the increasing availability of low cost, high throughput DNA sequencing methods (see D.4). Current DNA sequencing methods such as pyrosequencing enable the sequencing of many thousands of PCR-amplified DNA segments from an environmental sample (see Appendix D, Question 28). Subsequent analyses of the resulting DNA sequencing data enables the relative abundance of different phylotypes to be determined for the microbial community captured in the DNA sample. These analyses are often much more comprehensive than methods such as T-RFLP and DGGE, and have the added bonus of providing the identity of microbes in addition to relative abundance and diversity. The low cost of these analyses is driven by not only rapidly decreasing DNA sequencing costs, but also by the automation of much of the analysis and by simultaneously conducting multiple analyses using barcoded PCR primers.

As with other EMD methods, however, these sequencing methods can have bias or errors. For example, during pyrosequencing error can be introduced within the DNA sequences generated, which can increase the potential to overestimate microbial diversity (Kunin et al. 2010). However, better bioinformatic tools continue to emerge to deal with and even eliminate the errors during sequencing and sequence data processing. (Quince et al. 2009; see Logares et al. 2012 for a review). Other "next-generation" high throughput sequencing approaches (such as, platforms known as Illumina®, SOLiD®, Ion Torrent™, PacBio, Starlight) are also being rapidly developed in parallel to pyrosequencing, often as research tools, but are also commercially available (see Glenn 2011 for a recent review).

5.4 Additional Information

Further reading on fingerprinting methods is provided in Appendix F.

6.0 MICROARRAYS

6.1 Summary of Microarrays

- Microarrays exploit the hybridization effect between DNA strands to detect the presence of DNA sequences for specific genes using short single-stranded DNA molecules, called "probes", with known nucleotide sequences.
- Microarrays can be fabricated in many forms and can contain up to hundreds of thousands of probes that target tens to thousands of genes. Multiple probes are typically designed for a target gene and the number of probes exceeds the number of target genes.

- Microarrays have been used for environmental research for at least 15 years but have only recently become widely commercially available.
- Microarrays can monitor changes in community structure and functional gene content in response to treatment such as biostimulation.

Additional information is available in the Microarrays Fact Sheet.

DNA typically is found as a double stranded molecule in which the sequence of nucleotide bases on one strand (for instance, ATCG) has a complementary sequence (TAGC) on the opposite strand. If two complementary DNA strands are mixed together in solution, they tend to join together (hybridize) and form a stable double-stranded molecule by hydrogen bonding. The degree of hybridization between the two strands is dictated by base-pairing rules (for example, A on one strand always binds to T on the other strand) and the specific sequence of bases in the two strands. Two DNA strands with complementary base sequences will bind together tightly, while dissimilar strands will bind together poorly, if at all.

Microarrays exploit this hybridization effect to detect the presence of DNA sequences for specific genes in environmental samples by using short, single-stranded DNA molecules (probes) with known nucleotide sequences. In practice, these probes are attached to a solid surface such as a glass slide or suspended in a gel. After extracting DNA from an environmental sample (and sometimes amplifying the DNA by PCR), the DNA sample is labeled with a fluorescent dye and applied to the array. When hybridization occurs, the labeled DNA that complements the microarray probes are retained on the slide, producing a characteristic fluorescent signal. DNA that does not have a complementary probe on the microarray slide does not bind (hybridize) and is removed in a washing step. Detection and relative quantification of specific genes in the DNA sample is based on the strength of the fluorescent signal that remains on the microarray after the washing step. Some microarrays use two fluorescent dyes (often red and green), which can be used to compare the range of genes present in more than one sample (Figure 6-1). Figure 6-1 shows a generic microarray analysis. DNA is isolated from groundwater collected within and outside of a contaminant groundwater plume. The DNA is labeled with two fluorescent dyes (green and red) and

hybridized to the microarray. After multiple washing steps, the microarray slides are analyzed with a high-resolution laser scanner.

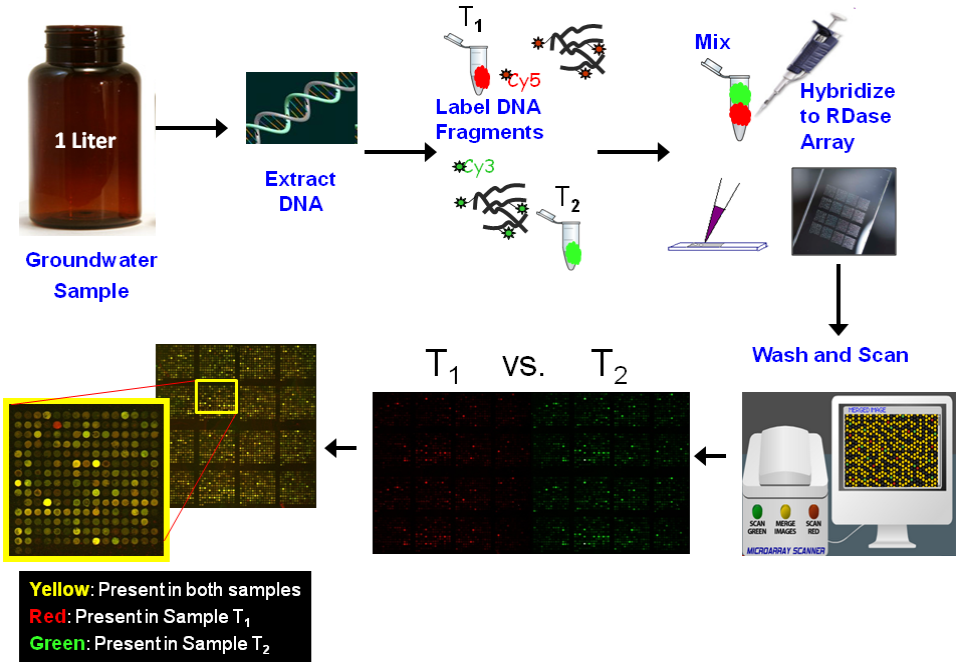

Figure 6-1. Generic microarray analysis.

Source: E. Padilla-Crespo and F. Löffler, Ph.D., University of Tennessee, 2012. Used with permission.

Microarrays can be fabricated in many forms and can contain up to hundreds of thousands of probes that target tens to thousands of genes. In high-density microarrays, the position and nucleotide sequence of each probe on the microarray is precisely known, and the fluorescence associated with each probe can be located and measured using laser excitation and detection. The benefit of high-density microarrays is that they can rapidly and simultaneously determine the presence and abundance of numerous genes in a single DNA sample using identical analytical conditions.

Microarrays have been used for environmental research for at least 15 years but have become commercially available to a wider community only recently. The commercially available Affymetrix Phylochip® microarray contains a comprehensive suite of probes for bacterial 16S rRNA genes, a stretch of DNA sequence that by convention acts as the "bar code" for bacterial identification. This microarray can therefore provide comprehensive information about microbial community structure and can answer the question “Which microorganisms are present?” (Conrad et al. 2010; Briggs et al. 2011; Cooper et al. 2011; DeAngelis et al. 2011; Mendes et al. 2011).

Another commercially available microarray, GeoChip, contains approximately 28,000 probes that target nearly 57,000 gene variants. These targeted gene variants are implicated in major microbial processes such as contaminant degradation, carbon, nitrogen, sulfur and phosphorous metabolism,

metal reduction and antibiotic resistance (He et al. 2007; He et al. 2010; He et al. 2011; Zhou et al. 2011). The GeoChip thus addresses the question "What can the microbes that are present potentially do?" and, if used as an expression array for transcript analysis, can answer the question: "What are the microbes that are present actually doing?".

If applied to DNA samples collected over time from the same sampling locations, DNA microarrays can monitor changes in community structure and functional gene content in response to treatment such as biostimulation. Microarrays can also be used to investigate which genes are being expressed by microorganisms. This information can be used to answer questions such as "Which organisms are active?" and "What metabolic pathways are these organisms using?" These questions are addressed using expression microarrays, which require extracting mRNA from environmental samples and then converting this mRNA to complementary DNA (cDNA) in the laboratory. The cDNA is then analyzed the same way as DNA directly extracted from an environmental sample. If applied to RNA samples collected over time from the same sampling locations, expression microarrays can also monitor changes in levels of gene expression in response to treatments such as biostimulation.

6.2 Applications

Site characterization, remediation, monitoring, and site closure require thorough examination of available chemical, geochemical, and microbiological data. The purpose of performing DNA or RNA microarray analyses is to provide the microbiological lines of evidence: the presence (semi-quantitative) and activity of microorganisms capable of biodegradation of the relevant contaminants or the biogeochemical processes that support biodegradation at a given site.

Table 6-1. Example applications of microarrays

Title	General information	Contaminants	EMDs used	Project life cycle stage
In Situ Uranium Reduction, CO (see description below)	In situ uranium bioremediation, key functional genes were studied with GeoChip	Uranium	GeoChip Functional genes that reflect redox conditions	Characterization and Remediation
High-density PhyloChip profiling of stimulated aquifer microbial communities (see description below).	Determine community membership for uranium bioremediation experiments	Uranium	PhyloChip	Characterization and Remediation
Deep Sea Oil Plume (see description below).	Understanding the microbial community functional composition	Crude Oil	GeoChip Functional Genes that reflect oil remediation	Characterization
Deep Sea Oil Plume (see description below)	Understanding the petroleum degrading microorganisms	Crude Oil	GeoChip and PhyloChip	Characterization

Table 6-1. Example applications of microarrays (continued)

Title	General information	Contaminants	EMDs used	Project life cycle stage
Monitoring microbial community structure (see Appendix A.10)	Application of a field-portable microarray system	Uranium	TruArray® BER	Characterization and Remediation

In Situ Uranium Reduction

To better understand the microbial functional diversity changes with subsurface redox conditions during in situ uranium bioremediation, key functional genes were studied with GeoChip, a comprehensive functional gene microarray, in field experiments at a uranium mill tailings remedial action (UMTRA) site in Rifle, CO (Liang et al. 2012).

High-density PhyloChip Profiling of Stimulated Aquifer Microbial Communities

Samples were taken from both laboratory and field experiments from the Department of Energy Integrated Field Research Challenge Site (IFRC) in Rifle, Colorado. The analyses were done to determine community composition and population patterns among a set of samples associated with uranium bioremediation experiments. The high-density microarray (PhyloChip) samples were collected from unstimulated and naturally reducing sediments, or collected during acetate donor stimulated Fe(III) and sulfate reduction (Handley et al. 2012).

Deep Sea Oil Plume

In this paper the authors showed how microbial community functional composition and population structure were altered in an oil spill in the Gulf of Mexico. Until this time the impacts on marine ecosystems were largely unknown. In the spill area a variety of metabolic genes involved in both aerobic and anaerobic degradation of petroleum hydrocarbons were highly enriched compared with areas outside the spill. This indicates potential for intrinsic bioremediation or natural attenuation in the deep sea (Lu et al. 2012).

Deep Sea Oil Plume (GeoChip and PhyloChip)

In this research effort the authors use various array data to show that the dispersed hydrocarbon plume stimulated deep-sea indigenous γ-Proteobacteria that are closely related to known petroleum degraders. The hydrocarbon-degrading genes identified coincided with the concentration of various oil contaminants. Incubation experiments with environmental isolates also demonstrated faster-than-expected hydrocarbon biodegradation rates at low temperatures (5°C) showing the potential exists for intrinsic bioremediation of the oil plume in the deep-water column (Hazen et al. 2010).

Microarrays can perform thousands to millions of hybridization reactions simultaneously with the same DNA or cDNA samples under identical conditions. Using microarrays thus provides comprehensive snapshots of the presence, abundance, and (potentially) the activity of many genes.

In general, microarrays can help to answer the following questions:

- Are specific organisms (16S rRNA genes) and pathways (functional genes) implicated in contaminant degradation present at the site?
- Does the community structure and functional gene abundance change over temporal and spatial scales?
- Which functional genes are active, and does gene activity change over time (such as following treatment)?

Microarray analyses are applicable to four key lines of investigation, including but not limited to:

- supporting the initial site investigation
- assessing the status of monitored natural attenuation (MNA) as a remedy
- evaluating bioremediation as an enhanced natural attenuation (ENA) option
- evaluating the ongoing requirements for biostimulation (such as electron donors to support reductive dechlorination) and bioaugmentation (such as addition of dechlorinating microorganisims)

Microarray support for site investigations is summarized in Section 6.1. Additional details regarding MNA and bioremediaton applications are provided in the following sections.

6.2.1 Monitored natural attenuation

MNA can be an effective site management strategy when microorganisms capable of degrading contaminants are present. When considering MNA as a remediation strategy, nucleic acid-based methods can be used to identify the presence of specific microorganisms already known to be able to degrade the contaminants. For some contaminants, a clear link between contaminant biodegradation and the presence of specific microorganisms or functional genes has been established, and EMD tools including both microarrays and quantitative PCR (qPCR) can be useful to determine and quantify the presence of the contaminant-degrading microbes. When evaluating sites for the potential to degrade contaminants with undefined degradation pathways (i.e., the genes encoding the pathway are not known) and contaminant-degrading microorganisms have yet to be identified, broad-spectrum microarray such as the Phylochip or GeoChip can potentially provide insights into the microorganisms and the functional genes involved in biodegradation. Microarrays thus establish an additional line of evidence (in addition to traditional contaminant concentration and geochemistry data) that can be used to establish links between microbial contaminant detoxification and effective cleanup strategies.

Microarray analysis may provide benefits when applied to mixed waste contaminated sites or sites with co-mingled plumes where a more comprehensive view of the microbial community is needed to assess MNA. For example, consider an industrial facility impacted by a mixture of chlorinated solvents and heavy metals. Under such a scenario, the feasibility of MNA or bioremediation as a remedial strategy will depend on the presence of several different microbial groups capable of degrading the major contaminants (PCE and TCE), co-contaminants (chlorinated ethanes and chlorinated methanes), and the resulting intermediate products. In addition, the microbiology must contribute to biological reduction of metals, which can be carried out by diverse microbial populations.

The advantage of microarray application is clear under such a scenario because many target genes can be monitored simultaneously in a single analysis.

6.2.2 Bioremediation

Bioremediation has two essential components - biostimulation and bioaugmentation. Biostimulation introduces a variety of additives and amendments to the aquifer to help the existing microorganisms transform and detoxify contaminants. For example, amendments for anaerobic bioremediation of chlorinated solvents include fermentable carbon substrates such as lactate, molasses, emulsified vegetable oil, slow release materials such as lactate polymers and refined plant fibers. The amendments enhance acetate and hydrogen fluxes, which serve as the direct electron donors for different chlorinated solvent-detoxifying bacteria. Amendments for aerobic bioremediation of petroleum hydrocarbons include electron acceptors such as oxygen.

Biostimulation triggers many responses of the microbial community, including relative changes in population abundances and the up-regulations and down-regulations in the expression of many genes. Microarrays that monitor many genes and transcripts simultaneously can monitor the impacts of biostimulation on the microbial community, the relative functional gene abundance, functional gene diversity, and gene activity. Such microarray measurements can document the success of biostimulation, monitor contaminant detoxification, and indicate the need for additional treatment in particular if combined with geochemical measurements. Such integrated analyses has the distinct value of reducing an over application of additives and reducing waste, secondary impacts to water quality and cost to closure.

Case Study A.10 describes a site impacted by uranium (VI). An electron donor, acetate, was injected into the subsurface to stimulate in situ microbial reduction of uranium and promote sequestration. Microarray analysis provided a means to track changes in microbial community structure following acetate injection. For example, acetate injection initially stimulated aerobic and nitrate-reducing bacteria. Once oxygen concentrations decreased, metal-reducing and finally sulfate-reducing bacteria became active and increased in abundance. Microarray analysis proved to be a valuable tool for monitoring and documenting these population shifts over the course of the bioremediation project.

6.3 Data Interpretation

The application, analysis, and interpretation of microbiology-based EMD methods differ from typical soil and groundwater geochemical measurement in a number of ways. For example, microbial biomarkers cannot easily be preserved, and sample handling and processing requires special care. The analysis of microbial parameters requires specific data quality considerations for sampling plans, sample collection and handling, quality control and laboratory procedures, and these are discussed in Section 10.0. Included below is a brief introduction to how microarray data are typically reported and some specific examples of how the data would be interpreted in answering the questions presented in Table 2-3.

6.3.1 How are the data typically reported?

Currently, a standard format for reporting microarray results obtained with environmental samples has not been adopted, but has been advised (Brazma et al. 2001). The analytical laboratory must have established Standard Operating Procedures (SOPs) that are uniformly applied to all samples, and a report should include information about sampling (location, methodology, handling, storage) and sample processing (such as nucleic acid extraction, labeling, reverse transcription, hybridization conditions). Microarray data are most informative when samples collected over temporal and/or spatial scales are compared to each other. As discussed elsewhere in this document, samples must be collected using the same methodology so that the microarray data obtained for different sampling events can be directly compared. The report should include information about the type of microarray used (e.g., a list of the all target genes) and document the differences in community structure and functional gene content and abundance (DNA microarrays), and gene activity (expression microarrays).

Included in Table 6-2 below is information that should be provided in laboratory reports of microarray EMD data including common laboratory report information, recommended information about the microarray method, and desirable information about the microarray method and results.

Additional information regarding sample handling and collection can be found in Section 10.0.

Table 6-2. Recommended and desirable information for microarray laboratory reports

Report information	Typical information or acceptable ranges
Common Laboratory Report Information	
Site Identifier	Needed to identify sample
Sample type or matrix	Groundwater, sediment, soil, other
Sample storage/transportation	4°C or field frozen (dry ice) overnight shipping, Chain of custody forms
Sample handling methods	Filtering methods, filters used, sediment washing, volume of water filtered, surface area swabbed
Specific Microarray Information	
Recommended	
Results	Identification and relative quantification of microorganisms (genus and species, if applicable) and functional genes; transcriptional activity of functional genes (gene expression)
Reporting Units	Signal intensity (e.g. raw signal, background corrected or log transformed) for each target gene; presence or absence of each target gene for DNA or cDNA
Limit of Detection	Varies, should be adequate for the application
Array targets	16S rRNA, functional gene name, probe sequence. This information may be proprietary to the manufacturer. The report should identify the specific product name, test, serial number and expiration date.

Table 6-2. Recommended and desirable information for microarray laboratory reports (continued)

Report information	Typical information or acceptable ranges
Hybridization conditions	Hybridization time, hybridization solution composition (e.g., probe concentration), and temperature. Some of this information may be proprietary to the manufacturer. Information that is provided on the product labeling and instructions for use may be a good source for this information.
Method of quantification	
QA/QC information	Laboratory positive and negative control results (non-sense probe)
Narrative of analyses	Describes interferences or quality control issues encountered
Desirable	
Photomicrographs	This image is the actual raw data.
Number of laboratory duplicates and replicate results	Each test, even if replicated, should have a unique ID.

6.3.2 How are the data interpreted?

Microarrays come in many sizes; low-density microarrays may have as few as 20 probes (a specific nucleic acid sequence) while others have several hundred thousand. Because of this range of sizes, microarrays can generate a large amount of data, which can complicate in-depth data interpretations. Because it is practically impossible to empirically develop and validate hundreds or thousands of probes against all possible environmental targets represented by an array (let alone all of the unknown nucleic acids in an environmental sample), individual probes are simply potential indicators of the cognate gene or organism.

Microarrays provide qualitative (presence or absence) and semi-quantitative information. Overall shifts in microbial community structure, functional gene abundance, and expression patterns can be easily displayed and interpreted via visual data tools; however, correlated samples are recommended for interpreting microarray data, in that it is the relative change in probe A versus probe A across the correlated sample set that carries the most biological or ecological information. That is, a comparison of the same probe on the array is compared to the same probe on the array over space or time (a correlated sample set). Interpreting microarray field data may require several levels of granularity, from fine-scale analysis of individual probe responses to summed intensities over genera to integrated intensities over wells and the entire site.

Since microarrays can perform many genetic tests in parallel, data reduction and statistical procedures may be required to extract meaningful information. While such approaches are now routinely applied in the medical field, standardized microarray data analysis pipelines and reporting formats for environmental microarray data sets is in its infancy. Since the demand for environmental microarray applications is expected to increase, the support structure for microarray application to environmental samples and associated data analysis pipelines and a robust framework for data interpretation are now becoming available.

Depending on site needs, microarray analysis may also be substantially simplified when reduced data sets are used or global pattern analysis is performed. For example, a functional gene analysis focused only on the relevant reductive dehalogenase genes may be appropriate at chlorinated solvent sites where anaerobic bioremediation has been implemented. Such a focused analysis based on a reduced microarray dataset may be sufficient for monitoring of chlorinated solvent bioremediation, and the examination of other functional genes included on the microarray may not be needed.To illustrate interpretation of microarray results, each of the questions relevant to microarrays posed in Table 2-3 is discussed.

6.3.2.1 Site Characterization

A) Are contaminant-degrading microorganisms present?

Microarrays can be used to target16S rRNA genes to evaluate the presence or absence of specific contaminant-degrading microorganisms. Microarrays can also be used to target functional genes encoding pathways involved in the degradation of a particular contaminant. As an example, at a site PCE and intermediate products TCE, DCE, and VC are present. Since microarrays can perform hundreds of parallel genetic tests in one assay, investigators could determine if any known dechlorinating bacteria are present by using 16S rRNA probes. Investigators could also use microarrays to verify the presence of pathways encoding specific contaminant degradation using structural gene targets (e.g. enzymes that catalyze reductive dechlorination). This resulting information could then be integrated with geochemical and contaminant data to provide a complete picture of the ongoing processes in the aquifer.

B) Are contaminant-degrading microorganisms active?

Microarrays can be used to determine if contaminant-degrading microorganisms are active. In order to accomplish this, RNA as opposed to DNA is extracted and then processed (converted into complementary DNA) for the microarray analysis. As an example, at a site PCE and intermediate products TCE, DCE, and VC are present. Investigators could verify the activity of dechlorinating bacteria by extracting RNA and analyzing the resultant cDNA targeting dechlorination pathways (e.g. enzymes that catalyze reductive dechlorination) via the microarray. These results would be supplemental to the contaminant concentrations trends data.

C) Are the microorganisms capable of complete degradation?

Microarrays can be used to assess if microorganisms are capable of complete degradation if the structural genes for the pathways of complete degradation are known. Using the example a site with PCE and intermediate products TCE, DCE, and VC, there are known structural genes responsible for steps along the reductive dechlorination pathway, and the final step (e.g. *bvcA* and *vcrA*) that code for the VC reduction to ethene.

6.3.2.2 Remediation

H) Are the numbers of contaminant-degrading microorganisms and/or genes changing?

Microarrays measure the presence or absence of target genes, and the analysis of samples collected over temporal or spatial scales can indicate if genes of interest are increasing or decreasing in abundance. As stated earlier, correlated samples are recommended for interpreting microarray data, since it is the relative change in probe A vs. probe A across the correlated sample set that carries the most biological or ecological information. For a site, array data could be correlated across the monitoring wells and time to discover where and what microorganisms or structural genes are present and in what relative proportions.

I) Is the remediation strategy affecting the numbers or types of contaminant-degrading microorganisms?

This question is addressed through the same approach described for the previous question, only varying with the remediation strategy in addition to space or time. If a carbon donor is being applied at the site with PCE, one would expect to see an increase in organisms, enzymes, and expression concomitant with that action. Since arrays can provide hundreds or thousands of genetic tests in a single analysis, they can be used to develop an overall picture of what is occurring in the microbial community.

J) Is there a biological basis for intermediates accumulating?

Microarray data can provide forensic information about the absence or relatively low number of bacteria otherwise needed to complete a degradation pathway. For the site with PCE, arrays could be used to diagnose that microbes capable of dechlorinating VC to ethene were either not present or were in very low abundance. Microarrays could also be used with RNA to investigate whether enzymes that code for specific reductive dechlorination reactions were active.

6.3.2.3 Monitoring

O) Does the microbial community composition support the remediation strategy?

Microarrays are the only EMD uniquely positioned to provide thousands of data points on the microbial community simultaneously. The information produced by microarray analysis can be used to support the remediation strategy by confirming the presence of known degraders and structural degradation pathways. For the site with PCE, array analysis can provide a very in depth analysis of literally thousands of specific microbial species (e.g. all known dechlorinators) or structural genes (reductive or other dechlorination pathways).

P) Do contaminant-degrading microorganisms continue to be sufficiently abundant?

Samples collected over temporal or spatial scales can indicate if genes of interest are increasing or decreasing in abundance. For the site with PCE, array data could be correlated across the monitoring wells and time to discover where and what dechlorinators or structural genes are present

and in what relative proportions. These data should also be analyzed in conjunction with geochemical and contaminant data to provide a complete assessment of remedial efforts.

Q) Are contaminant-degrading microorganisms remaining active?

This question is answered by relative gene abundances using DNA results from the arrays or directly by using RNA.

R) Is there a biological basis for intermediates accumulating?

Microarray data can provide forensic information about the absence or relatively low number of bacteria otherwise needed to complete a degradation pathway. For the site with PCE, arrays could be used to diagnose that microbes capable of dechlorinating VC to ethene were either not present or were in very low abundance. Microarrays could also be used with RNA to investigate whether enzymes that code for specific reductive dechlorination reactions were active.

6.3.2.4 Closure

EMD data provide relevant lines of evidence that microbial processes contribute to contaminant concentration reductions and to achieving the applicable closure levels. EMD data can reveal whether biodegradation processes are occurring, have sufficiently proceeded, are likely to continue, or if microbial activity will not be a major contributor to contaminant attenuation. Microarrays are suitable EMD tools to provide information about the current status of the microbial community and its activity and can provide some information about a site's trajectory with regard to decreasing contaminant concentrations. Thus, at sites that are nearing the concentration-based goals for site closure, with limited information about natural attenuation mechanisms, microarray application can provide crucial information about the microbiology and its role for continued contaminant attenuation.

W) Is contaminant degradation likely to continue?

In order to determine if degradation is likely to continue, there may be parameters required in addition to microbial community composition and population information like that provided by arrays. As an example for chlorinated solvent degradation it would be critical to understand organic carbon dynamics, and for hydrocarbons the availability of terminal electron acceptors. These parameters are used to build a conceptual narrative or in some cases used as model inputs. Array data can be integrated with these geochemical parameters to provide a more complete picture of contaminant degradation and answer the question if that degradation is likely to continue. The answer to these types of questions can be complicated and no one type of data can stand alone.

AA) Does the microbial community composition suggest that sufficient contaminant degradation has occurred?

To date, microarrays have seen limited application for site assessment and bioremediation monitoring (He et al. 2011; Zhou et al. 2011; Chandler et al. 2010). The reductive dehalogenase

(RDase) array contains probes targeting many of the known *Dehalococcoides* (*Dhc*) RDase and hydrogenase genes and has been used to demonstrate changes in gene abundance following biostimulation treatment. Unfortunately, function has been assigned to only a few of the hundreds of RDase genes, limiting detailed functional analysis. At one site, groundwater samples were collected from a PCE/TCE-contaminated site from the same well prior to bioremediation and at three time points following biostimulation during the course of seven months. Total DNA was extracted and the same amounts of labeled DNA were hybridized to the RDase array.

As shown in Figure 6-2, the numbers of "bright spots" increased over time, indicating that the abundance of *Dhc* RDase and hydrogenase genes increased following biostimulation. In other words, this analysis demonstrates that microbes (*Dhc*) carrying these RDase genes multiplied in response to biostimulation. Identical DNA amounts extracted from groundwater from the same monitoring well were labeled with the fluorescent dye Cy5 and hybridized to the microarray. After washing, the slide was scanned with a laser at a wavelength of 635 nm and red fluorescence was recorded. A red signal indicates the presence of a target gene in the sample and the color intensity can be used to infer relative abundance.

These data demonstrate the utility of the RDase microarray for monitoring *Dhc* populations at sites undergoing bioremediation. Since *Dhc* strains can only grow with the chloro-organic contaminants as electron acceptor and hydrogen as electron donor, the detection of fewer target genes or a decrease in color intensity without reaching contaminant cleanup goals may suggest that *Dhc* activity is limited by electron donor availability. Thus microarrays may be a powerful tool to determine if additional electron donor additions are needed and guide decision-making.

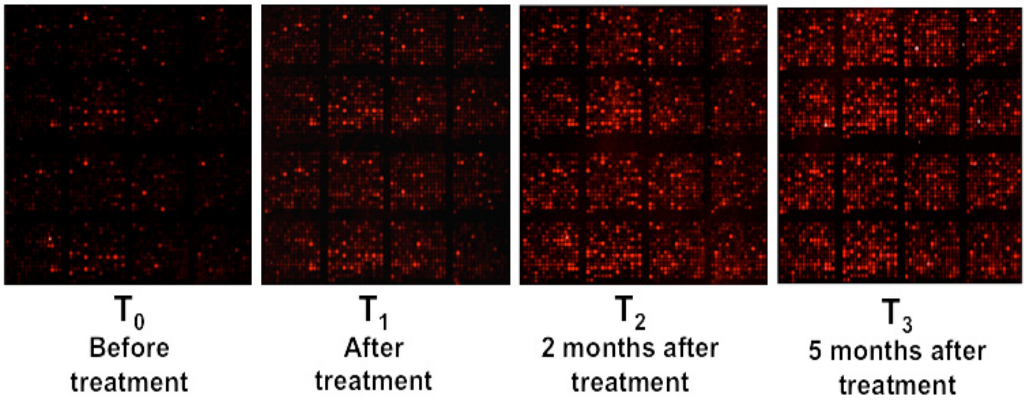

Figure 6-2. Microarray visualization of DNA samples from a chlorinated solvent contaminated site prior to bioremediation and at three time points following biostimulation.

Source: E. Padilla-Crespo and F. Löffler, Ph.D., University of Tennessee, 2012. Used with permission.

Among the genes that increased in abundance after biostimulation were RDase genes with assigned function such as the *pceA* gene implicated in PCE/TCE-to-*cis*-DCE reductive dechlorination, *tceA* responsible for TCE-to-VC reductive dechlorination, and *vcrA* and *bvcA* implicated in VC reductive dechlorination. Figure 6-3 below, depicts the relative increase in the abundance of

pceA, *tceA*, *vcrA*, and *bvcA* genes, determined with the RDase microarray. The gene abundance was calculated by taking the SNR (signal to noise ratio) of "x" spot at time T_X and divided by the values of that spot at T_0; this value is then "a fold change in SNR". A targeted qPCR approach could then be used to enumerate the RDase genes of interest and more accurately measure gene abundances. The intensity values of all probes targeting RDase genes with assigned function (*pceA*, *tceA*, *vcrA*, and *bvcA* genes) were individually grouped and averaged. For example, the microarry included probes targeting the *pceA* genes included genes of *Sulfurospirillum*, *Dhc*, *Desulfitobacterium*, and *Geobacter* spp.

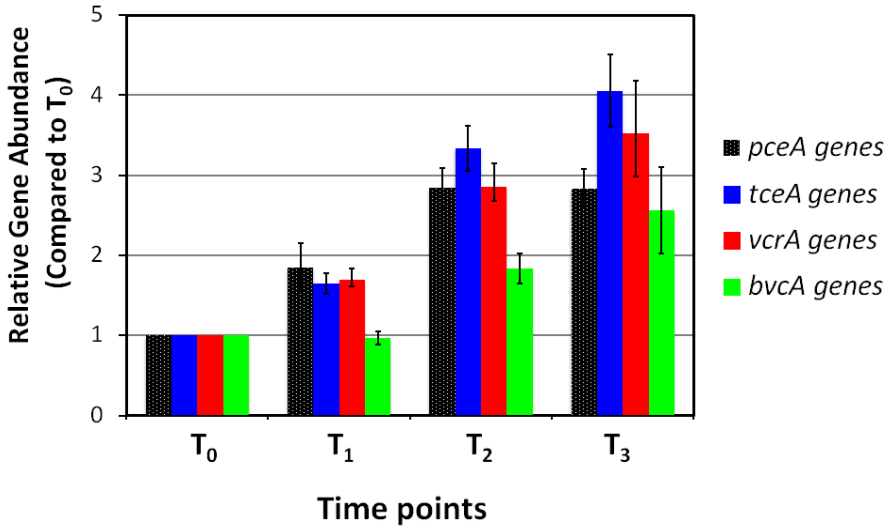

Figure 6-3. Relative RDase gene abundance detected with the RDase microarray. Error bars represent the standard error.

Source: E. Padilla-Crespo and F. Löffler, Ph.D., University of Tennessee 2012. Used with permission.

6.3.3 Practical considerations

6.3.3.1 Sampling

Information about sampling considerations and protocols is presented in Section 10.0.

For microarray groundwater sampling, 1 liter groundwater samples can be shipped directly to the laboratory. In-field filtration can greatly reduce shipping costs and is highly recommended. Another mode of collection is to use colonizable surfaces on passive collection devices that can be deployed in a well, such as the BioTrap®.

A time-dependent colonizable system gives an integrated result over time. Prior to sampling, the well should be purged in accordance with regulatory guidelines and standard operating procedures. For Sterivex® the inlet of the cartridge is attached to the pump tubing (1/4 to 5/16 in. inner

diameter) using a Luer-Lock® and a hose clamp. The filter is placed in a receiving container so that the volume of water filtered can be measured and recorded. In general, 1 to 2 liters should be filtered for microarray analysis. After sample collection, cap the filter on both ends, place in a Falcon™ or other tube, label appropriately, and place on ice.

Soil and Sediment Sampling: For microarrays, soil and sediment samples are typically collected in sterile Whirl-Pak™plastic bags or plastic containers. Microarrays need a minimum of 2-5 micrograms of DNA; otherwise the sample must be amplified prior to analysis.

6.3.3.2 Implementation

The advent of microarray applications in bioremediation monitoring programs is just beginning, and the examples cited here indicate the potential value of microarrays for successful remediation of contaminated sites. As with all of the EMDs, an understanding of the site contaminant concentrations and trends is important and the microarray data are used as supplemental information to answer questions that are not answered with traditional data. A standardized framework for reporting microarray data is needed, which will facilitate microarray data interpretation and linking microarray information with bioremediation processes.

Microarrays allow the simultaneous detection of tens of thousands to millions of target genes in a single analysis. A powerful application is the comparative analysis of samples collected over temporal scales from the same locations, which can reveal global shifts in gene abundance and gene activity (if expression arrays are used). Microarrays are highly adaptable platforms, and probes for new phylogenetic and functional genes can be readily added to existing microarrays to expand the applicability of the technique to a broader range of microorganisms and biodegradation pathways of interest.

One limitation of the microarray technology is limited sensitivity for targets present in low abundance. An amplification step can be included; however, this step adds to the cost and an unbiased amplification of all DNA in an environmental sample is difficult to achieve (Vora et al. 2004; Gao et al. 2007). Other shortcomings of the microarray approach include a narrow dynamic range for quantification (i.e., only semi-quantitative information can be obtained for the most abundant gene targets) and cross-hybridization issues (i.e., false positive signals are generated). These limitations are particularly problematic when applied to nucleic acids derived from environmental samples.

The scientific literature has reported on a variety of microarrays specifically designed to address individual research questions from pathogen detection in clinical (Wong et al. 2007) and microbial ecology applications (Vora et al. 2004; Gentry et al. 2006). Microarray design and target choice greatly influences the quality and clear interpretation of hybridization results and affects the utility of the array for the analysis of environmental samples. Microarrays that specifically address questions of importance for bioremediation have not been commercialized yet, and the meaningful interpretation of microarray data requires experience in performing the appropriate quality control and standardization of the approach.

If the target genes are present in sufficient abundance, microarrays provide excellent qualitative results and can determine whether or not a gene is present. Although recent studies have demonstrated relationships between signal intensity and target gene abundance, at this time the dynamic range is limited, and only semi-quantitative information can be obtained.

Probes are based on known gene sequences; hence, novel or as yet undiscovered genes cannot be detected. Since the gene content in environmental samples is unknown, cross-hybridization (i.e., gene fragments unrelated to the targeted gene function hybridize to the probe) can lead to false-positive signals and erroneous conclusions.

The scientific literature has reported on a variety of different microarray designs to address specific research questions. Microarray design greatly influences the quality and clarity of the hybridization results, and affects the utility of the array for the analysis of environmental samples. The meaningful interpretation of microarray data requires experience and may not be easily accomplished, in particular with environmental samples. Currently, few microarrays relevant to environmental monitoring and bioremediation are commercially available, and standardized pipelines for data analysis and results interpretation have yet to be developed. Efforts to make microarray technology more accessible for environmental applications are underway.

Finally, microarrays provide information about many genes and can facilitate the identification of indicator genes for the process of interest at a given site. For example, microarray application may attribute transformation of the primary contaminant to a specific reductive dehalogenase gene. A more targeted follow-up analysis with qPCR will provide more detailed information about the abundance of this gene and, if combined with RT-qPCR, detailed information about gene activity can be obtained.

6.4 Additional Information

Further reading on microarrays is provided in Appendix F.

7.0 STABLE ISOTOPE PROBING (SIP)

7.1 Summary of SIP

The stable isotope probing (SIP) method includes a family of techniques that all use individual contaminants (probes) that are enriched with stable isotopes (such as ^{13}C-labeled benzene or ^{15}N-labeled RDX) to characterize contaminant-specific biodegradation processes. The underlying principle of all SIP techniques is that biodegradation of isotopically-enriched contaminants results in the selective labeling of biomolecules such as DNA, RNA, or phospholipid fatty acids (resulting in ^{13}C- or ^{15}N-lableled DNA or ^{13}C-labeled fatty acids) in organisms that are responsible for degradation. A flow diagram showing the basic protocol for SIP studies is provided in Figure 7-1.

Unlike most other EMDs, SIP techniques can directly and unequivocally establish whether biodegradation of a specific contaminant is currently occurring in a contaminated environment or sample from that site. SIP techniques can also be used to investigate whether changes in environmental conditions are likely to enhance or inhibit contaminant biodegradation. SIP approaches are unique in that they can also detect and identify microorganisms responsible for biodegrading specific contaminants, even if the microorganisms, enzymes, and genes involved in these biodegradation processes are presently unknown.

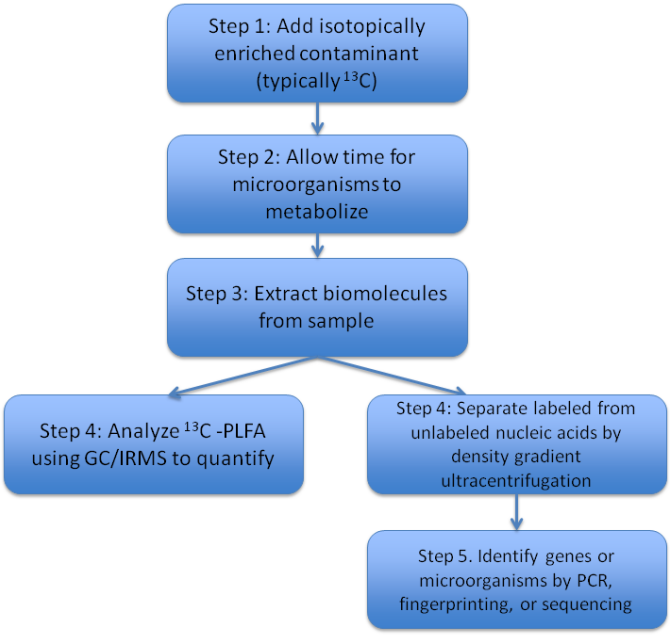

Figure 7-1. SIP Flow Diagram.

All SIP methods detect individual biodegradation processes by analyzing for the incorporation of stable isotopes from individual isotopically-enriched contaminants into either structural biomolecules in living microorganisms or terminal products (such as CO_2 or CH_4) released by

contaminant-degrading microorganisms. Even though the name "stable isotope probing" suggests some similarity with another important EMD, "compound specific isotope analysis" (CSIA), these two techniques are fundamentally different in two key respects. First, SIP uses individual contaminants that have been chemically synthesized to have artificially elevated levels of a particular stable isotope (such as ^{13}C). In contrast, CSIA detects process-specific changes in the natural-occurring levels of stable isotopes in individual contaminants. Second, SIP analyzes the isotopic composition of biomolecules generated by the microorganisms responsible for biodegrading isotopically-enriched contaminants. In contrast, CSIA analyzes the impacts of biological and abiotic process on the isotopic composition of individual contaminants However, despite these important differences, the information generated by these distinct techniques can also be complementary, since both techniques can provide unequivocal evidence for biodegradation process and insights into the mechanism and microorganisms involved in these processes.

Relationships between SIP analyses and other EMD methods depend on the type of SIP approach used. For example, an SIP analysis involving isotopically-enriched DNA can often involve use of PCR and fingerprinting techniques such as DGGE. In this instance, PCR can be used to amplify 16S rRNA gene sequences using SIP-generated ^{13}C-enriched DNA as a template. These amplified genes are then separated and visualized using DGGE. Similarly, SIP analysis of PLFAs can also involve fingerprinting analyses as well as measurements of isotopic enrichment in specific PLFAs using mass-spectrometry. If both ^{13}C-CSIA and ^{13}C-SIP-based field studies are proposed for a site, the CSIA-based studies should always be conducted first to avoid prior introduction of a ^{13}C-labeled contaminant. This is because the levels of ^{13}C-enrichment required for ^{13}C-SIP studies are many orders of magnitude greater than those that are generated by biodegradation processes and measured by CSIA.

Additional detailed information about the types of SIP analyses and their methodologies is available in the SIP Fact Sheet and in Section 7.4

7.2 Applications

Most current applications of SIP technique make use of contaminants that have enriched levels of ^{13}C. ^{13}C-based SIP techniques can potentially be applied to any type of environmental sample and can also be used to examine the biodegradation of any compound that is used by microorganisms as a sole (or predominant) source of carbon for growth. However, in current practice, SIP is used for two main purposes; (a) demonstrating that a specific contaminant undergoes biodegradation in a specific environment (PLFA-SIP) and (b) identifying organisms involved in specific biodegradation processes (DNA/RNA-SIP).

For example, a PLFA-SIP study using ^{13}C-enriched contaminants could be used to determine whether often slow anaerobic biodegradation of contaminants like methyl tertiary butyl ether (MTBE) or benzene is currently occurring at a gasoline-impacted site. A DNA-SIP study at the same site could not only provide the same information about the biodegradation process, but also identify the organisms responsible for this activity. Identification of these organisms could subsequently lead to the development of qPCR approaches that would enable these organisms to be

quantified at the same or other sites.

Although SIP approaches are typically thought to provide unequivocal evidence for biodegradation of the contaminant under investigation, there are two important and related caveats that can sometimes impact the interpretation of results from SIP studies. First, biomolecules could potentially become isotopically-labeled if the contaminant under investigation undergoes abiotic chemical degradation (e.g. hydrolysis, chemical reduction/oxidation) at a significant rate. In this case the labeled biomolecules detected could be derived from microorganisms that have assimilated the abiotic degradation products derived from the contaminant rather than the parent contaminant itself. Interpretation of SIP studies therefore needs to recognize and address the stability of the contaminant under investigation. Similarly, in some instances the microorganisms that are responsible for directly biodegrading a contaminant can excrete partially degraded metabolites. These metabolites can then be assimilated by secondary microorganisms that are otherwise unreactive towards the parent contaminant. This second process is called "cross-feeding". Cross-feeding can potentially strongly impact the interpretation of DNA/RNA-SIP studies which are typically used to identify organisms responsible for initiating the biodegradation of a contaminant. In contrast, cross-feeding has a much smaller impact if the aim of an SIP study is simply to demonstrate the biodegradability of a contaminant (e.g. PLFA-SIP).

Examples of several diverse applications of PLFA and DNA-SIP studies are provided in Table 7-1. This table is followed by a brief explanation of the major findings of several studies.

Table 7-1. Example SIP applications

Title	General information	Contaminants	EMDs used	Project life cycle stage
Hydrogenation Plant, Germany (see description below)	Anaerobic	BTEX	PLFA-SIP (CSIA)	Research (Natural attenuation)
Bioreactors for Ex Situ Treatment, NY (see description below)	Aerobic	TBA	DNA-SIP (PCR-DGGE)	Remediation Monitoring (Pump and treat)
RDX biodegradation in microcosms; Picatinny Arsenal, NJ (see description below)	Aerobic conditions	RDX	DNA-SIP PCR	Research (Fingerprinting)
Uranium Plant; Rifle, CO (see description below)	Anaerobic	Uranium	PLFA-SIP DNA-SIP (PCR-DGGE)	Feasibility/Research (Biostimulation)

Table 7-1. Example SIP applications (continued)

Title	General information	Contaminants	EMDs used	Project life cycle stage
SIP Case Study –Air Force Plant 44; Tucson, AZ (Appendix A.8)	Aerobic	TCE 1,1-DCE 1,4-dioxane	qPCR PLFA-SIP EAPs	Remediation Selection (Natural attenuation)
SIP Case Study– Fuel Compounds; NJ (Appendix A.9)	SIP was used to evaluate impacts of remediation and whether the remedial process could be sustained	Naphthalene	PLFA-SIP, RT-qPCR, qPCR	Remediation

Hydrogenation Plant, Natural Attenuation of Benzene

This study was conducted at the Zeitz aquifer in Germany. The site groundwater was largely anaerobic and contained high levels of dissolved benzene (~850 ppm) and toluene (50 ppm). A prior study at this hydrogenation plant (oil processing) site provided strong evidence for anaerobic toluene oxidation under sulfate-reducing conditions, but evidence for anaerobic benzene biodegradation was inconclusive. Bio-Traps® amended with ^{13}C-benzene (98% ^{13}C-enrichment) were deployed to conduct PLFA-SIP analyses of microorganisms present in several wells in the contaminated aquifer. An analysis of extracted PLFAs revealed substantial incorporation of ^{13}C into specific PLFAs for the ^{13}C-benzene-amended Bio-Traps®. However, this analysis was unable to further identify the organisms responsible for anaerobic benzene oxidation. Total PLFA measurements suggested a substantial microbial population (>10^7 cells/ bead) developed on Bio-Traps® amended with either toluene or benzene. Overall, the PLFA-SIP analysis provided strong evidence for anaerobic benzene oxidation that was not discernable through an analysis of the contaminant concentrations alone (Geyer et al. 2005).

Bioreactors for ex situ treatment of tertiary butyl alcohol

A DNA-SIP analysis using TBA (99% ^{13}C) was conducted on samples from several aerobic bioreactors designed to treat TBA-contaminated groundwater. In all cases, the reactors were self-inoculated with indigenous microorganisms present in the groundwater undergoing treatment. The study was designed to identify the native organisms responsible for TBA biodegradation on the reactor as a prelude to developing molecular probes to detect and quantify these native organisms in the groundwater environment. A PCR-DGGE analysis of 16S rRNA genes in ^{13}C-enriched DNA demonstrated that several TBA-metabolizing bacteria were present and active in these reactors and that these organisms that were similar but far from identical to other TBA-oxidizing organisms previously identified and characterized in pure culture. Another PCR-DGGE analysis of specific genes present in ^{13}C-enriched DNA demonstrated that several genes previously implicated in TBA oxidation in pure cultures were also highly conserved in the native TBA-oxidizing bacteria identified through DNA-SIP. Overall, the results of this study suggested that the full diversity of aerobic TBA-oxidizing organisms is large, although the enzymes and pathway of TBA oxidation may be highly conserved in these diverse organisms (Aslett, Haas, and Hyman 2011).

Microcosm-based ^{15}N-DNA-SIP Analysis of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)-Utilizing Microorganisms

In this study, ^{13}C-based SIP analyses were used to investigate the biodegradation of compounds that serve as carbon sources for microbial growth. In contrast, ^{15}N-DNA-SIP can be used to investigate the biodegradation of compounds that can serve as nitrogen sources for microorganisms. In these types of studies, both an ^{15}N-labeled compound and an unlabeled ^{12}C compound were supplied simultaneously as N and C sources, respectively. In the study described here, ^{15}N-DNA SIP was used to investigate the diversity of RDX-utilizing microorganisms in aquifer sediments and groundwater from Picatinny Arsenal. This site had a history of soil and groundwater contamination with explosives. Microcosms were supplied with cheese whey as a C source and ^{15}N-RDX as an N source. Fifteen 16S rRNA gene sequences were amplified from purified ^{15}N-DNA fraction and ten of these sequences were novel and unrelated to previously described RDX-degrading microorganisms. Six sequences of the *xplA* gene associated with RDX degradation were detected that were >96% similar to the *xplA* gene of a *Rhodococcus* strain previously shown to degrade RDX. It should be noted that when SIP is conducted for complex molecules with differing routes of metabolism, such as RDX (which may be used as a N or a C source), that it may not be possible to distinguish organisms performing an initial reaction on the parent molecule from those that incorporate C or N from metabolites. However, all of these organisms are part of the microbial community involved in degrading the compound in the environment (Roh et al. 2009).

Uranium Plant, Biostimulation of Uranium Reduction

This research project evaluated the use of Bio-Traps® (see Section 10.4 on sampling devices) amended with ^{13}C-acetate as a method to conduct PLFA-and DNA-SIP analyses aimed at identifying microorganisms responsible for the biological reduction of U(VI) to U(IV) (Lovley et al.1991, 1992). A variety of bacteria, including some sulfate-and iron-reducing organisms, can reduce U(VI) using different electron donors, including acetate. Conventional geochemical measurements indicated stimulation of anaerobic respiration (iron-, sulfate- and U- reduction) followed acetate amendment. A PCR-DGGE analysis of 16S rRNA gene sequences amplified from ^{13}C-enriched and total DNA suggested many members of the microbial community consumed acetate. Sequences similar to iron- (and U) reducing *Geobacter* sequences were identified in several samples close to the point of acetate injection and U reduction. Sequences representing sulfate-reducing bacteria were detected in samples down gradient from the acetate injection point. PLFA-fingerprinting conducted by GC/MS and determination of ^{13}C-incorporation into specific PLFAs using CSIA provided results that largely agreed with DNA-SIP results. Overall, DNA-SIP and PLFA-SIP analyses both demonstrated a widespread ability of native organisms to assimilate acetate. Moreover, both SIP analyses provided distinct evidence for changes in microbial community composition that were consistent with observed reduction of dissolved uranium at the site (Chang et al. 2005).

7.3 Data Interpretation

The application, analysis, and interpretation of microbiology-based EMD methods differ from typical soil and groundwater geochemical measurement in a number of ways. For example, microbial

biomarkers cannot easily be preserved, and sample handling and processing requires special care. The analysis of microbial parameters requires specific data quality considerations for sampling plans, sample collection and handling, quality control and laboratory procedures, and these are discussed in Section 10.0. Included below is a brief introduction to how SIP data are typically reported and some specific examples of how the data would be interpreted in answering the questions presented in Table 2-3.

7.3.1 How are the data reported?

Included in Table 7-2 below is information that should be provided in laboratory reports of SIP data including common laboratory report information, recommended information about the SIP method, and desirable information about the SIP method and results.

Additional information regarding sample handling and collection can be found Section 10.4 and Section 10.5

Table 7-2. Recommended and desirable information for SIP laboratory reports

Report information	Typical information or acceptable ranges
Common Laboratory Report Information	
Site Identifier	
Sample type or matrix	Groundwater, sediment, soil
Sample storage/transportation	4°C, overnight shipping, chain of custody forms
Sample handling methods	Filtering methods, filters used, sediment washing, volume of water filtered
Specific SIP Information	
Recommended	
Results	• DNA-SIP and PLFA-SIP: ^{13}C biomass enrichment, contaminant loss, terminal metabolite accumulation ($^{13}CO_2$, $^{13}CH_4$, DIC) • PLFA-SIP: Total biomass and community structure • DNA-SIP: physiological status, presence and abundance of target species or functional genes
Reporting Units	Varies depending on analyses selected: • DNA-SIP and PLFA-SIP: mass of contaminant per volume of water, mass of soil, or bead; metabolite accumulation ($^{13}CO_2$, $^{13}CH_4$, DIC) with ^{13}C δ values • PLFA-SIP: ^{13}C-enriched cells per volume of water, mass of soil, or bead; ^{13}C δ values; total cells per volume of water, mass of soil, or bead; percent of community structure; ratio of various fatty acids • DNA-SIP: gene copies per volume of water, mass of soil, or bead; identity and relative proportion of taxa identified
Typical Reporting Limits	• DNA-SIP and PLFA-SIP: GC limits for specific compounds • PLFA-SIP: 1,000 ^{13}C enriched cells per sample
Limit of Detection	Varies, should be adequate for the application
SIP Probe	Contaminant name, Isotope type (^{13}C, ^{15}N, ^{18}O), Isotopic composition (% enrichment/dilution)
Probe exposure	In situ (such as Bio-Trap®), ex situ (such as microcosm), time of incubation

Table 7-2. Recommended and desirable information for SIP laboratory reports (continued)

Report information	Typical information or acceptable ranges
Sample processing	
Method of quantification	
QA/QC information	Laboratory positive and negative control results
Results, Narrative of analyses	Indicating interferences or quality control issues encountered
Desirable	
DGGE	photographs or densitometric analysis
DNA	separation photographs
PLFA	profile (GC or GC/MS)
Laboratory Results	Number of duplicates and replicate results

Different SIP techniques report data differently. The data generated from a PLFA-SIP analysis can include an estimate of the total amount of biomass (determined from total PLFA amounts), the bulk ^{13}C enrichment of the total PFLAs extracted from a sample (reported as per mil or parts-per-thousand [‰]), the relative abundance of individual PLFAs, and the isotopic enrichment of individual PLFAs. Additional data can include the level of $^{13}CO_2$, ^{13}C-dissolved inorganic carbon, or $^{13}CH_4$ detected. In some studies involving Bio-Traps®, the amount of ^{13}C-enriched contaminant that has been degraded can also be reported based on the amount of ^{13}C-enriched contaminant remaining in the Bio-Trap® after deployment in the field. However, if amounts or rates of biodegradation are desirable from Bio-Trap® SIP studies, in addition to data on labeled PLFAs or microorganisms, adequate controls must be included in a study to estimate contaminant losses from the Bio-Traps® due to desorption and diffusion, especially if the contaminant under investigation is highly water-soluble (such as MTBE, 1,4-dioxane).

A measurement of total PLFAs can provide an estimate of the numbers of microorganisms present in an environmental sample, even though this method does not rely on the use of isotopically-enriched contaminants. Changes in the total amount of PLFAs can be directly related to changes in the size of a microbial population. The detection of elevated (above background) levels of ^{13}C in either bulk PLFAs, individual PLFAs or terminal products (CO_2, DIC, CH_4) are all unequivocal indicators that biodegradation of the ^{13}C-enriched contaminant has occurred under the conditions examined in the study. However, the level of ^{13}C-enrichment in these analytes is strongly dependent on at least two issues:

- the initial level of ^{13}C enrichment of the labeled contaminant (e.g.,percent of ^{13}C-MTBE versus ^{12}C-MTBE)
- the background levels of biodegradable carbon within the sample

The effect of the level of ^{13}C-enrichment in the labeled contaminant is straightforward. If a labeled contaminant contains only low levels of ^{13}C-enrichment, a high level of ^{13}C-enrichment in PLFAs or terminal products will indicate extensive biodegradation of the contaminant. Conversely, lower levels of biodegradation would be inferred if low levels of ^{13}C-enrichment in PLFAs or terminal products are observed when using contaminants that are highly enriched with ^{13}C. The effect of

background contaminants (either the contaminant of interest or co-contaminants with similar core structures, such as benzene and toluene) is more complex, as concurrent biodegradation of these predominantly ^{12}C compounds can dilute signals from ^{13}C-enriched contaminants. For example, if a sample contains a high level of the contaminant of interest, the incorporation of ^{12}C from the background contaminant into biomolecules or terminal products will effectively decrease the ^{13}C signal obtained from the concurrent biodegradation of the ^{13}C-enriched version of the contaminant. Knowledge of the background levels of contaminants and the concentration of contaminant probe used in an SIP study are therefore important considerations in data interpretation.

The results of DNA/RNA-SIP studies typically include identification of the "active" microorganisms detected in a sample based on an analysis of PCR-amplified 16S rRNA genes (such as DGGE). These identifications are based on comparisons of 16S rRNA gene sequences to sequences available in national databases. Other data might include estimates of the specific or relative abundance of individual organisms or functional genes based on fingerprinting (T-RLFP) analysis of purified ^{13}C-enriched DNA. Additional data could include the level of $^{13}CO_2$, ^{13}C-dissolved inorganic carbon or $^{13}CH_4$ detected.

In SIP studies of contaminant biodegradation, the similarity between 16S rRNA gene sequences is often used to infer the physiological capabilities of microorganisms detected within a sample by comparing them to the sequences of organisms that have been physiologically characterized in pure culture. For example, detecting a 16S rRNA gene sequence that is similar to *Desulfovibrio* sequences likely implies the detected organisms are also sulfate reducing bacteria (SRB). This type of comparison becomes less reliable when investigations focus on individual organisms with specific capabilities. For example, different *Dehalococcoides mccartyi* strains can have different contaminant-degrading capabilities although they may have very similar, if not identical, 16S rRNA gene sequences. Currently, a working rule of a minimum of 97% sequence identity based on a comparison of the entire 16S rRNA gene sequence (~1540 base pairs) is regarded as a the threshold for identifying similar species. However, DGGE-based analyses often use much shorter DNA fragments (≤650 base pairs) for species comparisons and even full sequence similarity does not ensure that the two organisms under comparison have the same contaminant-degrading capabilities.

7.3.2 How are the data interpreted?

For the most part, SIP techniques are not inherently quantitative and their primary use in contaminant biodegradation studies is to demonstrate whether the contaminant biodegrades under a given set of environmental conditions. More elaborate studies can refine this information and identify which organisms are responsible for any observed biodegradation activity. To illustrate interpretation of SIP results, each question relevant to SIP in Table 2.3 is discussed.

7.3.2.1 Site Characterization

A) Are contaminant-degrading microorganisms present?

In most cases, SIP analyses examine the biodegradation of organic compounds that are used by microorganisms as carbon and energy sources. Consequently, SIP would not be appropriate to

determine the role of microorganisms in the fate of contaminants that are biologically transformed primarily through their use as electron acceptors (for example chlorinated solvents, PCBs, metals and others because organisms do not incorporate C or N into their DNA, RNA or lipids during this process). However, PLFA-SIP studies using field-deployed Bio-Traps® could be used to determine whether an important specific petroleum hydrocarbon (such as benzene) can be biodegraded at a site. PLFA-SIP can also determine what environmental modifications (such as addition of alternative electron acceptors) might promote the biodegradation of petroleum hydrocarbon compounds. Microcosm-based DNA-SIP studies could also be used identify native microorganisms responsible for the biodegradation of more unusual compounds such as explosives (RDX or TNT) or emerging contaminants like 1,4-dioxane. Like many other explosives, RDX is a nitrogen-containing contaminant. ^{15}N-DNA-SIP could be used to determine whether RDX or other explosive compounds can be biodegraded. The organisms detected by this approach can be identified as capable of using explosive-derived nitrogen, but they are not necessarily directly biodegrading the explosives as growth-supporting carbon sources.

B) Are contaminant-degrading microorganisms active?

Both PLFA- and DNA-SIP intrinsically rely on the metabolic activity of specific contaminant-degrading organisms. A positive signal from either approach would confirm the activity of bacteria capable of biodegrading the specific contaminants used as SIP probes. These contaminants would include petroleum hydrocarbons, and possibly other compounds such as explosives, propellants, emerging contaminants. Typically SIP techniques would not be appropriate for demonstrating active biodegradation of contaminants that are typically used as terminal electron acceptors (such as chlorinated solvents, PCBs because, as previously noted, organisms do not incorporate C or N into their biomolecules during this process).

C) Are the microorganisms capable of complete degradation?

SIP approaches can be used to answer this question in two ways. First, if a specific organism or type of organism that completely degrades (mineralizes) a specific contaminant as a carbon source is already known, then either PLFA- or DNA-SIP can be used to demonstrate the presence and activity of that type of organism at a site. The second alternative does not involve an analysis of the traditional isotopic enrichment of biomolecules such as lipids or nucleic acids but rather examines the isotopic composition of terminal carbon-containing microbial metabolites, such as CO_2 and CH_4. Complete microbial degradation of a contaminant implies that biodegradation proceeds to the level of CO_2 or CH_4. The isotopic composition of these gases (the ratio of $^{13}C/^{12}C$ in CO_2) can be readily determined and quantified by GC/MS and these data can be used to determine whether full or substantial mineralization of a ^{13}C-enriched contaminant has occurred. This is the less direct and precise of the two methods for determination of complete degradation, and this approach requires prior knowledge of both degradation pathways and the stoichiometry of CO_2 or CH_4 production.

D) Is biodegradation occurring?

Both PLFA- and DNA-SIP intrinsically rely on the metabolic activity of specific contaminant-degrading organisms. A positive signal from either approach would unequivocally confirm the activity of bacteria capable of biodegrading the specific contaminant.

7.3.2.2 Remediation

H) Are numbers of contaminant-degrading microorganisms and/or genes changing?

This question can be answered by using PLFA-SIP and assuming that the contaminant under consideration acts as a carbon and energy source for the contaminant-degrading organisms at a site. A time series of field-deployed Bio-Traps® using a ^{13}C-enriched contaminant would enable a study of the changes in the microbial community over time. This approach would detect a change in the number of contaminant-degrading microorganisms through changes in the PLFA profile and the relative abundance of specific ^{13}C-enriched PLFAs.

I) Is the remediation strategy affecting the numbers or types of contaminant-degrading microorganisms?

This question can be answered by using PLFA-SIP and assuming that the contaminant under consideration acts as a carbon and energy source for the contaminant-degrading organisms at a site. A time series of field-deployed Bio-Traps® using a ^{13}C-enriched contaminant would enable a study of the changes in the microbial community over time. This approach would detect a change in the number of contaminant-degrading microorganisms through changes in the PLFA profile and the relative abundance of specific ^{13}C-enriched PLFAs.

M) Is biodegradation occurring?

Both PLFA- and DNA-SIP intrinsically rely on the metabolic activity of specific contaminant-degrading organisms. A positive signal from either approach would confirm the activity of bacteria capable of biodegrading the specific contaminant used as an SIP probe.

N) What is the rate of biodegradation?

Estimates of biodegradation rates can be determined from some common PLFA-SIP applications using Bio-Traps®. These estimates are based on the amount of SIP probe depletion from the Bio-Trap® matrix as well as the accumulation of terminal metabolites such as CO_2 and methane. These rate estimates are not based on incorporation of ^{13}C from the labeled contaminant into microbial biomass, but rather on loss of the labeled contaminant from a Bio-Trap® and extracellular accumulation of labeled metabolites. As previously noted, if a rate determination is needed, controls must be used to account for losses due to abiotic processes, particularly desorption from the Bio-Trap® media.

7.3.2.3 Monitoring

O) Does the microbial community composition support the remediation strategy?

The use of PLFA-SIP using field-deployed Bio-Traps® can partially answer this question on a contaminant-specific basis. The principal limitation of PLFA analysis is that physiologically distinct types of bacteria can produce the same phospholipids. Unless there are distinctive PLFAs

associated with microorganisms responsible for a particular activity, the resolution of PLFA-SIP is limited.

P) Do contaminant-degrading microorganisms continue to be sufficiently abundant?

A time series of field-deployed Bio-Traps® using a ^{13}C-enriched contaminant and PLFA-SIP would enable a study of the changes in the microbial community over time. This approach could detect a change in the number of contaminant-degrading microorganisms through changes in the PLFA profile and the relative abundance of specific ^{13}C-enriched PLFAs.

Q)Are contaminant-degrading microorganisms remaining active?

Both PLFA- and DNA-SIP intrinsically rely on the metabolic activity of specific contaminant-degrading organisms. A positive signal from either approach would confirm the activity of bacteria capable of biodegrading the specific contaminant used as an SIP probe.

U) Is biodegradation occurring?

See response in Question M.

V) What is the rate of biodegradation?

See response in Question N.

7.3.2.4 Closure

Some variability in site closure requirements exists among states and programs. However, in many situations, EMD data could serve as an additional line of evidence for understanding what processes are important in reducing concentrations and reaching the applicable closure levels. The evidence provided by EMD data would reveal whether biodegradation processes are occurring, have sufficiently proceeded, and are likely to continue. The following questions can be addressed for site closure.

W) Is contaminant degradation likely to continue?

Both PLFA- and DNA-SIP intrinsically rely on the metabolic activity of specific contaminant-degrading organisms. A positive signal from either approach would suggest the activity of bacteria capable of biodegrading the specific contaminant used as an SIP probe would continue. A time series of field-deployed Bio-Traps® using a ^{13}C-enriched contaminant would enable a study of the changes in the microbial community over time and support and assessment of the likelihood of degradation continuing.

Y) Is biodegradation occurring?

See Question M.

Z) What is the rate of biodegradation?

See Question N.

7.3.3 Practical considerations

If the exposure of a sample to a ^{13}C-enriched contaminant probe occurs in a laboratory microcosm-based system, biodegradation rates can be determined, especially if continuous sampling for contaminants or terminal products is possible. Under these circumstances, SIP studies should include replicate samples, control samples with unenriched contaminant, as well as suitably poisoned controls. Samples should also be analyzed at locations within and outside the area impacted by the contaminant.

Since SIP involves analysis of biomolecules, particularly phospholipid fatty acids and DNA, avoid contamination of samples with other microorganisms and store samples so that the biomolecules present in the sample are not destroyed or altered until they have been extracted. For example, field samples should be stored and transported on ice (but not frozen) and extracted promptly. During extraction of DNA from environmental samples, all forms of DNA will be collected in the extract (including DNA present in microbial cells, as well as potentially large amounts of free DNA released from dead organisms). While free DNA from dead cells can be problematic for some molecular studies, DNA-SIP focuses on the analysis of ^{13}C-enriched DNA obtained from living cells that have grown on the ^{13}C-enriched contaminant probe. Free DNA present in the sample under analysis will be predominantly ^{12}C-enriched and will therefore not be detected. This may not be true of other EMDs that simply analyze total DNA extracted from a sample.

Extraction efficiency of the biomolecule under investigation, is important since the concentration of ^{13}C-enriched contaminant and micronutrients in microcosm-based SIP analyses can affect PLFA-SIP and DNA/RNA-SIP results. While chloroform/methanol extraction is a well-characterized process for extracting microbial lipids, the efficiency of DNA extraction from soils and other media can be highly variable, even when using commercial kits. Sequential extractions can greatly increase the overall yield of DNA, although the composition of the microbial community reflected in each extraction step does not significantly alter the overall species diversity detected by DNA-SIP (Jones et al. 2011).

Another important consideration is the concentration of ^{13}C-enriched contaminant used in SIP studies. If samples are exposed to abnormally high concentrations of contaminants, the detected microorganisms may not reflect the organisms capable of degrading the contaminant at lower, more relevant in situ concentrations. At best, this effect may provide misleading information about which organisms are involved in a particular biodegradation process. At worst, it may suggest that a contaminant that does not biodegrade at low concentrations actually does biodegrade at the higher concentrations used in the SIP analysis. The inclusion of micronutrients in microcosm-based SIP analyses can also alter the biodegradability of a contaminant because the nutrients may be limiting in the environment from which the sample was taken.

7.4 Additional Information

7.4.1 Method Details

All PLFA-SIP studies involve three key sequential steps:

1. exposure of a microbial community in the field or laboratory to an individual isotopically enriched contaminant (usually ^{13}C-enriched)
2. subsequent extraction of the total phospholipids from a sample using standard chemical extraction techniques
3. characterization of the level of ^{13}C enrichment in either total or phospholipid fatty acids. Depending on the protocol that is used, these studies can also provide further evidence for biodegradation based on the production of terminal products such as $^{13}CO_2$ or $^{13}CH_4$.

In many instances PLFA-SIP studies have been conducted in the field using Bio-Traps® (see EMD Sampling Methods Fact Sheet and Section 10.4.5 for more information concerning the use of Bio-Traps) amended with ^{13}C-enriched contaminants. Total lipids are extracted from samples using chloroform/methanol and then converted to fatty acid methyl esters (FAMEs) using tri-methylchlorosilane. Absolute concentrations of PLFAs are determined by GC/MS using spiked internal standards. Membrane-derived PLFAs are obtained from total lipid extracts by separation into neutral, glycol, and polar fractions. The polar fractions (PLFAs) are then converted to fatty acid methyl esters (FAMEs) using trimethylchlorosilane. Individual FAMEs are then quantified by GC/MS analysis. The ^{13}C-enrichment of individual PLFAs is determined using GC-IRMS. Production of $^{13}CO_2$ or $^{13}CH_4$ is also determined by GC/MS analysis. Additional details on methods used for SIP are provided in Busch-Harris et al. 2008.

One important constraint on the use of DNA/RNA-SIP in field studies of contaminant biodegradation is that this form of SIP requires contaminants with high levels of isotopic enrichment. In the case of ^{13}C-enriched compounds, this requirement comes from the need to separate ^{12}C- and ^{13}C-labeled forms of nucleic acids using density gradient centrifugation. The degree of separation of isotopically distinct nucleic acids is directly impacted by their level of ^{13}C-enrichment. This separation and the value of the information derived from this technique is maximized with high levels of ^{13}C-enrichment (as high as 100% ^{13}C). In general, the cost of even simple and widely available ^{13}C-enriched compounds (e.g. $^{13}CH_4$) increases as the level of enrichment increases. Less common organic compounds with the high levels of ^{13}C-enrichment often require custom synthesis, and these compounds can therefore be expensive. Because field SIP studies often require relatively large amounts of labeled compounds, DNA/RNA-SIP has most often been used in laboratory-based settings using microcosms containing appropriate samples (soil, water) and the isotopically-enriched contaminant. After biodegradation of the contaminant has been observed, total DNA is typically extracted using commercial kits (such as the PowerMax® Soil DNA isolation kit from MoBio Laboratories, Inc, Carlsbad, CA). ^{12}C-DNA is then separated from ^{13}C-DNA by CsCl density gradient (140,000 x g for 69 hours at 20°C). Separated DNA fractions are visualized with UV light and removed from each centrifuge tube using sterile needles or by displacement and fraction collection. The resulting DNA is then isolated from each fraction by n-butanol extraction and

ethanol precipitation. Purified ^{12}C- and ^{13}C-DNA samples from CsCl gradients are then typically used as templates in PCR reactions and subsequent analyses by DGGE or other fingerprinting methods.

7.4.2 Permitting and Regulatory Issues Specific to SIP

Since in situ SIP techniques involve the introduction of a small amount of stable-isotope labeled organic contaminant (generally mg quantities) into the subsurface, regulatory agencies may have specific regulatory requirements (see Section 11.0) for SIP above and beyond the traditional work plan approval process. Involve the appropriate regulatory agency early in the site investigation/remediation process, and ensure that the regulatory agency has a good understanding of the SIP technique to be used at the site. Since SIP is a relatively new technology to site investigation/remediation, the regulatory requirements may vary widely, with some regulators incorporating the approval of SIP within the overall project approval process, while other regulators may have a separate permitting process.

7.4.3 Variations or newer methods that the user may encounter

Various recent modifications to the SIP techniques have been described in the scientific literature and these predominantly focus on DNA-SIP rather than PLFA-SIP.

One variation of DNA-SIP combines the conventional sample exposure to ^{13}C-labeled contaminants and subsequent separation of ^{12}C- and ^{13}C-enriched DNA with a subsequent quantitative fingerprinting procedure based on terminal restriction fragment length polymorphism (T-RFLP) analysis. Following purification of ^{13}C-enriched DNA, a TaqMan®-based qPCR amplification of 16S rRNA genes is conducted using both fluorescently-labeled primers (needed to generate terminally-labeled amplicons for T-RFLP analysis) and a fluorescently-labeled probe (for quantitative aspect of the PCR amplification). This combined analysis enables the total number of copies of the gene to be determined in a sample (for example, 16S rRNA gene copies/ml of sample) based on the fluorescence generated from the PCR amplification. The resulting terminal fluorescently labeled PCR amplicons are then digested as part of a conventional T-RFLP analysis. This analysis enables the relative abundance of individual ribotypes to be determined in the original ^{13}C enriched DNA. If the individual T-RFLPs can be matched to individual 16S rRNA gene sequences, this type of analysis can provide a quantitative estimate of the relative contribution of individual types of organisms to specific biodegradation processes.

A second modification of DNA-SIP addresses the fact that conventional DNA-SIP analyses often use concentrations of ^{13}C-enriched contaminants that are far in excess of ambient or environmentally relevant concentrations. In practice, these artificially high contaminant concentrations are used because lower concentrations do not generate sufficient ^{13}C-enriched DNA for further molecular characterization. This is especially true when the objective of the study is characterize not only 16S rRNA genes (which can be amplified by PCR using specific primers) but also other unknown genes for which primers are not known. To generate sufficient DNA for these types of metagenomic analyses, ^{13}C-enriched DNA obtained from DNA-SIP incubations conducted with

low concentrations of ^{13}C-enriched contaminants can be amplified using multiple displacement amplification (MDA). This type of amplification does not use the combination of gene-specific primers and a thermotolerant DNA polymerase used in conventional thermocycling PCR but instead relies on the room temperature activity of a DNA polymerase from a bacterial virus (bacteriophage Φ 29), that replicates long strands of new DNA using random primers. This type of approach may be particularly useful in future DNA-SIP-based studies of contaminant biodegradation if there are concerns that the high concentrations of contaminants used in some more conventional studies detect microorganisms that are not representative of those active in environments containing low contaminant concentrations (see Dumont and Murrell 2005).

Techniques involving unstable (radioactive) isotopes: By definition SIP techniques do not use contaminants that have been enriched with intrinsically unstable (radioactive) isotopes. However, radioactive isotopes have been used for a long time in biodegradation studies and historically have two predominant uses. First, the most frequent use of radiolabeled compounds have been studies in which individual contaminants that have been synthesized to contain radioactive isotopes such as ^{14}C can be used to detect biodegradation processes in microcosm-type studies. These studies can be conducted with very low contaminant concentrations because there are very sensitive techniques such as liquid scintillation counting that can be used to detect terminal biodegradation products such as $^{14}CO_2$ or $^{14}CH_4$.

The second and less frequent type of study uses the same detection techniques to discriminate between the biodegradation of "modern" ^{14}C-containing organic materials and chemicals that either contain, or are derived from, "radiocarbon dead" carbon sources. This second application relies on the fact that like ^{13}C, there is a small and relatively constant amount of ^{14}C in the overall pool of biologically available CO_2. Organisms that either directly (plants) or indirectly (animals) obtain carbon from atmospheric CO_2 always contain low and constant but readily detectable amount of ^{14}C. While the organism is alive, this carbon is continuously replaced while it also undergoes radioactive decay. However, when these organisms die, they cease to assimilate new carbon while the ^{14}C already present in these organisms undergoes radioactive decomposition. As this radioactive decay occurs with a precise half-life, the level of remaining radioactive ^{14}C can be used to date the age of an organic material (radiocarbon dating). Petroleum hydrocarbons are mainly derived from decayed plant and animal remains and typically contain no discernable residual ^{14}C because they were formed sufficiently long ago that the vast majority of the original ^{14}C in these materials has undergone radioactive decay. These compounds, and materials derived from these compounds, are therefore regarded as "dead" in terms of radioactivity. When these radiocarbon dead materials are biodegraded in the environment, the terminal metabolites generated from these materials (e.g. CO_2 or CH_4) also reflect this lack of radioactivity. Consequently, in environments that are impacted by petroleum hydrocarbons, ongoing biodegradation of these compounds can be determined by examining the level of radioactivity in terminal products such as CO_2 or CH_4. When petroleum biodegradation is occurring, the level of ^{14}C in these terminal products will be low compared to the levels detected when "modern" organic materials are undergoing biodegradation. In contrast, if petroleum hydrocarbon biodegradation is not occurring, the levels of radiocarbon detected in these ter-

minal products will be higher and comparable to the levels encountered when modern organic compounds are being biodegraded.

Radioactive materials can be used to study other specific biodegradation processes by combining autoradiography with various EMDs. For example, microautoradioography can be combined with FISH by first exposing a sample to a radiolabeled compound that can be biodegraded. Using the same principle exploited in SIP approaches, the radioactive elements derived from the radiolabeled contaminant are incorporated into newly synthesized biomolecules. The organisms that have assimilated the radiolabeled probe can be localized by fixing them on a microscope slide that is then treated with a autoradiography emulsion. The presence of silver grains generated by radioactive decay can be detected by microscopy and the identity of these organisms can then be detected by conducting a conventional FISH analysis. This type of analysis can enable researchers to identify specific microorganisms with specific metabolic capabilities. A drawback of this technique compared to ^{13}C-DNA-SIP is that the technique requires the use of radiolabeled compounds and the detection of metabolically active organisms is limited by the availability of appropriate radioactive contaminants and appropriate FISH DNA probes. A second variant of this approach is called an isotope array (a type of microarray). In this technique a sample is incubated with a radiolabeled contaminant (often ^{14}C). After exposure, total RNA is extracted from the sample and then labeled with a fluorescent dye. The total RNA is then hybridized with a DNA array that contains DNA probes for specific 16S rRNA genes. The range of microorganisms that are present in the sample can be determined from an analysis of the hybridization of the fluorescently-labeled RNA. The subset of microorganisms that directly metabolized the radiolabeled contaminant can then be determined from an autoradiogram of the DNA array. Again, the limitations of this approach are that the techniques requires the use of radiolabeled contaminants and the diversity of organisms that can be detected is limited by the range of DNA probes included on the microarray.

7.4.4 Additional Information

Further reading specific to SIP is provided in Appendix F.

8.0 ENZYME ACTIVITY PROBES (EAPS)

8.1 Summary of EAPs

Enzymes are typically proteins and they are responsible for catalyzing all of the biochemical reactions brought about by microorganisms. Enzyme-catalyzed reactions all convert one or more starting compounds (substrates) into one or more products. However, enzymes are rarely absolutely specific and they can often transform compounds other than their physiologically relevant substrate. The enzyme activity probes (EAPs) capitalize on this lack of enzyme specificity and act as alternative or surrogate substrates for specific enzymes involved in contaminant degradation processes. EAPs are useful because they can be added to environmental samples, where they are transformed by their target enzymes into distinct and readily detectable products. These products can be detected through fluormetric, colormetric or analytical methods, which determine if there is a positive response. Most contaminant-degrading enzymes are only active in intact microbial cells, so a positive response to an EAP can indicate the presence of microorganisms that possess active forms of the target enzyme. A positive response also suggests that contaminant biodegradation is therefore possible at the site and may even indicate biodegradation is ongoing.

An important distinction in EAPs is that the EAPs themselves are not catalyzing reactions (the target enzymes do that), EAPs are chemicals that are transformed by the target enzymes (which catalyze the reactions) and generate specific products that are assessed through fluorometric, colormetric, or analytical methods. Another question might be are EAPs transformed by non-target enzymes to generate the same products? For the EAPs discussed herein, there is no evidence that non–target enzymes will transform the substrate(s) into the same detectable products.

EAPs rely on the metabolic activity of single cells present in the subsurface. As such, any geochemical or environmental condition that could impact the overall physiological status or activity of cells could directly impact EAPs and include metrics as pH, temperature, and redox conditions. In particular, metals and other chemicals if present at high enough concentrations, have the potential to inhibit the metabolic activity, and the detection of activity with EAP, of target cells. The concentration of organic matter does not seem to impact the detection of activity with EAPs. For example, at several contaminated sites where the concentration of organic matter is below quantifiable amounts (for instance, large oligotrophic plumes), EAPs will detect active oxygenase enzymes. Most EAP inhibitors are other chemicals that should not be found at high enough concentrations in groundwater (such as acetylene and 1-pentyne). In cases where multiple contaminants are co-mingled, those contaminants which provide the cells with carbon and energy for growth will likely out-compete the EAPs but will not inhibit them. In general auto fluorescence and other background fluorescence which occurs naturally in groundwater, surface water and in soils and sediments can be problematic with some EAPs.

Additional introductory information is available in the EAP Fact Sheet. Figure 8-1 illustrates the analytical process for EAPs.

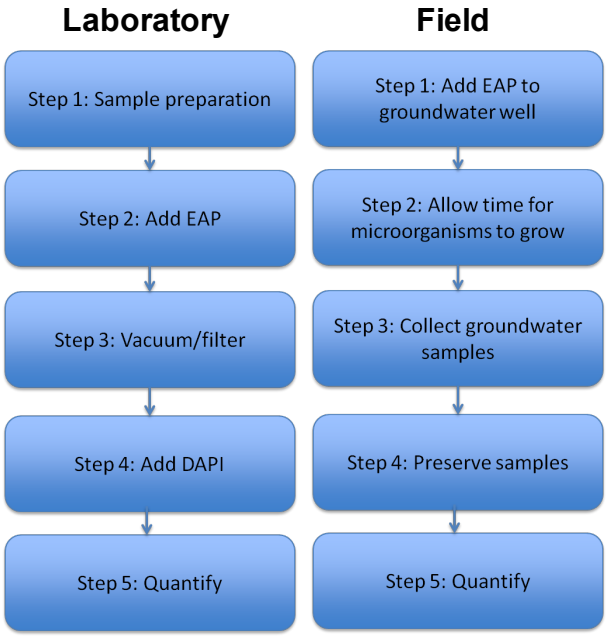

Figure 8-1. EAP Process Flow Diagram

8.2 Applications

Microbiologists used various forms of EAPs for decades prior to applying these methods to detecting contaminant-degrading microorganisms. For example, the acetylene reduction assay (a long-standing EAP) can quantify aspects of the nitrogen cycle (Dilworth et al. 1966; Hardy et al. 1968, 1973; Stewart et al. 1967). Some bacteria can reduce atmospheric nitrogen gas (N_2) into ammonia (NH_3), which is then used as a nitrogen source to support growth (Bergersen 1970). This process is of central importance to the biological nitrogen cycle. The activity of nitrogenase, the key enzyme responsible for this activity, can be determined by its ability to reduce acetylene (C_2H_2) to ethylene (C_2H_4) by gas chromatography. This quantitative analysis has been used in numerous studies of the environmental distribution of nitrogen-fixing microorganisms (Hardy et al. 1968, 1973; Howarth et al. 1988).

Over the last thirty years, several EAPs have been developed for enzymes involved in anaerobic and aerobic contaminant biodegradation processes. These EAPs have subsequently been used to evaluate biodegradation at sites with contaminants including chlorinated solvents and petroleum hydrocarbons. Table 1 in the EAP Fact Sheet lists several currently recognized EAPs for various oxygenase and dehalogenase enzymes involved in specific biodegradation processes. Note that EAPs are enzyme specific rather than contaminant specific. For instance, coumarin is an EAP used to detect the activity of soluble methane monoooxygenase (sMMO) found in methane-oxidizing (methanotrophic) bacteria. sMMO can oxidize a wide range of pollutants including chlorinated solvents such as TCE and ethers such as 1,4-dioxane. The same contaminants can also be oxidized by some of the several forms of toluene monooxygenase found in aerobic toluene-oxidizing bacteria. These enzymes are detected using a different type of EAP (such as phenylacetylene) even

though the contaminants degraded by these enzymes can be the same as those degraded by sMMO.

Examples of diverse applications of EAPs are provided in Table 8-1. A brief explanation of several key studies and their findings follows the table.

Table 8-1. Example EAP applications

Title	General information	Contaminants	EMDs	Project life cycle stage
Test Area North, Idaho National Laboratory, ID (see summary below)	Evaluated aerobic co-metabolism targeting aerobic oxygenases using the following EAPs: coumarin, hydroxyphenylacetylene, 3-hydroxy-phenylacetylene	TCE	EAP, CSIA, PCR, qPCR	Remediation
Chemical Manufacturing Plant, CA (see summary below)	Evaluated anaerobic metabolism targeting TCE reductase using the TCFE EAP	TCE	EAP, PCR, SIP	Site Characterization
Former Cement Company, NY (see summary below)	Evaluated aerobic co-metabolism targeting aerobic oxygenases using the following EAPs: coumarin; naphthalene, hydroxyphenylacetylene, 3-hydroxy-phenylacetylene; trans-cinnamonitrile	1,1,1-TCA; TCE	EAP, PCR, qPCR	Site Characterization, Remediation
SIP Case Study - AFP 44, AZ (see Appendix A.8)	Evaluated aerobic co-metabolism targeting aerobic oxygenases with the following EAPs: coumarin, hydroxyphenylacetylene,3-hydroxy-phenylacetylene	TCE	SIP, qPCR, EAP	Site Characterization, Remediation
EAP Case Study - Paducah Gaseous Diffusion Plant, KY (see Appendix A.7)	Evaluated aerobic co-metabolism targeting aerobic oxygenases using the following EAPs: coumarin, hydroxyphenylacetylene, 3-hydroxy-phenylacetylene, trans-cinnamonitrile	TCE	EAP, CSIA, qPCR	Site Characterization, Remediation

Test Area North, Natural Attenuation of TCE through Aerobic Co-metabolism

This research project was conducted at the TCE-contaminated Test Area North (TAN) site at the Idaho National Laboratory. EAPs and other EMDs (see Section 9.2) were used to investigate the co-metabolic oxidation and natural attenuation of TCE by methane-oxidizing bacteria using coumarin as an EAP to detect sMMO activity (Wymore et al. 2007) and by aromatic-degrading bacteria using phenylacetylene and 3-hydroxy-phenylacetylene (M. H. Lee et al. 2008). Groundwater samples were obtained from various depths. Aerobic oxidation analyses were conducted with a suite of EAPs, either directly on groundwater or using or cells concentrated by filtration.

For the coumarin analysis, the generation of fluorescent coumarin-derived oxidation products (such as 7-hydroxycoumarin) was determined using a fluorescence spectrophotometer (data recorded as relative fluorescence units, RFU, over background values). For the aromatic oxygenases analyses, the number of cells, active enzymes, that transformed the EAP into quantifiable fluorescent products was determined by microscopy (data recorded as cells by volume or weight). Fluorescent product generation was widespread among the samples examined and was typically decreased by selective inhibitors. The presence of active enzymes was confirmed using other EAPs, enrichment cultures of bacteria with oxygenases enzymes, and PCR-based analyses for distinctive genes involved in aerobic bacterial oxidation. Collectively the results of these various analyses support the suggestion that both methane- and aromatic-oxidizing bacteria are present and active in the groundwater at the TAN site and are contributing to the natural attenuation of TCE at this site.

Chemical Manufacturing Plant, In situ Evaluation of TCE Reduction

Trichlorofluoroethylene (TCFE) is an EAP that can be used to determine the activities of PCE- and TCE-reducing microorganisms. This particular EAP is useful because the fluorine atom is not removed during reductive dehalogenation of this compound. In this study, TCFE was used to determine rates of chlorinated solvent reduction (PCE and TCE) at a former chemical manufacturing plant. A push-pull test system was used to introduce TCFE into groundwater. Samples were recovered over time (≤3 months) and were analyzed by GC/MS. Fluorinated reduction products were generated from TCFE in TCE-contaminated portions of the site but not in uncontaminated areas. The results demonstrated that TCFE biodegradation occurred at comparable rates to TCE. Furthermore, TCFE was also shown to have similar in situ transport properties to TCE (Hageman et al. 2001).

Former Cement Company, Natural Attenuation of Co-mingled Plume of 1,1,1-TCA, TCE, and PCE

Aerobic oxygenase EAPs were applied to soil cores from a former cement factory in upstate New York. Historical contaminants at the site included 1,1,1-TCA, petroleum hydrocarbons, and chlorinated solvents. Carbon in the form of methane and the petroleum hydrocarbon contaminants were present across the site and suspected to be stimulating the aerobic oxygenases in situ. EAPs were applied to six soil cores from within and outside the contaminated area and sampled for at least two depths, specifically above the mapped contaminated zone. The methods for analyzing the samples were similar to the example above, except cells were first washed from the solid materials prior to exposure to the EAPs. Fluorescence was recorded in all of the samples, either in solution (RFUs) for the methane monooxygenase or enumerated by microscopy (active cells per g of soil) for aromatic oxygenases.

The presence of active enzymes was confirmed by inhibition analyses and qPCR amplification of target enzymes. Inhibition analyses were conducted to verify that EAP products formed were from reactions with sMMO and not other oxygenase activity. Assays using phenylacetylene and methane were conducted as described in Wymore et al. 2007. The methane study is particularly important because it is a reversible inhibitor; once the enzyme is saturated with methane, it will not be able to transform the EAP, however when the methane is removed, the cells react equally with the

EAP and methane and a fluorescent product is measured. Collectively the results suggested that aerobic oxidizing bacteria were present and active in the soils at the site and likely involved in the attenuation of the contaminants, thus minimizing vapor intrusion issues at the location.

8.3 Data Interpretation

The application, analysis, and interpretation of microbiology-based EMD methods differ from typical soil and groundwater geochemical measurement in a number of ways. For example, microbial biomarkers cannot easily be preserved, and sample handling and processing requires special care. The analysis of microbial parameters requires specific data quality considerations for sampling plans, sample collection and handling, quality control and laboratory procedures, and these are discussed in Section 10.0. Included below is a brief introduction to how EAP data are typically reported and some specific examples of how the data would be interpreted in answering the questions presented in Table 2-3.

The range of EAPs that have been developed to date is focused mainly on enzymes involved in aerobic oxidation of contaminants and reductive dehalogenation of chlorinated ethenes. Currently no EAPs exist for investigating metal-transforming microbial processes or microbial process directed at explosives, PCBs, or flame retardants. EAPs would not be appropriate for determining the presence of organisms capable of transforming these types of contaminants. Information for EAP data quality is included in Section 10.0.

8.3.1 How are the data typically reported?

EAPs used in laboratory analyses are often fluorescent. In some EAP applications, cells that transform an EAP internally accumulate fluorescent products and can then be enumerated by epifluorescent microscopy. The number of fluorescent cells is then compared to the total number of cells stained with a universal DNA-reactive stain such as acridine orange (AO) or DAPI (4.6-diamindino-phenylindole). These types of EAP results are typically presented as either relative fluorescence units (RFU) or the number of target cells (fluorescent cells) per volume of groundwater or per weight of soil. Results can also be presented as the fraction of the total cells that are active (active cells/total cells) and recorded as the percent of total. In other cases, the fluorescent products are detected in the reaction medium and can be used to determine relative rates of EAP transformation. These results can then be correlated with other independent approaches for determining cell numbers or the abundance of the genes that encode the target enzyme in the sample. Field applications of EAPs that target contaminant-degrading enzymes are relatively limited and have focused on chlorinated solvents such as TCE. In these studies, the rate of reduction of compounds such as TCFE can be stated and compared to site-specific rates of TCE reduction.

Included in Table 8.2 below is information that should be provided in laboratory reports of EAP data including common laboratory report information, recommended information about the EAP method, and desirable information about the EAP method and results.

Additional information regarding sample handling and collection can be found in Section 10.0, the data quality section.

Table 8-2. Recommended and desirable information for EAP laboratory reports

Report information	Typical information or acceptable ranges
Common Laboratory Report Information	
Site Identifier	Location, depth
Sample type or matrix	Groundwater, sediment, soil
Sample storage/transportation	4°C, overnight shipping, chain of custody forms
Sample handling methods	Filtering methods, filters used, sediment washing, volume of water filtered
Specific EAP Information	
Recommended	
Results	Fraction of the total cells that are active; rate of EAP transformation
Reporting Units	Total cells and active cells per volume of water or mass of soil; fraction of total cells that are active; transformation per unit time
Typical Reporting Limits	100 cells per sample volume or mass
Limit of Detection	Varies, should be adequate for the application
EAP	Contaminant name, probe type/target
Probe exposure (in situ EAPs)	Exposure time of probe in situ; concentration of added probe
Sample processing	Time to processing after sampling Purge parameters prior to sampling
QA/QC information	Positive and negative control analyses Inhibition testing; Dilutions, Spike, Trip Blank(s)
Narrative of analyses	Discussion of inhibition testing, interferences or quality control issues encountered
Desirable	
Number of laboratory duplicates and replicate results	• Laboratory EAP: At least one duplicate field sample is taken for every 8 samples collected. If less than 8 total samples are collected at least one sample is duplicated. Duplicate samples are sampled in the field and analyzed in the laboratory blindly. • All EAPs require a minimum of triplicate analyses for each probe; each triplicate is composed of a minimum of 20 individual, independent counts for a total set of values of 60 (minimum). • Field EAP: The premise of these EAPs also requires numerous samples over-time and a minimum of triplicate analyses for the EAP quantification. The number of replicates vary for the field EAPs and ranges from 1-5 samples. In most cases these probes are sampled at high frequency and with a multitude of triplicate or more samples at each time point.

8.3.2 How are the data interpreted?

Laboratory EAP data are used most often to determine the presence and activity of organisms and/or enzymes and to demonstrate the possibility of current biodegradation of specific

contaminants. These analyses can be useful for site characterization, remediation, and monitoring. The presence of active enzymes/microorganisms indicated by EAPs can then be used in combination with other conventional data and EMD data to provide a line of evidence for current or potential biodegradation activity at a site. Although less frequently deployed, EAP-based field studies using approaches such as push-pull tests can also provide direct estimates of contaminant biodegrading activities in contaminated groundwater environments. To illustrate interpretation of EAP results, the questions relevant to EAPs in Table 2.3 are discussed below.

8.3.2.1 Site Characterization

A) Are contaminant-degrading microorganisms present?

The range of EAPs that have been developed to date is limited mainly to enzymes involved in (a) aerobic aromatic hydrocarbon oxidation, (b) aerobic methane oxidation and (c) reductive dehalogenation of chlorinated ethenes. If the contaminants at a site can be transformed by key enzymes that involve enzymes such as toluene-oxidizing oxygenases, methane monooxygenase or chlorinated ethene reductases then EAPs can be used to answer whether contaminant-degrading microorganisms are present. Laboratory-based EAP studies could be used to demonstrate the presence (or absence) of specific contaminant-degrading enzymes at a site. For instance, at a site contaminated by petroleum hydrocarbons or BTEX-containing LNAPLs, EAPs such as 3-hydroxyphenylacetylene could be used to detect and quantify toluene-oxidizing organisms and toluene-degrading activity in aerobic areas. Alternatively, coumarin could be used to detect sMMO activity in site samples contaminated by poorly characterized xenobiotics. This information could be useful if the purified sMMO enzyme system or sMMO-expressing bacteria had previously been shown to degrade the xenobiotic under investigation. Field-derived estimates of chlorinated ethene degradation rates could also be determined using push-pull tests using EAPs such as TCFE.

B) Are contaminant degrading microorganisms active?

EAPs are currently the most direct method for determining whether "active" microorganisms are present in samples from a site. EAPs are surrogate substrates specific for an enzyme, which when transformed into a detectable product, signifying that the degrading enzymes in the given sample are active at the time of analysis. However, incubation conditions used in laboratory-based studies may not always accurately reflect in situ conditions. For example, many of the current EAPs are for aerobic enzyme systems (Table 1, EAP Fact Sheet). The detection of an EAP-transforming activity in samples incubated in the presence of saturated oxygen concentrations in the laboratory may not accurately reflect in situ conditions; carefully plan both field sampling and laboratory analysis to ensure that accurate data are obtained. Many EAPs also interrogate co-metabolic biodegradation processes that rely on the presence of a growth supporting substrate to support microbial growth and activity of the EAP-target enzyme. For example, TCE is co-metabolically biodegraded by sMMO in bacteria that grow on methane under aerobic conditions. The laboratory detection of sMMO activity in a sample using an EAP such as coumarin may indicate the presence of sMMO in microorganisms in the site sample. However, the activity of these microorganisms and/or sMMO may be constrained in situ by ambient methane concentrations. Some of these

ambiguities associated with the use of EAPs can be lessened if the EAP analyses are carefully and thoughtfully designed, and EAPs are used for in situ studies where the EAP exposure occurs under the prevailing environmental conditions at the site.

D) Is biodegradation occurring?

As indicated for Question B, EAPs can provide some of the most direct EMD-based evidence for ongoing biodegradation. Most EAPs determine the activity of degrading enzymes of interest; most analyses provide quantified information regarding the total number of organisms with active enzymes as a fraction of the total number of organisms in a given location. However, the interpretation of laboratory-based EAP studies must carefully consider the effects of differences between in situ conditions and the incubation conditions used in laboratory measurements. Changes in temperature, dissolved oxygen concentrations, and variables such as pH and the presence of naturally occurring enzyme inhibitors or other alternative substrates can impact both cellular activities and the activities of EAP-targeted enzymes. Examples of naturally occurring enzyme inhibitors for sMMO include copper, nickel and zinc (Jahng 1996), and acetylene (Prior and Dalton 1985). In general, high concentrations of metals, often indicative of anaerobic conditions, limit or inhibit the activity of oxygenase EAPs. Other EAPs for reductive dehalogenases are inhibited by high concentrations of oxygen >1 mg/L whereas the oxygenase EAPs are inhibited under very low concentrations of oxygen < 0.5 mg/L. To date, there are no known alternative probes for these enzymes that would produce a detectable product.

8.3.2.2 Remediation

H) Are numbers of contaminant-degrading microorganisms and/or genes changing?

If a suitable EAP is available for a specific process, then both laboratory- and field-based EAP studies can provide quantitative evidence for increases in the numbers of specific microorganisms. For example, in laboratory studies, changes in the numbers of EAP-transforming cells can be determined by comparing the numbers of fluorescent-labeled cells per gram of soil or milliliter of groundwater. Changes in these quantities can be evaluated over time from a baseline during transformation of contaminants or compared between test sets where different conditions are applied (e.g. biotic control compared to adding primary substrates or nutrients to accelerate biodegradation rates). While less direct than a cell-enumerating laboratory study, a field study using the same EAP at the same site at two different times could potentially demonstrate changes in the in situ rate of EAP transformation.

I) Is the remediation strategy affecting the numbers or types of contaminant-degrading microorganisms?

This question could be addressed through the same approach described for Question H.

M) Is biodegradation occurring?

See response for Question D.

N) What is the rate of biodegradation?

EAP-based analyses can be quantitative and can provide an estimate of the rate of biodegradation of a specific contaminant. However, various environmental factors can potentially influence the quantitative use of laboratory EAP studies and the uncertainty associated with these impacts can be amplified in biodegradation rate estimates if these factors are not adequately accounted for. Factors such as differences in geochemical conditions (e.g. oxygen concentrations and pH), ratio between soil and groundwater quantities, representativeness of environmental samples used in the laboratory studies, differences in static (e.g. static microcosms) versus dynamic (e.g. flowing aquifer) can all result in differences in the rates of biodegradation and EAP response measured in the laboratory compared to the field. While less intensively studied, in situ measurements of biodegradation processes using EAPs can potentially provide more accurate estimates of contaminant biodegradation rates than laboratory EAP studies (Vancheeswaran et al. 1999; Hageman et al. 2001; Pon and Semprini 2004; Ennis et al. 2005; Taylor et al. 2007; M. H. Lee et al. 2008).

8.3.2.3 Monitoring

O) Does the microbial community composition support the remediation strategy?

Whether EAPs can answer this question is dictated by the type of remediation strategy used and the type of contaminant under consideration. For example, both laboratory and field-based EAP studies can potentially establish that remediation approaches (such as addition of electron donors or acceptors) alter the abundance of specific types of microorganisms over time. EAP studies can also provide supporting evidence for monitored natural attenuation, although it must be established that in situ environmental conditions are likely to support specific activities detected in laboratory studies. For instance, an in situ source of methane must be present in the field if methane-oxidizing bacteria are implicated in contaminant biodegradation by EAP analysis.

P) Do contaminant-degrading microorganisms continue to be sufficiently abundant?

Laboratory-based EAPs can be used to directly enumerate microorganisms that possess specific enzyme activities. In contrast, field-based EAP studies would only be able to demonstrate this indirectly by determining changes in EAP transformation rates under similar conditions.

Q) Are contaminant-degrading microorganisms remaining active?

See response for Question B.

U) Is biodegradation occurring?

See response for Question D.

V)What is the rate of biodegradation?

See response for Question N.

8.3.2.4 Closure

Some variability of closure requirements exists among states and programs. However, in many situations, EMD data could serve as an additional line of evidence for understanding what processes are important in reducing concentrations and reaching the applicable closure levels. The evidence provided by EMD data would reveal whether biodegradation processes are occurring, have sufficiently proceeded, or are likely to continue.

W) Is contaminant degradation likely to continue?

The presence of microorganisms with specific enzyme activities can be assessed by both laboratory and field-based EAP studies. Assuming that in situ conditions do not change over time (in particular the geochemistry and availability of carbon, and a downward contaminant trend), EAP techniques can provide some confidence that degradation is likely to continue and contribute to the attenuation of the contaminant. EAP study results, such as temporal EAP analyses in the laboratory (for instance, Test Area North data was collected over several years at same MW), or field (collection of samples from same MW after injection of probe, such as TCFE) can be used as part of a multiple lines of evidence approach and as part of long term monitoring strategy to demonstrate that a specific microbial activity can occur and continues to occur at a site.

Y) Is biodegradation occurring?

See response for Question D.

Z) What is the rate of biodegradation?

See response for Question N.

8.3.3 Practical considerations

EAPs provide the most direct measurement of the number of contaminant degrading organisms, both in situ and in laboratory studies. However, laboratory studies need to be carefully designed in order to minimize the artificial conditions typically present when removing the microbial populations from the subsurface to examine them in the laboratory. For example, the EAPs specific for the aromatic oxygenase genes (M. H. Lee et al. 2008) require specific bottleware, which is sterile and minimizes the diffusion of oxygen across the bottle interface during shipment. In addition, a specific procedure is provided to the sampler which requires less than 1 mL headspace volume in the bottle for shipping and parafilm or tape sealing the caps prior to placing the bottles in the cooler for transport.

Groundwater samples are shipped overnight to arrive less than 24 hours after sampling at the field site. In addition to the metrics that minimize artificial induction of the oxygenase enzymes during the sampling and shipping, the laboratory procedures are also designed to minimize the exposure of the samples to the saturated oxygen conditions present in the laboratory atmosphere. Analyses are

less than 15 min total time from breaking the seal on the bottle to sample preservation. These practices are critical for clear and definitive results for this class of EAPs.

8.4 Additional Information

8.4.1 Method details

EAPs are alternative or surrogate substrates that are transformed by the target enzyme into stable products which are readily detectable. Some EAPs are initially colorless compounds that are transformed to strongly fluorescent products. As these products slowly diffuse out of cells, they accumulate internally and "color" the organism. The organisms that contain the active enzyme can then be detected, discriminated and quantified using microscopy and cell counting. Other EAPs contain unusual chemical signatures, such as fluorine atoms, that can be monitored and more precisely measured in the presence of high concentrations of contaminants, such as chlorinated solvents.

The EAP Fact Sheet includes a detailed description of these methods, as well as a discussion of their benefits and limitations.

8.4.2 Permitting and regulatory issues specific to EAPs

The laboratory EAPs have been validated and applied without regulatory concerns for over ten years. Thus the permitting, regulatory, and technical risks are relatively low for this class of EAPs.

The field EAPs involve the introduction of the probe chemicals (generally in mg quantities) into the subsurface. Regulatory agencies may have specific requirements (such as a permit; see Section 11.0) for EAPs above and beyond the traditional work plan approval process. For this reason it is important to involve the appropriate regulatory agency early in the site investigation or remediation process and ensure that the regulatory agency has a good understanding of the EAP technique to be used at the site. Since EAPs are a relatively new technology to site investigation and remediation, the regulatory requirements may vary widely. Some regulators may incorporate the approval of the use of EAPs within the overall project approval process, while other regulators may have a separate permitting process.

8.4.3 Additional Information

Further reading specific to EAPs is provided in Appendix F.

9.0 FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

9.1 Summary of FISH

Fluorescence in situ hybridization (FISH) is a molecular biology method used to visualize and enumerate specific types of microorganisms or groups of microorganisms in an environmental sample. The method does not require isolation or cultivation of microorganisms and allows for examination of microorganisms in complex environmental samples with minimal disruption of the natural microbial community. Since its introduction in the late 1980's, FISH has been used in medical and developmental biology and environmental bacteriology (Amann et al. 1995). Today, FISH is considered to be a powerful tool for phylogenetic, ecological, diagnostic, and environmental microbiology studies.

Within complex mixtures of microorganisms, the FISH method can target both general groups of microorganisms (such as methane-producing organisms) and specific degrading species of interest such as *Dehalococcoides mccartyi* (*Dhc*). Further, FISH can provide some information about the general activity of the microbial populations of interest. FISH does not require cultivation of the organisms or any technology-based signal amplification. This method also allows for study (visualization) of whole cells from their natural environment, typically containing unknown or non-culturable microorganisms. The FISH technique requires the insertion of a probe inside a microbial cell (in situ) that recognizes a specific DNA sequence and allows direct counting of the number of cells that are degrading the contaminant of interest. Therefore, FISH avoids cultivation issues, DNA extraction efficiency concerns, and PCR amplification biases (such as failure of the PCR reaction by the presence of PCR inhibitors, lack of primer specificity, and variable number of 16S rRNA operons (see Section 10.8).

FISH is the method of choice over other EMDs when other EMDs are not technically feasible (for instance, if qPCR primers are not available) or when information on mixed microbial communities structure is necessary to evaluate the occurrence of biodegradation.

Visualization of whole cells by FISH can provide information on the following:

- abundance of microorganisms or genes of interest
- cell morphology and growth characteristics
- spatial distributions and associations with other microorganisms
- microbial community structure and architecture

These characteristics can help to interpret microbially-mediated processes in soils, sediments, or groundwater. For example, some microorganisms may exhibit differential gene expression when part of bioflocs (or biofilms) versus when they are in a planktonic existence. Spatial distribution may be an important considerations for the functioning of syntrophic population of microorganisms, including contaminant degrading consortia; for instance, such associations may facilitate the transfer of electron donors and metabolites between microorganisms (Duhamel and Edwards

2007). Such information cannot be gained from the analysis of genes alone (i.e., PCR). FISH is best used when combined with other EMD tools, which by themselves may not always provide straight-forward or definitive information about contaminant degradation processes, or to provide additional resolution to understanding of a contaminated site.

In general, detection limits of 100 cells or lower in a sample can be achieved with FISH analyses (Moreno et al. 2011). In general, sampling procedures for FISH analysis are straightforward and are readily integrated into existing monitoring protocols.

The basic FISH procedure includes:

1. fixation and permeabilization
2. hybridization
3. washing
4. microscopy (for counting and visualization) or flow cytometry (for high speed counting)

In certain types of samples the FISH procedure may be preceded by a cell isolation (such as in sediments) or concentration method (low biomass samples). This basic principle and the steps involved in FISH are shown in Figure 9.1 and described in detail in Section 9.4.1.

Additional introductory information is available in the FISH Fact Sheet.

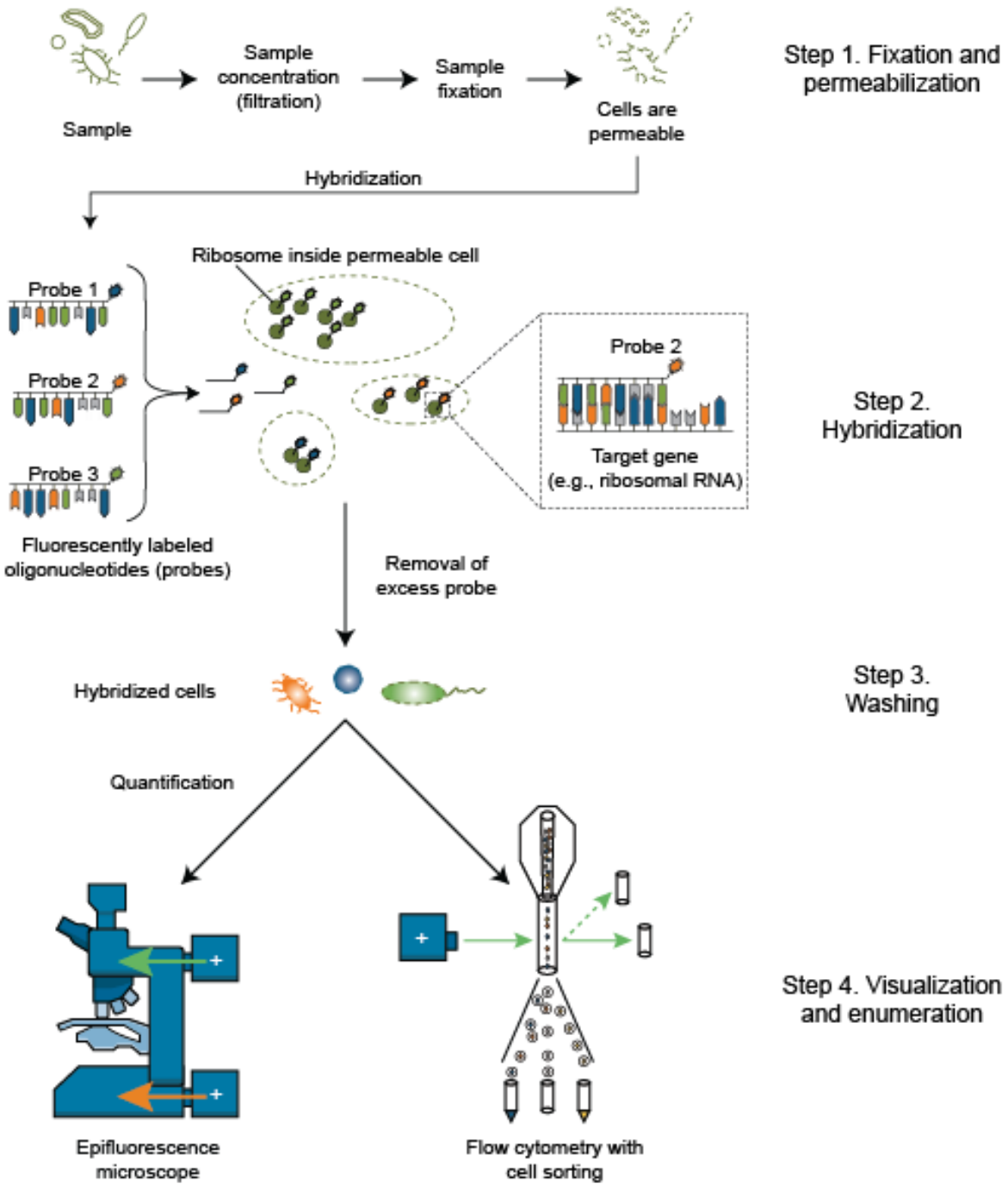

Figure 9-1. Diagram of the FISH method.

9.2 Applications

The results of FISH analysis of environmental samples are typically used in combination with a suite of analytical methods and field observations to generate a lines-of-evidence argument

regarding biological degradation of contaminants. For example, FISH was recently used to provide evidence of intrinsic remediation or natural attenuation of PAH compounds in the coal-tar impacted aquifer underlying the Cherokee former manufactured gas plant (FMGP) site in Northwestern Iowa, in combination with analysis of redox indicators and groundwater contaminant concentrations (Rogers, Ong, and Moorman 2007). In soils, FISH has been used to evaluate the PAH associated microbial community in soils from a former coal gasification plant in Denmark (Uyttebroek et al. 2006) and the atrazine transforming community colonized on BioSep® beads in atrazine exposed soils (Ghosh et al. 2009). Examples of several diverse applications of FISH studies are provided in Table 9-1. This table is followed by a brief explanation of several key studies and major findings.

Table 9-1. Example FISH Applications

Title	General information	Contaminants	EMDs used	Project life cycle stage
Test Area North, Idaho National Laboratory, ID (see description below)	Anaerobic	Chlorinated solvents (TCE)	FISH, qPCR, EAPs	Remediation Monitoring and Optimization
Ft. Lewis, WA (see description below)	Anaerobic	Chlorinated solvents (TCE)	FISH, qPCR	Feasibility/Research
Cherokee former manufactured gas plant, IA (see description below)	Aerobic and Anaerobic	PAH, coal-tar	FISH $^{14}CO^2$ generation Note 1	Remediation Selection (Natural attenuation)
Pesticide degradation in soils; Lodi, Italy (see description below)	Aerobic	Pesticides (Simazine)	FISH	Feasibility/Research
Bitterfeld, near Leipzig, Germany (see description below)	Groundwater megasite; aerobic and anaerobic	Chlorobenzenes	FISH	Feasibility Studies/Research (Bioaugmentation/ Biostimulation)

Note 1: ^{14}C radiolabeled compounds biodegradation was confirmed in a microcosm in which $^{14}CO_2$ was generated.

Test Area North, ID

In situ bioremediation was conducted at the Test Area North site at the Idaho National Laboratory. FISH analyses were used to monitor the progress of the remediation, along with EAPs and qPCR (ICP 2007, Wymore et al. 2007, M. H. Lee et al. 2008). The EAP results are discussed in Section 8.2.

Fort Lewis, WA

FISH was used to identify, and quantify, key microbial populations including *Dhc* species and methanogens during implementation of enhanced anaerobic bioremediation for a chlorinated solvent source area located at Landfill 2, Joint Base Lewis McCord, Washington. FISH was used to track microbial population dynamics prior to injection of bioremediation amendments (whey powder and bicarbonate buffer), and during and after amendment injection and bioaugmentation over the course of approximately nine months. FISH was used to correlate changes in quantities of these populations with geochemical changes to verify controlling parameters, such as groundwater pH, and to understand competitive relationships between *Dhc* and methanogens. FISH data were also compared to similar data collected using quantitative polymerase chain reaction (qPCR) targeted for *Dhc* and methanogens. Conclusions from this comparison were that FISH was redundant with qPCR and generally more labor intensive and expensive. Also, methods were not yet developed for evaluating mRNA for *tceA*, *bvcA*, and *vcrA* and/or other strains of *Dhc*. FISH, however, was much better for evaluating methanogenic populations (Macbeth and Sorenson 2011).

Cherokee Former Manufactured Gas Plant

This feasibility study used a combination of FISH on aquifer sediment samples, laboratory microcosm studies, and analysis of aqueous geochemistry to determine the natural attenuation of PAH's in coal-tar DNAPL-impacted groundwater. Groundwater sampling at the site indicated anaerobic conditions predominate downgradient of the coal-tar DNAPL source area as indicated by the presence of ferrous iron, manganese (II) and hydrogen sulfide. Laboratory microcosms showed degradation of naphthalene and phenathrene under aerobic conditions as well as under anaerobic conditions, although at a slower rate. PAHs were shown to degrade in the laboratory microcosms at a slower rate in the sulfate- and nitrate-reducing microcosms.

FISH analysis on sediment samples showed that PAH-contaminated sediments contained three orders of magnitude higher concentrations of microorganisms as compared to uncontaminated sediments. FISH analysis of the sediment microbial community indicated that β- and γ-*Proteobacteria*, *Actinobacteria*, and *Flavobacteria* were dominant in the aerobic sediments (similar to results found in the laboratory microcosm studies). FISH results also showed that sulfate-reducing bacteria dominated (>37%) the microbial community in the sediments of the sulfidogenic region of the aquifer (Rogers, Ong, and Moorman 2007). This study provided evidence of natural attenuation of PAHs in the aquifer sediments and indicated which groups are abundant in microbial communities involved in PAH degradation.

Pesticide Degradation in Soils; Lodi, Italy

A laboratory study was conducted to determine the following:

- degradation of the pesticide simazine (a triazine herbicide) in the absence and presence of urea (used to represent co-application of nitrogen fertilizer with herbicides)
- the changes overall microbial community during simazine biological degradation
- the abundance of ammonia oxidizing bacteria.

Microbial community abundance was determined by FISH methods and the study quantified total bacteria and the α, β, and γ-*Proteobacteria*subgroups as well as ammonia oxidizing bacteria. Microcosms were setup in the laboratory and contained soils historically treated with simazine and fertilized with nitrogen. Herbicide degradation was observed according to the SETAC guidelines for assessing the environmental fate of pesticides in laboratory and soil degradation studies (SETAC 1995). The results of this study showed that simazine half-life was approximately 39 days when urea was absent and 32 days when urea was added to the treatment. FISH results indicated that bacterial abundance increased during simazine degradation with or without the presence of urea, although the presence/absence of simazine and urea affected the relative abundance of different groups. Additionally FISH results indicated that ammonia oxidizing bacteria may be involved in the degradation of simazine due to changes in abundance of the bacteria (Caracciolo et al. 2005).

Bitterfeld, Chlorobenzene Degradation in Groundwater

This feasibility study was undertaken to evaluate biological degradation of chlorobenzene, and 1,2- and 1,4-dichlorobenzene by various *Pseudomonas putida* microorganisms. A set of microcosms were developed in the laboratory containing groundwater from the contaminated site, one of three *P. putida* microorganisms previously shown to degrade chlorobenzenes, and additional concentrations of the three contaminants. Concentrations of the three organisms over time were monitored by FISH and the total microbial community was evaluated using single-strand polymorphism (SSP) analysis of the 16S rRNA gene from the total microbial community. The results showed that bioaugmentation with two cultures *P. putida* GJ31 and a genetically modified microorganism *P. putida* F1ΔCC were capable of degrading 30 mg/L of chlorobenzene, 2 mg/L of 1,2 dichlorobenzene, and 2 mg/L of 1,4-dichlorobenzene to less than 1 mg/L. Further the study showed that these organisms were capable of degrading the chlorbenzenes under both aerobic and nitrate oxidizing conditions (Wenderoth et al. 2003; Wenderoth et al. 2002).

9.3 Data Interpretation

The application, analysis, and interpretation of microbiology-based EMD methods differ from typical soil and groundwater geochemical measurement in a number of ways. For example, microbial biomarkers cannot easily be preserved, and sample handling and processing requires special care. The analysis of microbial parameters requires specific data quality considerations for sampling plans, sample collection and handling, quality control and laboratory procedures, and these are discussed in Section 10.0. Included below is a brief introduction to how FISH data are typically reported and some specific examples of how the data would be interpreted in answering the questions presented in Table 2-3.

9.3.1 How are the data typically reported?

Included in Table 9-2 below is information that should be provided in laboratory reports of FISH data including common laboratory report information, recommended information about the FISH method, and desirable information about the FISH method and results.

Additional information regarding sample handling and collection can be found in Section 10.4 and Section 10.5.

Table 9-2. Recommended and desirable information in FISH laboratory report

Report information	Typical information or acceptable ranges
Common Laboratory Report Information	
Site Identifier	
Sample type or matrix	Groundwater, sediment, soil
Sample storage/transportation	4°C, overnight shipping, chain of custody forms
Sample handling methods	Filtering methods, filters used, sediment washing, volume of water filtered, cell separation methods
Specific FISH Information	
Recommended	
Results	Total cell and active cells counts including standard deviations of multiple fields; spatial distribution; biofilm, floc, or planktonic state
Reporting Units	Cells per volume of water or mass of soil
Typical Detection or Reporting Limits	100 cells per sample volume or mass (e.g., filtered groundwater); 104 cells/ml (e.g., unfiltered wastewater sample)
Limit of Detection	Varies, should be adequate for the application
FISH target	Ribosomal gene (e.g., 16S rRNA), functional gene name, probe sequence
FISH probe fluorescent marker	Fluorescein, Alexa Fluor®
Sample fixation method	Chemicals used for fixation (e.g., paraformaldehyde)
Hybridization conditions	Hybridization time, hybridization solution composition (e.g., probe concentration), and temperature
Method of quantification	Manual • Counts of cells (30 to 200 cells per field) including number of fields counted (e.g., minimum of 20 fields), • equipment used for quantification (epi-fluorescent microscope used and filter sets) Automatic • flow cytometry conditions and instrumentation (e.g., post processing software)
QA/QC information	Laboratory positive and negative control results (non-sense probe)
Narrative of analyses	Indicating interferences or quality control issues encountered
Desirable	
Photomicrographs and graphs from flow cytometry analyses	
Number of laboratory duplicates and replicate results	

9.3.2 How are the data interpreted?

Interpretation of FISH data varies depending on the site microbiology, the degradation pathways

present, and the contaminants. The following overview illustrates the selection of an appropriate analysis and integration of the subsequent results with site monitoring plans. Each bioremediation strategy (e.g., monitored natural attenuation, biostimulation, and bioaugmentation) will be discussed as well as specific information given for common contaminants.

FISH data are used to determine the presence, spatial relationship and (in some cases) activity of microbes of interest in a sample. Visualization of whole cells by FISH can provide information on cell morphology and growth characteristics, spatial distributions and associations with other microorganisms, and microbial community structure and architecture—which may be important for interpreting microbially-mediated processes in soils, sediments, or groundwater. FISH signals can provide some information about activity of the target organisms, although no rate information can be obtained. Presence of various microorganisms or genes of interest should be used in combination with other data (e.g., contaminant concentrations) to provide a lines of evidence argument regarding biodegradation potential and activity. To illustrate interpretation of FISH results, each question relevant to FISH in Table 2.3 is discussed.

9.3.2.1 Site Characterization

A) Are contaminant-degrading microorganisms present?

FISH results can be used to determine the presence of contaminant- degrading organisms in water samples and in some cases in soil and sediment samples. Depending on which FISH probes are used, FISH data can quantify total bacteria, organisms with specific metabolic capabilities (such as sulfate reducers) or concentrations of organisms known to degrade contaminants (such as chlorinated solvent degraders or naphthalene degraders).

For example, at a site where the shallow aquifer is contaminated with heating oil and low concentrations of oxygen are present in the plume. Monitored natural attenuation parameters were collected (groundwater monitoring data, geochemical characterization) and an evaluation of the potential for biological attenuation performed. FISH was performed to determine the abundance of both sulfate reducers and methanogens during concomitant degradation of petroleum hydrocarbons. Sulfate reducers can degrade and grow on petroleum hydrocarbons (Kleikemper 2002). Methanogens do not directly degrade petroleum hydrocarbons in low oxygen systems, rather they facilitate the fermentation of petroleum hydrocarbons by making the conditions conducive to growth and activity of petroleum hydrocarbon fermentors (i.e., hydrogenotrophic methanogens keep H_2 and CO_2 low and acetoclastic methanogens degrade end products of fermentation) (Kleikemper 2005).

FISH analytical results included cells/ml groundwater or cells/g aquifer material of the following microorganisms:

Total microorganisms

- Total bacteria
    - Sulfate reducing bacteria
- Total *Archaea*
    - Methanogens

FISH results showed that sulfate reducers consisted of a relatively large percentage of the bacteria present (45%), and the concentration of methanogens in groundwater was greater than the concentrations extracted from soil as normalized by total microorganisms.

B) Are contaminant degrading microorganisms active?

With emerging approaches, such as quantifying the amount of mRNA for a degradation genes with FISH, one can examine the activity of microorganisms performing important biodegradation functions. If the mRNA detected by FISH correlates to a functional gene for a biodegradation process, then biodegradation of a contaminant is potentially occurring in the contaminated matrix. Additionally, the percentage of the total versus active microbial population containing the functional gene could also potentially be quantified.

For example, at a site that contains a coal-tar impacted groundwater aquifer naphthalene is one contaminant. Microbial metabolism of naphthalene begins with dioxygenase-mediated transformations and is encoded for by naphthalene dioxygenase genes in *Pseudomonas putida*. Groundwater samples from the site could be evaluated for the presence of naphthalene dioxygenase mRNA by FISH (Bakermans and Madsen 2002; Wilson, Bakersman, and Madsen 1999). Detection of naphthalene dioxygenase mRNA by FISH would suggest that microorganisms are biologically degrading naphthalene. For example, samples could be analyzed for the following targets and reported as cells/ml groundwater:

Total microorganisms

- Total active microorganisms
    - Total bacteria
        - Naphthalene dioxygenase mRNA

The FISH results could show:

1. the percentage of cells that were bacteria versus *Archaea* in the groundwater sample
2. if mRNA encoding for naphthalene dioxygenase is present at the site and how it changes with time

3. what percentage of the native microbial population contains mRNA encoding for naphthalene dioxygenase by comparing total active microorganisms by acridine orange counts as compared to naphthalene dioxygenase mRNA counts.

C) Are the microorganisms capable of complete degradation?

See Question A.

9.3.2.2 Remediation

H) Are numbers of contaminant-degrading microorganisms and/or genes changing?

FISH can be used to track the number of contaminant-degrading microorganisms and/or genes changing in groundwater, soil, or water environmental samples. For example, a site has s-triazine contaminated soil (i.e., simazine) that is undergoing natural attenuation. FISH could be used to determine the concentration of the atzB gene which has been linked to biological degradation of s-triazines by hydrolytic deamination over time. These atzB genes have been found in soils that have been historically exposed to triazines, but not in soils that have not been exposed previously. Additionally, the atzB gene has been found to be correlated to the mineralization rate of simazine (Martin et al. 2008). Therefore detection of the atzB gene in the soils at the area contaminated with triazines could provide another line of evidence that biological degradation of triazines is occurring.

I) Is the remediation strategy affecting the numbers or types of contaminant-degrading microorganisms?

FISH can be used to track the number of contaminant-degrading microorganisms and genes changing in groundwater, soil, or water environmental samples. For example, a site is contaminated with chlorobenzenes such as 1,2-dichlorobenzene, 1,4-dichlorobenzene, and chlorobenzene. The site has undergone bioaugmentation with a mixed culture of*Pseudomonas putida* GJ31 and *Pseudomonas putida* F1DCC. After bioaugmentation, one portion of the site is sparged with air and the other site is left under anaerobic conditions. Over time, degradation of chlorobenzenes is observed and concentrations of the *P. putida* species are monitored by FISH. Concentrations of *P. putida* as measured by FISH could show an increase in concentration with time during the simultaneous degradation of chlorobenezenes as has been shown previously (Wenderoth, et al. 2003). However, less significant growth of *P. putida* could be observed under aerobic conditions and could correlate with decreased rates of chlorobenzene degradation as compared to the portion of the plume undergoing bioremediation under anaerobic conditions. Therefore, FISH results when used in combination with traditional groundwater monitoring methods could reveal which bioremediation strategies resulted in optimal growth of bioaugmentation cultures and suggest which approach resulted in increased chlorobenzene degradation rates.

J) Is there a biological basis for intermediates accumulating?

FISH can be used to determine if the right microorganisms are present to completely degrade contaminants to non-toxic byproducts. For example, FISH could be used to determine if appropriate dehalogenating communities are present at a site which is contaminated with chlorinated ethene and their approximate concentrations. Only *Dhc* has been shown to completely degraded chlorinated ethenes (e.g. PCE) to non-toxic byproducts (i.e., ethene). Therefore FISH probes for *Dhc* (such as *Dhc*1259t) could be used in combination with other probes for total bacteria and total *Archaea* to determine the abundance of this important dechlorinating community. However if *Dhc* is not present, but the total biological community is fairly robust, then degradation of PCE may be stalling at *cis*-DCE or be degrading by different mechanisms. Currently FISH methods cannot adequately distinguish between *Dhc* that degrade PCE to ethene and other strains in the genus which do not completely degrade chlorinated solvents. Continued research may develop probes specific to the various *Dehalococcoides* , thus discriminating between the various contaminant degrading organisms and degradation pathways.

9.3.2.3 Monitoring

O) Does the microbial community composition support the remediation strategy?

Based on the combination of probes used, the FISH method can indicate which specific microorganisms are present, whether functional genes associated with biodegradation are present, or the percentage that larger groups of microorganisms constitute of the total biomass. For example, at a site where groundwater is contaminated with uranium and is undergoing bioremediation to reduce soluble U(VI) to insoluble U(IV). Specifically, *Desulfotomaculum* sp., a sulfate reducer, is using U (VI) as the sole electron acceptor and precipitating U(IV). FISH can be used to determine the concentration of sulfate reducing bacteria present in the groundwater during the precipitation of uranium.

P) Do contaminant-degrading microorganisms continue to be sufficiently abundant?

See Question A.

Q) Are contaminant-degrading microorganisms remaining active?

See Question B.

R) Is there a biological basis for intermediates accumulating?

FISH can be used to determine if the right microorganisms are present to completely degrade contaminants to non-toxic byproducts. For example, FISH could be used at a site contaminated with chlorinated ethenes to determine if appropriate dehalogenating communities are present and their approximate concentrations. Only *Dhc* has been shown to completely degraded chlorinated ethenes (e.g. PCE) to nontoxic byproducts (i.e., ethene). Therefore FISH probes for *Dhc* (e.g., Dhe1259t) could be used in combination with other probes for total bacteria and total *Archaea* to

determine the abundance of this important dechlorinating community. However if *Dhc*is not present, but the total biological community is fairly robust, then degradation of PCE may be stalling at *cis*-DCE.

9.3.2.4 Closure

Some variability of closure requirements exists among states and programs. However, in many situations, EMD data could serve as an additional line of evidence for understanding what processes are important in reducing concentrations and reaching the applicable closure levels. The evidence provided by EMD data would reveal whether biodegradation processes are occurring, have sufficiently proceeded or are likely to continue.

W) Is contaminant degradation likely to continue?

FISH can be used to track the concentrations of microorganisms of interest over time. In particular the concentration of microorganisms known to biodegrade contaminants could be monitored over several sampling events to show the sustained presence of contaminant degrading microorganisms. Demonstrating steady state concentrations of biodegrading organisms at a site could provide additional lines of evidence, along with geochemical analyses and downward trends in contaminant concentrations, that biological natural attenuation processes may continue and that residual contamination will not pose a threat to human health or the environment following closure.

9.3.3 Practical considerations

A study conducted by Robertson et al. in 2002 highlighted some potential issues associated with using FISH counts of organisms of interest for bioremediation activities. First, the team found that although the sulfate reducing organism (*Desulfosporosinus meridiei*) associated with aromatic hydrocarbon biodegradation under anaerobic conditions in the laboratory was present in groundwater at the Eden Hill site, there were no differences in the concentration of the organism inside or outside the plume. The team also found that the organism was not correlated with hydrocarbon concentrations or with indications of sulfate reduction. Second, the team found that autofluorescence of the sample and poor nutritional state of the groundwater environment lead to problems with quantification of the microorganism of interest. Poor nutritional state of the environment will lead to lower concentrations of 16S rRNA as this gene is only present in high numbers in actively replicating microorganisms, which requires a nutritionally rich environment. The poor fluorescence of the samples was further confounded by the presence of autofluorescent particles in the samples.

The authors concluded that FISH may not be an appropriate method for quantifying bacteria in nutritionally poor environments or when the organism is slow growing (Robertson et al. 2002). Many of the issues raised by the Robertson, et al. (2002) study regarding the FISH method can be addressed using newer, more sensitive detection techniques such as those described below in Section 9.4.2.1(CARD-FISH). Others have suggested that FISH can be used to monitor dynamic temporal changes in intrinsic biodegradation activity when specific probe sets are used and where the target bacteria has been definitively linked to contaminant degradation at the site of interest (Rogers, Ong, and Moorman, 2002; Yang and Zeyer 2003). When specific probes are not

available, FISH can be used in combination with isotopic techniques (CSIA, SIP, or MAR-FISH; see Section 9.4 below) to show which organisms are actively degrading contaminants of interest.

9.3.3.1 Limitations

The FISH method is not widely available commercially. Currently, only specialized research laboratories are performing these analyses. Validated probes and procedures are not available for a wide range of organisms. While several databases provide access to over 2600 rRNA targeted oligonucleotide probes (probeBase, SILVA rRNA database project), there are numerous additional 16S rRNA or functional gene sequences of environmental remediation significance that could be used for FISH probe design, yet have not been developed and validated. The lack of validation for these sequences may be related to the expense of the FISH method and associated with the expertise and labor needed for direct microscopic counting. FISH can be automated to some extent by using flow cytometry to count target cells more efficiently, thus reducing the analysis costs, but information regarding spatial relationships among and between the cells in the sample is lost in the process. Finally, standard protocols for sample collection, storage and analysis have not yet been developed for many degradation processes.

9.3.3.2 Ribosomal content

As a majority of FISH applications target the ribosomes of microbial cells, variations in the concentration of ribosomes in a cell can affect the sensitivity of a FISH method. For example the average number of ribosomes in a microbial cell can change over time. During exponential growth, *E. coli* can contain upwards to 72,000 ribosomes. In contrast after reaching a stationary phase of growth, *E. coli* cells have been shown to contain only 6,800 ribosomes (Bremer and Dennis 1996). In relatively smaller microorganisms (for instance, *Dhc* with cell diameters of 0.5 um) there are significantly fewer ribosomes, a few hundred, due to physical space restrictions. It is difficult to detect smaller cell with fewer ribosomes by fluorescently labeling. However, new methods to detect cells with fewer ribosomes have overcome some of these limitations include most commonly, CARD-FISH. This method includes the use of horseradish peroxidase-labeled oligonucleotide probes, in combination with catalyzed reported deposition (CARD) of fluorescently labeled tryamides (Fazi et al. 2008; Hoshino et al. 2008). Multiple tryamides react with the peroxidase enzyme to amplify the fluorescent signal and increase the sensitivity of hybridization method (lower detection limit).

Several new technologies are emerging that will advance the detection of even individual genes in bacteria by FISH and automate this method (for reviews see Amann and Fuchs 2008; Czechowska et al. 2008; Lee et al. 2011).

9.4 Additional Information

Example FISH probes and cellular stains used for environmental remediation studies or activities are also included in the FISH Fact Sheet. Further reading specific to FISH methods is provided in Appendix F.

9.4.1 How does it work?

This method has been used for the identification, quantification and characterization of microbial communities or their degradation associated genes of interest in complex environmental samples.

The basic principle of FISH is to bind (hybridize) a target reporter (fluorescently labeled oligonucleotide probe) to a sequence of interest (such as 16S rRNA) inside a microorganism and visualize or count the resulting fluorescent signal by micrsoscopy or other method. The basic FISH procedure includes: 1) fixation and permeabilization; 2) hybridization; 3) washing;and 4) microscopy (for counting and visualization) or flow cytometry (for high speed counting). In certain types of samples the FISH procedure may be preceded by a cell isolation (e.g., sediments) or concentration method (e.g., low biomass samples). This basic principle and the steps involved in FISH are shown in Figure 9.1 and described in detail below. Independent of the specific FISH approach applied, only cells that contain the target DNA are recognized by the probe and will be fluorescently labeled when visualized with appropriate techniques.

Fixation and Permeabilization. Fixation and permeabilization of microbial cells is required to 1) preserve the integrity and shape of all cells; 2) prevent cell loss through lysis; 3) allow penetration of the fluorescent FISH probes into the cell; and 4) protect the target gene (typically RNA) from degradation during storage and analysis. Typically a sample, such as groundwater or other water sample, is settled on membrane filters and covered with a fixing agent or the sample itself is mixed with fixing agent. Formaldehyde and ethanol are typically used for fixation of cells (Roller et al. 1994). The fixing agent serves to permeabilize as many cells as possible to allow the large labeled oligonucleotides entry to the cells and subsequent diffusion of the probes to their intracellular rRNA targets. After fixation and several steps to remove residual fixative, the sample is transferred to a microscope slide and the microorganisms are dehydrated by washing with ethanol.

Hybridization. During hybridization, a target reporter such as a fluorescently labeled probe is bound to the sequence of interest. Only organisms containing the target genes incorporate the fluorescent label, so they can be directly visualized and counted using a microscope or other technique. In environmental microbial ecology studies, FISH applications have targeted ribosomal RNA (rRNA), particularly the 16S gene, with oligonucleotide probes. The rRNA molecule is targeted for identification of microorganisms (Bacteria and Archaea) as all cells required ribosomes for translation and growth. Since each cell contains many ribosomes, there can be 100 to 100,000 targets per cell. Additionally, the rRNA of each species of microorganism contains short signature sequences that are unique to each group of microorganisms (for example, *Dhc* specific 16S rRNA sequences). Oligonucleotide probes are molecules composed of 15 to 30 nucleotides and are covalently linked to a fluorescent dye. Other types of cell labeling techniques that are used less commonly than oligonucleotide probes include combinations of reporter molecules (dioxygenin), fluorescent antibodies (horseradish peroxidase) and enzymatic signal amplification (tryamide), or polyribonucleotide probes.

Hybridization of probes to the target sequence must be carried out under stringent conditions to ensure proper annealing of the probe to the target sequence. During this crucial step of FISH, pre-heated hybridization buffer containing fluorescently labeled probes complementary to the target gene is applied to the sample and incubated in a dark, humid chamber at exact temperatures for 30 minutes to several hours.

Washing. During the washing step the microscope slides containing the fixed and hybridized cells are briefly rinsed to remove unbound probe that would interfere with quantification of the target microorganisms. The slides are then dried, mounted with an anti-fading agent and visualized.

Visualization or counting. Hybridized cells are visualized or counted by epifluorescence microscopy or by flow cytometry. Fluorescence miscroscopy uses a high intensity light to illuminate a sample, which excites fluorescence species bound to the olidonucleotide probes, resulting in an emission of a longer wavelength light. The image magnified in an epifluorescence microscope is actually an image of the light emanating from the fluorescent molecules bound to the oligonucleotide probes rather than illuminated light as in light microscopy. Cells are enumerated by the laboratory technician or by an automated counting programs (Pernthaler, Pernthaler, and Amann 2003; Selinummi et al. 2005). More efficient counting of labeled cells is achieved with flow cytometry (Porter et al. 1997; Vives-Rego, Lebaron and Nebe-von Caron 2003). In flow cytometry, labeled cells are diluted so that individual cells pass through a laser beam, which detects and counts fluorescently labeled cells. Various cell staining procedures are sometimes combined with FISH probes to allow quantification of all microorganisms (see Table 1, FISH Fact Sheet).

9.4.2 Variations or Newer Methods Becoming Available

9.4.2.1 CARD-FISH

As mentioned previously, this method is used to increase the FISH signal intensity thus allowing the quantitation of low activity microbial assemblages or organisms with low ribosomal contents (Fazi et al. 2008).

9.4.2.2 MAR-FISH

This technique involves the uptake of radioactively labeled substrates into cells, which can be detected by microautoradiography (MAR) with simultaneous identification of the cells by FISH. Unlike with stable isotope probing (see Section 7.0), where the labeled substrates contain non-radioactive isotopes, MAR-FISH requires the use of radioactive isotopes such as ^{14}C, ^{32}P or ^{3}H (Wagner et al. 2006). This technique has been widely used (Wagner et al. 2006) and recently has been automated (Alonso and Pernthaler 2005; Cottrell and Kirchman 2003).

9.4.2.3 Raman-FISH

This technique involves the uptake of stable isotope tracers into microbial cells, determination of uptake of the stable isotopes by Raman microspectroscopy, and identification of the microbial cells

by FISH (Huang et al. 2007). This method overcomes the equipment costs and reduces the resolution that has limited SIMS and MAR-FISH applications (Amann and Fuchs 2008).

9.4.2.4 NanoSIMS-FISH

Nano-scale secondary-ion mass spectrometry (SIMS) allows multiple-isotope imaging in cells and identification of the microbial cells by FISH with resolutions down to 50 nm. The advantage of this technique is that one can potentially detect metabolic activities, such as contaminant degradation, in single cells (Musat et al. 2012).

10.0 DATA QUALITY, SAMPLING, QA/QC, AND PROCEDURES FOR BIOLOGICAL EMDS

EMDs can provide unique information about biological activity at a site that can be used along with other lines of evidence to support decision making. In most cases, no formal standardized methods exist for the EMD technologies described in this document because the science of EMDs is developing rapidly. However, some common methodologies can contribute to the successful description of microbial activity at a site using EMDs. For instance, quality control procedures for CSIA are similar to procedures for traditional chemical analytical techniques and are detailed in Section 3.3. This section focuses on biological and biochemical EMDs. These EMDs are also referred to as molecular biological techniques (MBTs).

10.1 Basic Concepts

USEPA defines data quality as: "A measure of the degree of acceptability or utility of data for a particular purpose" (USEPA 2002b). Acceptability and utility are determined by sampling design and execution, as well as by laboratory practices. Some common approaches contribute to the successful qualitative description and quantitative measurement of site biological properties. This section describes the critical components of EMD approaches so that project managers can make informed decisions in the design of effective sampling plans. Using the guidance presented here, regulators can also evaluate those plans, and stakeholders can evaluate the quality of vendors' EMD analyses and data.

Current practices for ensuring and measuring data quality occur at several steps throughout the sampling and analysis process. Data quality assessment is not solely dependent on the use of sound methodologies but also contingent on the accurate use of qualified equipment and good laboratory practices that directly impact the accuracy of the method (USEPA 2004a). Furthermore, documenting protocols and schedules for verification of the equipment performance and appropriate procedures is essential to ensure that the analysis is acceptable and will increase the confidence in a particular EMD methodology.

10.2 Project Life Cycle Stages

EMD sampling can occur at any point in the project life cycle: site characterization, remediation, monitoring, or closure. Therefore, quality considerations are critical in the remedial investigation (RI)/site assessment, feasibility study (FS)/corrective action planning, site monitoring, and closure request processes. General guidelines for sampling and QA/QC procedures will not vary between the different phases of the project life cycle, and Sections 10.3 through 10.14 are applicable regardless of life cycle phase. Some considerations specific to individual project phases are discussed in Section 10.15.

10.3 Sampling Plan

The collection of quality data begins prior to field or laboratory sampling. The hypothesis to be tested must be carefully considered and a plan which addresses that hypothesis formed.

10.3.1 Number of Samples

Statistical comparisons of data typically require a minimum of three or more samples for analysis. If three or more samples have been collected, numerous statistical methods (for example, t-test, analysis of variance (ANOVA) are available to determine differences in the means of datasets. Each method of difference determination has a corresponding formula for calculating the number of samples needed to detect differences at a pre-determined level of significance.

Determining the minimum number of qualitative analyses, such as the presence or absence of a functional gene, is more challenging. Results should be expected to be reproducible. In a limited number of cases, one sample may be construed as sufficient, but should be viewed in the context of the question being asked and the potential value of repeating the result from the same location or other locations at the site. A negative conclusion (such as 'the microbial community at the site is NOT sufficient to support biodegradation') may be supported with a single result, whereas a positive conclusion might require replicate analyses. Another approach may be to look at false positive and negative rates for a particular analysis and conduct a sufficient number of analyses to have a pre-determined confidence (α) that the results obtained are not false.

Replicate values are rarely obtained for EMDs due to the limited sample numbers and high cost of the analyses. This data gap may compromise data utility. For example, sample variability is likely to be high relative to other analytical processing steps and critical to understanding and predicting the performance of EMDs, and thus replicate samples are required to quantify this variability. Data quality samples such as field and equipment blanks, or matrix and trip spikes, should be added to sampling programs to detect sampling and analysis errors and cross-contamination.

Across a site, the number of samples needed will be a function of site conditions, including geology, hydrogeology, geochemistry, and contaminant distribution. A successful sampling program will incorporate these site conditions in a site specific sampling plan and consider the CSM. Greater heterogeneity (variability) in each of these site conditions will require more samples to sufficiently characterize a site.

10.3.2 Sample Locations

For EMD analysis of groundwater samples, contaminant concentrations and distribution, as well as plume size and shape, each contribute to the selection of sample locations. As with many environmental measurement techniques, multiple samples from the same location can yield differing results. These differences can be due to environmental heterogeneity, as well as variations in the sampling and measurement operations. As with the number of samples, locations for sampling the

microbial community are driven by site geology, geochemistry, hydrogeology, and contaminant concentration and distribution. The contaminant concentration in groundwater, in particular, likely influences the microbial community at that location. Therefore, in addition to identifying which areas are suitable as background, the source zone and leading edge of the plume are valuable areas to apply EMDs. In these cases, contour maps and contaminant distribution isopleths can be useful resources in selecting monitoring wells for groundwater sampling. Similarly, the vertical separation of distinct aquifers and site geology (stratigraphy and lithology) is expected to impact microbial ecology and should therefore influence the selection of sample locations. The selection of sampling locations should be consistent with the CSM and the results of the EMD analysis in turn should be used to update or refine the CSM.

The rationale for selecting soil/sediment sample locations is similarly driven by variations in geology, soil chemistry, and contaminant concentration and distribution. Generally, vadose zone biology is more location-specific, because microbial transport (and thus distribution) depends on excess water. With less water available, more samples may be required to characterize a smaller area. Since microbial diversity in soils and sediments can vary on micro-to-millimeter scales, homogenization and multiple samples are desirable.

Variation can be characterized through replicate data collection at multiple analysis points. Once replicate samples are collected and analyzed, variation at the specific locations can be estimated. In some cases, standardization may reduce variation and thus reduce the need for replication. In other cases, replicate sample analysis will be identified as an important data quality assessment tool. These data provide insights into where variability occurs, and the degree of replication needed to provide meaningful information can be estimated using statistical tools such as power calculations. Once true variation in target microorganism concentrations can be separated from sampling and analysis variation, meaningful correlating EMD information between sites/locations becomes possible. This correlation supports the evaluation of EMD information as predictive or performance measures at bioremediation sites.

10.3.3 Sampling Design Summary

Table 10-1 presents a summary of sampling design considerations.

Table 10-1. Suggestions for sampling plans for number of samples, sample locations, and sampling frequency (from Lebrón et al. 2011)

Components of sampling plan	Factors to consider	Explanation	Guidance
Number of Samples	Vertical and aerial extent of plume. Border of compliance. What are the goals: source zone remediation, establishment of a biobarrier, or treatment of the entire plume?	The number of MBT samples is partially dependent on the volume/size of the plume and the remedial goals (for example, the cleanup target area).	The goal of the MBT analysis must be clearly defined. The number of samples should be sufficient to clearly establish cause-and-effect relationships, guide site management decisions, and to be accepted by regulatory agencies as a line of evidence for attenuation.
	Variability of data used to characterize and delineate plume.	The variability of the VOC data across the plume may be an indicator of the expected variability of the MBT sample results.	The number of MBT samples should be sufficient to document expected variability in MBT results.
Sample Locations	Plume shape and expansion in relation to source area.	Does the plume have a simple elliptical shape emanating from a single source area or does it have an irregular shape with one or more source areas?	MBT samples should be collected from locations so that the results are representative of the area targeted for remediation.
	Distribution of contamination within stratified/heterogeneous aquifers.	Is the plume contained in one homogenous aquifer or is it contained in multiple stratified aquifers separated by low permeability units.	MBT samples should be collected from each aquifer/unit containing the plume.
	Distribution of indicator parameters throughout the target area (such as biodegradation products, DO, ORP).	Does the distribution of indicator parameters, such as biodegradation products, oxygen, and ORP, indicate that there are distinct biodegradation zones in the plume? Are there distinct biodegradation zones in the plume?	MBT results must be performed in an integrated manner and include site geochemical parameters collected simultaneously. At least one sample should be collected from each distinct biodegradation zone.

Table 10-1. Suggestions for sampling plans for number of samples, sample locations, and sampling frequency (from Lebrón et al. 2011) (continued)

Components of sampling plan	Factors to consider	Explanation	Guidance
Sample Frequency	Seasonal variability of groundwater data (VOCs, oxygen, ORP).	Is there seasonal variability of the existing groundwater data, such as VOC concentration, oxygen, and ORP, that indicate the potential for seasonal variability of the MBT data?	The sample frequency should be sufficient to document expected seasonal variability of MBT results.
	For active remediation systems, frequency of electron donor injection, observed biodegradation rates, location of monitoring wells relative to injection points and groundwater flow velocity, and remediation goals.	For enhanced bioremediation performance monitoring, a baseline should be established prior to any treatment. Donor injection will rapidly affect the richness and eveness of the microbial community.	Sampling should be conducted more frequently (monthly or quarterly) following bioaugmentation to monitor the distribution of dechlorinators and the establishment of dechlorinating activity in the target area.

10.4 Sampling

Biomass in environmental matrices can be collected in a variety of ways for EMD analysis. This section discusses general considerations for EMD sampling, the application of active and passive approaches, as well techniques specific to different matrices. Health and safety considerations for EMD sampling are similar to soil and groundwater sampling for conventional parameters and should be included in the site health and safety plan.

10.4.1 Aseptic Sampling and Sterility

Sampling materials (such as instruments, passive sampling devices, filters) should be sterilized prior to use in the field. Field personnel should be adequately trained in maintaining the sterility of sampling devices and practicing aseptic technique. In cases where it is impractical to maintain sterile conditions, effort should be taken to minimize the introduction of microbial contamination. Samples should also be handled accordingly and shipped on ice as soon as possible to prevent or minimize changes in microbial abundances or activities during the time interval between sample collection and analysis.

Steps to ensure that field sampling equipment does not contribute contamination include:

- Use bleach solution as part of the decontamination for soil sampling equipment such as augers.

- Use disposable or dedicated tubing/samplers.
- Use lab-sterilized bottles and sampling devices.

10.4.2 Active Groundwater Sampling

To monitor microbes of interest such as *Dhc* (a species of bacteria that dechlorinate chlorinated solvents) in aquifer formations, groundwater samples are typically collected due to the practical limitations to routinely collecting soil samples from the saturated zone. In the case of *Dhc*, a significant fraction (over a third) of the total *Dhc* are found in the aqueous phase, in addition to *Dhc* cells attached to the solids (Frank Löffler, University of Tennessee, personal communication; Amos et al. 2009; Schaefer et al. 2009). Most EMDs call for on site filtration to collect biomass from groundwater (Ritalahti et al. 2010a). A guidance protocol providing a step-by-step approach for groundwater sampling using field filtration methods was developed as part of the Environmental Security Technology Certification Program (ESTCP) Project ER-0518 and is available in Lebrón et al. (2011) and Ritalahti et al. (2010b).

Note that other subsurface microorganisms may form biofilms and are primarily attached to aquifer solids; groundwater analyses alone may not reflect the true abundance of such organisms in the aquifer formation (Alfreider et al. 1997; Griebler et al. 2002, 2009; Hazen et al. 1991; Thomas et al. 1987). In these cases, sampling aquifer solids may be necessary, or use of passive sampling devices with solid matrices to encourage microbial biofilm development may be more appropriate (Sublette et al. 2006). Although the analysis of groundwater and solid samples is required to obtain the total numbers of target cells in the aquifer formation, groundwater samples alone will be useful for estimating cell numbers of organisms that occur in the non-attached state (for example, *Dhc*).

10.4.3 Passive Groundwater Sampling

Passive microbial sampling devices, also called retrievable media devices, are typically deployed in purged groundwater monitoring wells located within and upgradient of the dissolved contaminant plume to compare results of analyses between impacted and background conditions (see Lebron et al. 2008).

Passive microbial sampling devices are incubated within the sampled environment for several weeks (typically 30-60 days or more) and rely on the formation and collection of biofilms on or within a solid matrix. See also Section 10.4.5 for more information about the devices themselves. Passive microbial sampling can provide a time-integrated sample of microorganisms from the sampled environment. Passive samplers can also be amended with electron donors or acceptors or with isotopically-labeled contaminant compounds (see Section 7.0) to assess in situ microbial activities (Lebron et al. 2008).

Advantages of passive microbial sampling devices include ease of storage and transport, capture of sufficient biomass for EMD analysis, and a potentially more accurate temporal and spatial representation of the subsurface microbial community surrounding the site of collection than can be

gleaned from groundwater samples (Peacock et al. 2004). A disadvantage of passive microbial sampling devices includes the uncertainty as to whether the sampling device represent groundwater geochemistry and the physical conditions of the aquifer matrix, and hence, truly represent the microbial community composition (i.e., richness and evenness) of the surrounding aquifer. These factors must be considered when interpreting the data, in particular when quantitative information is evaluated. Questions surrounding suitable incubation times are another issue with passive microbial sampling devices that have not been thoroughly addressed.

10.4.4 Soil Sampling

Soil samples can contain high concentrations of diverse bacteria and other biota. A soil sample, much like a single groundwater sample, can be viewed as a 'snapshot' in both time and space. Do not necessarily assume that the sample is in biological or chemical equilibrium. Avoid contamination of the sample with bacteria from other locations and sampling devices. Field blanks can offer a quality control check to detect the presence of interfering bacteria.

10.4.5 Sampling Devices

Various active and passive microbial sampling devices have been developed to collect microorganisms from an environment (typically groundwater) for analysis using EMDs. Active microbial sampling devices are used to collect a grab sample of the microbial community from a particular point in time. Passive microbial sampling devices provide a time-integrated sample of the microbial community. Both methods, when combined, can be used to assess monitored natural attenuation and evaluate enhanced bioremediation alternatives.

Active: Various biomass extraction/filtration approaches are available for collecting active microbial biomass from environmental media. Collecting biomass samples using filtration techniques is preferable to the common practice of collecting and shipping chilled groundwater samples for most EMD techniques (Ritalahti et al. 2010b). Field filtration approaches involve low-flow groundwater purging and sampling from monitoring wells, using the same methods that are generally recommended when sampling for volatile organic compounds. In this process, representative groundwater is passed through a filter (for example, Sterivex™), which isolates biomass from the sample. The filter (not the sampled groundwater) is then shipped overnight on ice to a laboratory for analysis. The ESTCP Guidance Protocol available under ER-0518 provides a step-by-step approach to groundwater sampling using field filtration methods (Lebron et al. 2011).

Passive: Passive devices are groundwater sampling tools (such as biofilm coupons, in situ microcosms, groundwater dialysis chambers, porous beads, Bio-Trap® samplers) that facilitate subsurface microorganisms colonizing onto a retrievable matrix. Biofilm coupons, are an artificial growth surface used to monitor biofilms in disparate environments. However, passive microbial sampling devices are not quantitative, since it is difficult to relate microbial concentrations in the groundwater or aquifer matrix to those detected on the passive devices because of biases imparted by the sampling media (Lebron et al. 2008).

EMD samples need to be transported in sterile containers, to the extent possible, to reduce the likelihood of biota associated with the container being detected in the sample.

Examples of sampling devices are included in Figures 10-1 and 10-2.

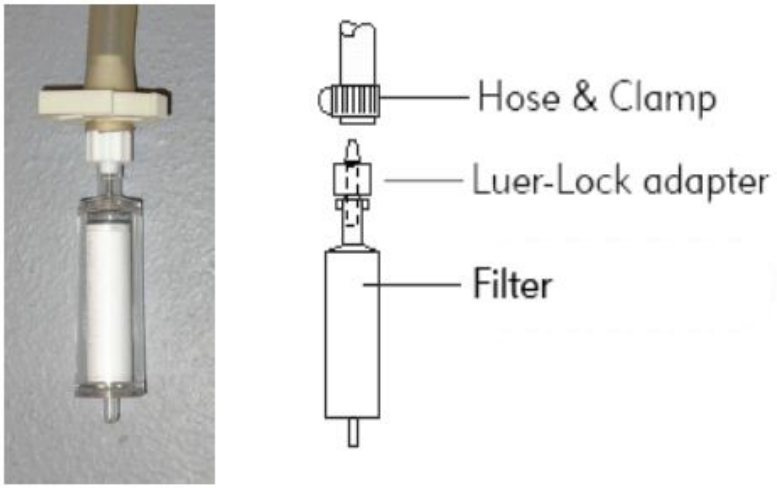

Figure 10-1. Groundwater sampling flow through filter.

Source: Microbial Insights 2012,http://www.microbe.com/images/stories/er/sampling/er_bio-flo_protocol_dna_mi.pdf.

Figure 10-2. BioTrap® passive microbial sampling devices.

Source: Microbial Insights 2012,http://www.microbe.com/index.php?option=com_content&view=article&id=76:bio-trap-samplers&catid=19:bio-trap-samplers&Itemid=33.

10.5 Sample Preservation

Sample preservation conditions upon receipt at the laboratory should be reported with any EMD analytical shipment. Sample preservation is critical for EMD analysis, as it is for chemical analysis. The approach to sample preservation depends on whether the material analyzed is DNA, RNA or whole cells (microbial activity).

10.5.1 DNA

While DNA is considered to be relatively stable, it is subject to degradation, which would impact the integrity of samples and the accuracy of subsequent testing in the analytical laboratory. In

general, DNA degradation is enhanced under high temperatures, acidic or alkaline conditions, and the activity of DNases, enzymes that specifically degrade DNA (Lebron et al. 2008).

Freezing of soil cores, including in situ freezing during core collection has been demonstrated effective for preserving DNA in environmental samples for analyses (Johnson 2012). Storage at -80°C was shown in the same study to produce statistically equivalent results from DNA analyses following a storage period of five months, and may be preferable for some applications.

Preservatives may increase the stability of DNA in groundwater, thereby increasing the accuracy of the downstream analysis and potentially allowing for increased hold times (i.e., storage of groundwater prior to biomass collection). However, this practice is not common, and preservatives are not currently used in direct groundwater sampling methods. If samples are not frozen, regardless of whether preservative is used, samples should be shipped cold (4°C) and received by the analytical laboratory the following day (i.e. guaranteed overnight delivery, confirm with the laboratory that staff will be able to process the shipment on the day delivered).

10.5.2 RNA

Samples collected for assessment of RNA activity should be preserved either with a commercially-available preservation solution, or by freezing at -80°C. Samples should not be frozen if preservative is added. Like DNA, freezing of soil cores, (including in situ) has also been demonstrated effective for RNA analyses (Johnson 2012). Storage at -80°C was shown in the same study to produce statistically equivalent results from RNA analyses following a storage period of five months. As with DNA, freezing groundwater samples in the field and storage at -80°C are logistically difficult. Alternative approaches should be carefully evaluated and at a minimum, samples should be shipped cold (4°C) by overnight delivery.

An exception to the preference for freezing of DNA or RNA samples involves the use of passive microbial sampling devices. Passive microbial sampling devices are typically deployed in groundwater monitoring wells for an incubation period of 30-90 days. Samplers are then recovered and shipped overnight on ice for analysis. Commercial active and passive microbial sampling devices are assembled under sterile conditions and shipped in sterile containers. After sampling, both types of samplers should be shipped cold (4°C) by overnight delivery to their respective locations for analysis. If recovered passive microbial sampling devices were naturally frozen, they must not thaw en route to the laboratory for analysis.

10.5.3 Whole cells

If whole cell samples are to be analyzed for microbial activity (such as enzyme activity probes) the samples should not be frozen or treated with preservatives as both can lead to lysis (destruction of living cells) with a concurrent loss of activity. Samples should be shipped cold (4°C) by overnight delivery. Transit times and time from receipt to analysis at the laboratory should be evaluated with data from whole cell analyses.

10.6 Complementary Traditional Site Characterization Data

Site specific data determined by more traditional analytical techniques are essential to interpretation of EMD results. EMD results that are consistent with site understanding based on traditional analytical techniques increases confidence in EMD data quality and appropriate technique selection. The traditional parameters that aid in interpretation of EMD data include site specific geology, hydrogeology, biogeochemistry, and water chemistry, as well as concentration trends for the contaminant.

10.6.1 Biogeochemical parameters

The success of nearly all in situ technologies depends on adequate characterization of site hydrogeology. Important determinations include which parts of an aquifer are connected, whether the aquifer is confined or unconfined, what the relative permeability and dispersivity of various portions of the aquifer are, the direction and flow rate of groundwater (gradient), and seasonal considerations (change in groundwater flow direction or influence of aquifer recharge events). For example, the EMD analysis of groundwater collected in one portion of an aquifer may be of limited use in assessing the microbiology of a different portion of the aquifer.

10.6.2 Hydrogeological parameters

Readily available groundwater chemistry tests of provide the context in which to understand EMD data. Biogeochemical parameters such as pH, oxidation-reduction potential (ORP), and dissolved oxygen complement EMD data by indicating whether or not conditions are favorable for the desired microbially-mediated reactions (ie., oxidation of fuel hydrocarbons or reductive dehalogenation of chlorinated organics) and microbial communities. Additionally, measures of dissolved species such as nitrate, nitrite, ferrous and ferric iron, sulfate, sulfide, and methane, can provide additional insight into the prevailing ORP and the abundance of terminal electron acceptors and electron donors.

10.6.3 Water quality parameters

Water quality parameters such as total dissolved solids (TDS), total organic carbon (TOC), and chloride content can also be indicators of the likelihood of enhancing microbial activity at a site.

10.6.4 Concentration trends

Knowledge of how the concentration or mass of the contaminant has changed over time can be used to evaluate the remedial approach, but it can be difficult to determine in the presence of separate phase (sorbed or non-aqueous liquid) materials. CSIA, as discussed in Section 3.0, can be an effective tool in this scenario. Where the contaminant is exclusively dissolved, the change in the aqueous phase concentration of the contaminant over time is an important factor in interpreting

EMD results at the site. Favorable EMD results typically coincide with or precede favorable contaminant concentration or geochemical trends.

As discussed previously, the location of the contaminant concentrations within the aquifer or soil provides the context for any EMD data which is collected. This effect may be particularly pronounced when there are separate phase (LNAPL, DNAPL) constituents or when the constituent has an inhibitory and/or toxic effect on the microbial population (for example, elevated 1,1,1-TCA concentrations). Contaminant concentrations can also enhance the distribution of some microorganisms. Other variables can also provide important spatial context for EMDs such as aquifer solids or locations of groundwater/surface water interactions.

10.7 Method QA/QC

In the field, current practices recommend, but do not require, data quality samples such as field blanks, equipment blanks, matrix blanks, and trip blanks. The inconsistent use of data quality samples creates problems in identifying erroneous data, such as samples affected by cross contamination or deterioration during shipping.

Of the biological EMDs, only PCR-based methods have USEPA guidance related to laboratory quality: "Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples" (USEPA 2004a). This document provides a good example of a detailed QA analysis for a particular laboratory method. The document's guidance could be extrapolated to those laboratories performing qPCR for environmental remediation applications. When considering those EMDs not based on PCR, this document still provides many meaningful QA/QC considerations. Additional QA/QC guidance can be obtained from the USEPA qPCR method for *Enterococci:* "Method A: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay" (USEPA 2010).

10.8 Known Biases (Extractions, PCR, Microbial Ecology)

Bias is a measure of agreement or disagreement between the concentration of an analyte as measured by a method and the true concentration in the environmental sample. Each EMD may have biases specific to the methodology. Please refer to the individual EMD sections for discussion of these biases. Because DNA extraction and amplification support several of the EMDs, some common biases are presented here.

Any analysis involving the extraction of DNA from an environmental sample should consider how selective or incomplete DNA extraction might impact results and their interpretation. The inability to accurately sample DNA from the entire microbial community in soil is generally accepted by the scientific community. Some DNA extraction methods tend to favor more abundant species over less abundant ones, or may be selective for particular groups while other groups may remain unnoticed (Feinstein et al. 2009; Inceoglu et al. 2010; Lipthay et al. 2004). Humic substances and other compounds common in soils and sediments may also inhibit PCR amplification or interfere with nucleic acid analyses (see Wilson 1997 for review). Moreover, DNA purification procedures

may also result in a loss of DNA or may impact the efficiency of subsequent amplification (Roose-Amsaleg et al. 2001). On the other hand, recent evaluation of SIP analyses indicates that "multiple DNA extractions on soil samples improves the extractable DNA yield and the number of quantifiable eubacterial 16S rRNA gene copies, but have little qualitative effect on the identification of the bacterial groups associated with the degradation of a given carbon source" (Jones et al. 2011). There is considerable scientific uncertainty about the value of the "rare but potentially important" species that may be under-represented with some EMDs (especially for long term natural attenuation). For example, it is possible that important contaminant degrading microorganism may not be dominant when EMDs are used to assess the microbial community at a contaminated site and may be overlooked.

Bias can also occur when using PCR to amplify certain target DNA sequences due to properties of the target (such as the concentration of DNA to be amplified), the flanking sequences, or the overall genome. Additional bias may also be introduced depending on the type of primers used, polymerases used, and the reaction conditions of the amplification process. The DNA replication process itself is not always perfect and may result in errors, such as deletions or additions of incorrect nucleotide bases. Bias also occurs when the amplification efficiencies of target sequences are not the same (that is, when some copies of PCR products are not amplified in subsequent cycles). Reporting sufficient information to address known sources of potential bias is recommended as part of QA/QC procedures. For additional information on recommended reporting procedures, see the recommended information requirements table contained in each EMD section (for qPCR example, see Section 4.3.1).

10.9 Blanks/Contamination Controls

Laboratory procedures can inadvertently introduce the substance to be detected into an otherwise uncharacterized sample. Such contamination, sometimes referred to as “cross contamination”, is of particular concern in DNA-based methods due to their sensitivity. Cross contamination is also a problem in methods involving live cells due to the possibility that those cells may replicate during transport and handling or that cells may lyse and have their cellular contents degraded (e.g., via the action of nucleases).

The ability to evaluate a blank and reference control is a critical QC criterion for all methods, and the extent to which the blank has followed the path of the uncharacterized sample can make results more convincing. Blanks should be reported with any analytical batch.

10.10 Positive Controls

A positive control shows that, in the presence of the substance to be analyzed, the test returns a positive result. Positive controls provide one indication that the test or measurement is working properly and can be relied on to detect the presence of a substance and potentially a measure of its quantity. This approach can be either qualitative or quantitative to evaluate the procedures employed. Positive controls should be reported with any analytical batch. Data from analyses

where any positive control fails should be discarded. As an example, positive controls for qPCR should include a qPCR amplification positive (a DNA source that can be amplified by the same primer as the target sequence, analogous to a known concentration gas chromatography standard), and matrix spike samples.

10.11 Negative Controls

A negative control shows that, in the absence of the substance to be analyzed, the test returns a negative result. Negative controls provide another indication that the test or measurement is working properly and can be relied on to provide a negative result or measurement of non-detect in the absence (or presence at a concentration below the detection limit) of a substance. Negative controls should be reported with any analytical batch. Data from analyses where any negative control fails should be discarded. As an example, negative controls for qPCR should include a DNA extraction negative (DNA-free water) and a qPCR amplification negative.

10.12 QA Metrics for Qualitative Analyses

The key metrics of qualitative analyses are the rates of false positives and false negatives. As a laboratory conducts positive and negative controls for a given analysis, it should track the rate at which those controls indicate the analysis has failed. In controlled laboratory conditions, these rates should be low, but they should be measurable. These rates should be available on request from the analytical laboratory.

The false positive and false negative rates may be used to estimate the number of results needed to confirm an initial result at a predetermined level of confidence. For example, if the combined false positive and false negative rates total 10%, multiple analyses should be required in order to reach a pre-determined confidence of 0.05 (5%).

10.13 QA Metrics for Quantitative Analyses

The acceptability and utility of quantitative analyses are routinely evaluated according to several standard measures. This section discusses how to apply the standard measures of precision, accuracy, completeness, representativeness, comparability, and sensitivity to EMDs.

10.13.1 Precision

Precision is the degree to which a set of observations or measurements of the same property, usually obtained under similar conditions, produce the same result—how reproducible is a measurement? (USEPA QA Glossary). Precision is usually expressed as standard deviation, variance, percent difference, or range, in either absolute or relative terms. Determining precision typically requires both field and laboratory duplicate samples (for example, minimum of one sample for each field sample and laboratory negative or positive reference controls). Additional considerations of precision for each EMD are discussed in the individual EMD method descriptions as needed.

10.13.2 Accuracy

Accuracy is the degree of agreement between an observed value and an accepted reference value. Accuracy includes random error (precision) and systematic error (bias or recovery) that are caused by sampling and analysis. Accuracy is typically reported as percent recovery of laboratory control samples, in relation to a method reporting limits. Additional considerations of accuracy for each EMD are discussed in the individual EMD method descriptions as needed.

10.13.3 Representativeness

The degree to which data reflects actual site condition may need to be considered. Field sampling biases and biases associated with laboratory methods may need to be addressed to maintain consistency with the data quality objectives, conceptual site models, or remedial objectives. Representativeness should be judged for the study design, specifically the location, number, and frequency of samples.

10.13.4 Comparability

Typically, data comparability includes correlation between field and laboratory data. However, it may also include correlation between procedures, quantitation units, and reporting formats between laboratories. Pay careful attention to the relevance of positive and negative controls to the field samples being tested. For example, is laboratory-grade water a valid substitute for site groundwater?

10.13.5 Completeness

Completeness can include the number of valid (usable or non-rejected) results in relation to the total number of results. Some regulatory agencies may have minimum completeness goal for each project (e.g., 85% minimum completeness). Completeness can be judged by comparing the planned number of samples and the valid results obtained. Failure to meet completeness criteria can lead to questions about whether the data obtained is sufficient to support the hypothesis.

10.13.6 Sensitivity

Sensitivity can involve limits of detection by the procedures employed (e.g., method detection limit, quantitation limit) and a determination if they meet project goals. Trip blank or lab blank results are often used to report/indicate quantitation limits. Sensitivity of the analysis should be reported in some way with each data package where relevant.

10.14 Reporting

Data from any analysis must be communicated in a manner that not only expresses the results of an analysis but also provides adequate supporting information to determine the acceptability and usab-

ility of the result. Where possible, EMDs should meet the same conceptual data quality standards as other environmental analyses.

10.14.1 Laboratory Reporting

The laboratory report should provide data to address the relevant quality control issues described in this chapter to the extent possible. Each method section of this guidance includes information about laboratory reporting requirements (e.g., for qPCR see Section 4.3.1). In addition, an example of a detailed checklist of report requirements is the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al. 2009). These guidelines "target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency" (Bustin et al 2009).

Reports typically include a laboratory data verification and quality assurance summary from the laboratory performing the analyses. For example, a qualitative analysis should report the associated blank, positive control, and negative control as well as, potentially, the overall false positive and false negative rates for a given analysis. Similarly for quantitative analyses, sufficient data should be provided so that the sensitivity, precision, and accuracy of that analysis can be determined.

10.14.2 Study Reporting

The study report should compile laboratory QC data and address all QA parameters related to the relationship among samples (i.e., representativeness, comparability, and completeness). Ideally, these factors will be presented in the relevant work plan and follow through the study report.

10.15 Project Life Cycle Specific Considerations

EMDs can improve site management at several points in the project life cycle. Any point where questions are raised regarding the site biological activity, EMDs should be considered.

10.15.1 Site Characterization

With EMDs, site characterization can include an appraisal of the site's microbial community (e.g., presence of degraders or overall community composition) and its real or potential microbial activity. When evaluating the spatial variation of a microbial species or activity using an EMD, it is important to collect a sample from an unaffected area for comparison. The USEPA document, “Guidance for Comparing Background and Chemical Concentrations in Soil for CERCLA Sites” (USEPA 2002a), provides guidance on selecting a reference area that can be applied to EMDs. The statistical methods detailed in this guidance can also be used for quantitative EMDs (e.g., qPCR). Statistical comparisons require that both background and on-site datasets are sufficiently large for the statistical tests. Biological systems also vary temporally (seasonal and even daily cycles) and sampling programs should reflect this by being contemporaneous if possible and avoiding seasonal extremes.

10.15.2 Remediation

EMDs can contribute lines of evidence to corrective action plans and FSs to support the selection of active bioremediation and/or natural attenuation strategies. Criteria for the number and location of samples are similar to those described for site characterization (Table 10-1).

Baseline. Similar to background, when evaluating a change in a given EMD over time or related to treatments that occur over time, it is important to have data that represent the range of values 'before' a treatment or event so that they may be contrasted with 'after'. Since samples are collected from single points in time, the data are representative "snapshots" of the microbial community. Thus, ongoing sampling events are typically used to describe how microbial conditions vary over time.

Assessing Progress at the Site. EMDs can be applied to answer site specific questions such as: Does this amendment stimulate microbial activity in the manner anticipated? Will an amendment promote biodegradation appreciably above what would be achieved using MNA? Did the bioaugmentation culture remain active? Was there a shift in the dominant members of the microbial community? While a detailed discussion of these possibilities is beyond the scope of this section, it is important to consider the specific question applicable to the site and determine how directly relevant the data are. In most cases, EMDs will provide inferential data about the biological status of a site that relate to site geochemistry and contaminant fate rather than provide conclusive proof of a particular activity or outcome. Consequently, EMDs are typically used in conjunction with other data to provide multiple lines of evidence for a decision.

Remedial Optimization. In pilot testing or in efforts to improve the performance of biologically-based, or chemically-based treatment systems, EMDs can provide key insights into the composition and response of the microbial community in key areas of the site. Criteria for the number of samples collected are similar to those described for site characterization and baseline measurements (Table 10-1). As previously stated, EMDs are not a substitute for collecting and evaluation of more conventional site metrics. Physical and chemical knowledge of the site is essential for interpreting EMD data. EMDs should complement existing chemical and physical information about the site.

10.15.3 Monitoring

When EMD data are being used to guide site activities, there may be an ongoing need for EMD testing to establish the stability or trend for an organism or degradation gene associated with site contaminants. Anytime testing is repeated, the frequency of that testing should be appropriate for the parameter being tested.

10.15.4 Closure

It may be possible for a site to achieve closure without meeting regulatory cleanup criteria if cleanup goals can be achieved in a 'reasonable' time frame, or there are no receptors or pathways

for contaminant exposure. Though predictions regarding biodegradation may be based on observed degradation rates, EMDs can add key supporting information. In addition to the current rates, estimates of the stability of those rates are implied in the closure scenario. The composition of the microbial community, as identified by various EMD data may complement other lines of evidence that suggest biodegradation is likely to continue, and that a biodegradative process are likely sustainable. As discussed in Section 10.15.2, the persistence of biodegradation is difficult to predict, but it may be critical to a prediction of the timeframe for remediation. However, it may be prudent to continue monitoring with EMDs for an extended period to demonstrate the stability of a microbial community or activity.

Some EMDs can aid in estimating biodegradation rates (CSIA and the Rayleigh equation in particular as discussed in Section 3.3.3.2, Question N). Care must be taken, however, in extrapolating rates both spatially and temporally. Although a change in number of gene copies, or number of microorganisms in a given sample, could be used to infer that biodegradation is occurring, currently there is no quantitative method to calculate a rate of biodegradation from a detected number of gene copies per liter.

10.16 Specialized Application Consideration

EMDs may also answer site-specific questions that do not fit in the preceding sections.

10.16.1 Presence, abundance, activity

Among the site management questions related to remedy selection and implementation, particularly monitored natural attenuation, there are some distinctions that must be drawn among the types of data that are available and whether they are related to presence, abundance, or activity.

Presence

When the question is simply whether or not a type of microorganism or a functional gene is present at a site, a qualitative test such as PCR, of the microorganism's presence or absence will suffice.

Abundance

If some indication of how much of a given microbe or gene is present at a site, a quantitative analysis is needed, such as qPCR.

If the question involves how much gene expression is occurring at a particular moment in time, quantitative activity analyses such as RT-qPCR and EAPs are needed. However, there are no quantitative methods to calculate a degradation rate from a detected number of gene copies per liter, though an emerging approach uses the number of gene copies, or counts of number of the organisms, to potentially infer degradation rates.

Activity

Contaminant fate and transport modeling, in addition to a scheduled time-series sample collection and analyses program can also help to predict contaminant fate or biodegradation rates.

Was there a shift in the dominant members of the microbial community? While a detailed discussion of these possibilities is beyond the scope of this section, it is important to consider the specific questions applicable to the site, and determine how directly relevant the data are. In most cases, EMDs will provide inferential data about the biological status of a site that relate to site geochemistry and contaminant fate rather than provide conclusive proof of a particular activity or outcome.

Monitoring through seasonal changes or changes in groundwater elevation along with known trends may be beneficial in predicting the stability of biological activity. Ultimately, ongoing monitoring suggests the stability of any measured effect, and it will be a matter of judgment whether anticipated changes in site conditions are likely to change biological activity.

10.16.2 Frequency of EMD sampling

The frequency of EMD sampling depends on the hypothesis being tested. Default quarterly and semi-annual groundwater monitoring schedules have been used and been able to show changes in microbial communities and activity. In some cases, more frequent sampling may be desirable.

As an example, bioaugmentation may be confirmed by sampling as little as a week after injection. It is recommended that EMD sampling be conducted more frequently (e.g., monthly or quarterly) immediately following bioaugmentation, "to monitor the distribution and proliferation of dechlorinating bacteria (i.e., *Dhc*) in the treatment area" (Lebron et al. 2011). Changes in amendments may also show results quickly. Considering seasonal changes in site characteristics (e.g., predominant groundwater flow direction) may also be appropriate. Regardless of sampling frequency, "two years of quarterly monitoring are recommended during bioremediation implementation" (Lebron et al. 2011). High costs associated with some methods (EAP, SIP, and FISH) may lead to less frequent sampling.

10.16.3 Trends in EMD data

A number of statistical approaches can be used to decipher trends regarding quantitative analyses (e.g., qPCR, EAPs, and FISH).These approaches are discussed in detail in “Guidance for Data Quality Assessment Practical Methods for Data Analysis: EPA QA/G-9” (USEPA 2000). Trend analysis is not possible for qualitative measures.

10.16.4 Low contaminant concentration sites

At sites where MNA is the chosen remedy for chlorinated ethenes, it is important to select groundwater sample locations "where total VOC concentrations are at least 100 µg/L" (Lebron et al.

2011). At locations where VOC concentrations are less than 100 µg/L, "organohalide-respiring bacteria may not be present in high numbers due to low electron acceptor (chlorinated ethenes) concentrations" (Lebron et al. 2011).

Therefore, immediately after bioaugmentation, "MBT sampling should be conducted more frequently (e.g., monthly or quarterly) to monitor the distribution and proliferation of dechlorinating bacteria" (i.e., *Dhc*) in the treatment area (Lebron et al. 2011). Regardless of sampling frequency, two "years of quarterly monitoring are recommended during bioremediation implementation" (Lebron et al. 2011).

10.17 Summary

The absence of standard methods for analysis of molecular EMD tools necessitates strict adherence to a rigorous QA/QC plan. Success of an EMD application will depend on several factors, including the following:

1. development of a site specific sampling plan for spatial extent and frequency that is developed in response to project goals
2. use of appropriate sampling methodologies, including selection of active or passive sampling, preventing sample contamination, and sample handling practices that preserve the microorganisms or nucleic acids depending on the selected EMD
3. appropriate use of blank, control and duplicate samples to provide controls of sources of error in sampling and analysis
4. adaptation of sampling and EMD tool selection to the site project life cycle

11.0 REGULATORY ACCEPTANCE AND ISSUES

Although EMDs have been used over the past 25 years in various scientific fields, particularly medical research and diagnostic fields, their application to environmental remediation management is relatively new and rapidly developing. One of the expected challenges in implementing EMDs for environmental remediation is acceptance by the regulatory community. The ITRC EMD team surveyed regulators, consultants, and stakeholders to determine the following:

- the current use and level of interest in EMDs
- regulatory or other constraints and barriers to using EMDs
- training needs of potential EMD end users
- who has experience with EMDs and may be willing to share case studies with a wider audience

Based on the survey results (see Appendix B), most regulators are not familiar with EMDs. Of the 54 regulators who responded, 65% were not familiar with or had not applied EMDs. Only four were identified as having applied EMDs in the field of environmental remediation. In the course of the survey, when provided with a description of each EMD, regulators identified key areas in environmental remediation where they thought EMDs could be used. Some thought different EMDs could be used as: a forensic tool; a way to evaluate remediation alternatives or feasibility studies; a monitoring tool for site management; a tool for site investigation/characterization; or a tool to expedite site closure. Although most regulators may currently be unfamiliar with EMDs and how to apply them in environmental remediation management, once the benefits of EMDs are better understood, acceptance is expected to increase.

In November 2011, ITRC published a series of EMD Fact Sheets (EMD-1) to provide introductory information about and promote awareness of a selection of EMDs applicable to environmental remediation management (including site characterization, remediation, monitoring, and closure). The EMD Fact Sheets, in conjunction with this document, should significantly improve EMD awareness not only among regulators, but also in the environmental community at large. This section provides guidance on involving regulatory agencies in the approval process for using EMDs and also describes regulatory permitting considerations.

11.1 Regulatory Approval Process

If EMDs will be proposed for a site, the regulator must be involved as early as possible in the EMD selection process. During the initial meeting, it is best to have a draft work plan for the EMD sampling, analysis and data use already prepared for discussion. The work plan can easily be adjusted to reflect all agreements reached during the meeting and can be submitted later as a final document. The work plan at a minimum should:

1. Clearly explain the site status based upon already existing traditional analytical chemistry methods.

2. Identify which EMDs are to be used (ITRC's EMD Fact Sheets (EMD-1) may be used as reference material).
3. Explain how EMD data can help to arrive at additional information to complement the existing data, or describe what is expected to be learned by using the EMD.
4. Identify at what stage of the life cycle process the EMD is to be used.
5. Identify sample locations.
6. Identify data quality objectives: type, quality, and quantity of data to be collected. Survey results showed that the lack of standardized QA/QC procedures was a main concern among regulators. To allay these concerns, both the standard operating procedures and internal QA/QC information of the laboratory performing the EMD analysis should be submitted as part of the work plan. Data quality information for EMDs is addressed separately in this document (see Section 10.0 and Section 3.3). Also, additional QA/QC and minimum reporting information is included with each method description in Sections 3 through 9.

All permitting requirements that are necessary for the use of the EMD must be identified to facilitate regulatory and stakeholder acceptance.

11.2 Permitting Requirements

The ITRC EMD team used survey results and results from a questionnaire completed by the states' POCs to identify permitting/regulatory concerns that may be raised when the use of EMDs is proposed. As expected, the responses varied from state to state. However, at a minimum, approval must be obtained for one or more of the following: notification, a work plan, a discharge permit or a Underground Injection Control (UIC) Permit. The use of amended EMD sampling devices, such as stable isotope probing (SIP) and in situ enzyme activity probes (EAPs) involve the introduction of contaminant-bearing materials into the subsurface. Although the introduced contaminants are small in quantity and are intended to stay in place, these in situ evaluations may require additional regulatory review and approval, or a UIC Permit. In cases where groundwater discharges to surface water, a discharge permit may be required. In cases where drinking water wells could potentially be impacted, it may be necessary to notify drinking water regulatory programs or even end-users or well owners. A thorough review of permitting requirements and regulatory approval is encouraged on a site-specific basis whenever the use of EMDs is proposed.

11.3 EMDs with No Permitting Requirements

Not all EMDs involve contaminant-bearing materials being introduced into the subsurface. Most are laboratory analyses only and therefore do not require permitting. These include CSIA, qPCR, microbial fingerprinting, microarrays, and FISH, but may also include all SIP and EAPs that are not conducted in situ. Although permits may not be required for laboratory analyses, prior approval from the regulatory agency may be needed. Therefore, at a minimum, a work plan as described above should describe the intended use and expected outcome.

11.4 Future Regulatory Considerations

Team members and most of those surveyed agree that education is the key to more widespread use of EMDs in the environmental remediation field. As regulators become better educated and more comfortable with their use, guidance and regulations specific to EMD use will be developed. Until state documents are developed, this ITRC document and ITRC's EMD Fact Sheets (EMD-1) serve as the most comprehensive resources available for regulators, consultants, and the general public.

12.0 PUBLIC AND TRIBAL STAKEHOLDER ACCEPTANCE AND ISSUES

Stakeholders include a broad array of people in communities living near contaminated facilities, site-specific advisory boards, restoration advisory boards, local governments, and a variety of non-governmental organizations. This section serves as a guide to public and tribal stakeholders who are dealing with contaminated sites where Environmental Molecular Diagnostics (EMDs) may be used.

This section addresses the concerns of those tribal governments and stakeholders affected by contaminated sites where EMDs may be used. EMDs can be classified into two major categories of analytical techniques: chemical techniques, specifically compound specific isotope analysis (CSIA), and a variety of molecular biological techniques (MBTs). MBTs evaluate the types, abundance and biochemical capabilities of microorganisms present in the environment. EMDs are relatively recent techniques that could be used to address important contaminated site management questions. The full list of questions is included in Table 2-3.

1. Are contaminant-degrading microorganisms present in the soil or groundwater that have the ability to degrade the contaminants in question? Several EMDs involve detection of genes and these techniques can determine whether the native bacteria at a site are capable of degrading specific contaminants. Some of these techniques are quantitative and can determine the abundance of specific contaminant-degrading bacteria. However, by themselves, EMDs that either detect or quantify specific genes typically do not provide unequivocal evidence that biodegradation is occurring at a site.
2. Are the bacteria active?
3. Is biodegradation or abiotic destruction occurring?
4. Are there multiple sources of contamination, and can they be distinguished? If a particular source is suspected to be the cause of the contamination, stable isotopic ratios can provide evidence to prove or disprove a particular source as the cause of the contamination.

Generally, stakeholders are favorable to advanced technologies that provide additional lines of evidence to site characterization, selection of remedial alternatives, and monitoring. Stakeholders, if properly informed, see the use of EMDs as an important addition to traditional analysis (see Table 2.1). However there are many concerns that should be addressed in proposals to use EMDs. Chief among them are the general concerns related to access to, and use of, these technologies. Specifically, EMD technologies potentially can be used to confirm or refute liability and also to support or disprove the viability of natural attenuation. Generally, concerns that EMD technologies will be used primarily to absolve a responsible party of blame or to provide a false proof that natural attenuation will occur are chief among those of stakeholders.

12.1 When and How are Stakeholders Involved?

Interested and affected stakeholders may be involved in all stages of a project, from site characterization through closure. There are important communication issues associated with applying

EMDs at any site, especially with nontechnical audiences. Stakeholders are generally comfortable with traditional sampling and analytical methods for groundwater, soil, sediment, surface water and air. When new sampling and analysis methods are used (such as EMDs), it is extremely important to effectively communicate the reason why a new technique is added to the suite of tests that stakeholders are already familiar with, and highlight the advantages and the limitations of the technique being used.

Many stakeholders (as well as many in the regulatory community) do not have the technical expertise to understand microbiology and a host of scientific terms used when describing EMDs (see Appendix C, Isotopic Chemistry and Appendix D, Microbiology FAQ). This lack of expertise increases the importance of effective stakeholder communication. When proposing EMDs, therefore, it is important to effectively educate and communicate with stakeholders when the results of a diagnostic test will lend weight to a position in dispute. For example, in one case the results of a CSIA approach were interpreted differently by a number of responsible parties, their consultants and regulatory agencies. This particular example was due to poor correlation between findings and conclusions, and would have benefited from a more rigorous work plan. In this case, some stakeholders remained skeptical that using new methods that are portrayed as improved scientific techniques will, in fact, provide definitive results. When proposing the use of EMDs for a site, take care to communicate the expectations for the results, carefully plan the data collection, and acknowledge that it is not always possible for the EMDs to provide definitive results. Information about developing plans for EMD data collection are included in Section 3.3 for CSIA, Section 10.3 for MBTs, and also in Section 11.0.

12.2 Issues Specific to Tribes

Tribes are different from public stakeholder groups because tribes have government-to-government relationships with federal, state, and local governments, and this status must be respected. There are 565 federally recognized tribes in the United States; the individual states also recognize additional tribes. Each tribe is a unique entity culturally, governmentally, and socially. This section provides some general guidelines and issues pertaining to tribes; the concerns of the affected tribes at specific sites need to be addressed on a case-by-case basis.

Tribes will share many of the same concerns with the public stakeholders. However, tribes have further concerns specific to their own interests that may not be shared with the public stakeholder groups. Some tribes view any level of contamination of their land and natural and cultural resources as a grave insult. Most tribes have areas that are culturally significant or sacred. Examples of these include springs, mountains, hunting areas, plant-gathering areas, or burial sites. Some tribes consider certain natural structures or features as a living being, to be protected and afforded all the rights of a human tribal member. As related to the use of EMDs, there is no unified "Native American" view or policy on laboratory techniques.

Because of prior negative experiences with government agencies, some tribes view both federal and state entities with distrust. It is important to communicate honestly about whether or not a par-

ticular EMD technology may actually help to address an environmental problem that the tribe is facing.

12.3 General Stakeholder Attitudes Towards the Use of EMDs

When using new EMD technologies that can lead to various interpretations, be aware of the following:

- Given the early stage of development of EMDs, data quality must be strict.
- Protocols for sample collection, storage of samples, and quality control are not standardized for many of the EMDs. Refer to data quality guidance provided in Section 3.0 (CSIA) and in Section 10.0.
- Stakeholders have a general distrust of MNA, and many view it as a "do-nothing" approach. Proving or disproving that biologically driven natural attenuation is occurring is a major purpose of many of the EMDs. EMDs should be used in conjunction with traditional lines of evidence to provide assurance that remediation is indeed occurring. EMDs should not be used in isolation.
- The burden of proof will be relatively high to convince stakeholders that the added knowledge derived from EMDs is reliable, particularly when the results support monitored natural attenuation (MNA).
- Conversely, stakeholders are generally very supportive of biodegradation and enhanced techniques that degrade and destroy contaminants. Evidence that there is a need to biostimulate or bioaugment is often a desirable outcome of using the EMDs presented in this guidance.
- Stakeholders also have interest in forensic applications, such as determining anthropogenic or synthetic sources of contaminants or identifying the different origins of comingled plumes.

12.4 Specific Stakeholder Concerns About EMDs

- Some stakeholders may object to placing microbial sampling devices in the subsurface (such as in situ passive sampling devices, in situ application of stable isotope probing (SIP), or enzyme activity probes (EAPs).
- While polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) indicate that a sample contains a specific microorganism, sampling strategies need to be developed to indicate if the organisms exist in other parts of the site being characterized, remediated, or monitored.
- Many of the EMDs are used to detect the presence of specific bacteria and microorganisms that have the potential to degrade contaminants. While this information is helpful, it does not necessarily indicate that the bacteria and microorganisms are actually degrading the compound. Thus, other information must be used in conjunction with the EMDs to confirm biodegradation.
- The terminology of some of the EMDs is often misunderstood. For example, some stakeholders may think that the word isotope in CSIA and SIP refers to a radioactive substance. Other misperceptions could be that some EMDs are used for cloning or genetic

modifications. While these examples may appear to be far-fetched, they are real concerns for laymen and can easily be allayed with a good and well-executed communication plan.

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APPENDIX A. CASE STUDIES

The case studies included in this appendix represent sites where EMDs have been used for site management decision making. References are included in each case study for more information. These case studies are provided in addition to the summaries of EMD use at more sites (included in each method section).

Table A-1. EMD Case studies summary

Case study no.	Complementary EMD	Environmental medium	Contaminants	Life cycle stage	State
CSIA					
A.1	None	Groundwater	Perchlorate	Site Char-acterization	NY
A.2	None	Vapor Intrusion	PCE, TCE, DCE	Site Char-acterization	CA
A.3	None	Groundwater	TCE	Site Char-acterization	NJ
qPCR					
A.4	EMD Sampling Methods	Groundwater	PCE, TCE, DCE	Remediation	NY
A.5	CSIA	Groundwater	PCE, TCE, DCE	Remediation	CA
RT-qPCR					
A.6	qPCR, EMD Sampling Meth-ods	Groundwater	BTEX and MTBE	Remediation	CA
EAP					
A.7	CSIA	Groundwater	TCE	Remediation	KY
SIP					
A.8	EAPs, qPCR	Groundwater	TCE, 1,4-diox-ane	Remediation	AZ
A.9	qPCR	Groundwater	Fuel oil com-pounds	Remediation	NJ
Microarrays					
A.10	qPCR	Groundwater	Uranium	Remediation	CO

A.1 CSIA for Perchlorate (NY)

Adapted with permission from: Böhlke, J.K.; Hatzinger, P.B.; Sturchio, N.C.; Gu, B.; Abbene, I.; Mroczkowski, S.J. 2009. Atacama perchlorate as an agricultural contaminant in groundwater: Iso-

topic and chronologic evidence from Long Island, New York. *Environmental Science & Technology*. 43: 5619-5625. Copyright 2009 American Chemical Society.

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- Compound Specific Isotope Analysis (CSIA)

Contacts

Dr. JK Böhlke
United States Geological Survey
(703) 648-6325
jkbohlke@usgs.gov

Dr. Paul Hatzinger
Shaw Environmental, Inc.
(609) 895-5356
paul.hatzinger@shawgrp.com

A.1.1 Site Background and Knowledge from Conventional Methods

Perchlorate (ClO_4^-) has been detected in groundwater at a number of different locations on Long Island, NY, and various sources may contribute to this contamination (Pokorny, 2003; Abbene, 2006; Munster, 2008; Bohlke et al. 2009). Possible sources, among others, include fireworks production and use, agricultural fertilizer application (historical and current), road flares, military facilities including missile launch sites, disinfection with bleach, and use of perchloric acid in manufacturing. Conventional methods for detection of perchlorate in groundwater include ion chromatography with conductivity detection (USEPA Method 314) and more recent methods using conventional mass spectrometry such as USEPA Method 331.0 (ion chromatography with electrospray ionization/mass spectrometry). These methods provide accurate concentration data, but do not yield relevant information on perchlorate sources (see USEPA 2009 for a summary of analytical methods).

CSIA was used to quantify ratios of the stable isotopes of chlorine ($^{37}Cl/^{35}Cl$) and oxygen ($^{18}O/^{16}O$ and $^{17}O/^{16}O$) in ClO_4^- using isotope-ratio mass-spectrometry (IRMS; Böhlke et al. 2005; Sturchio et al. 2006, 2011a; Böhlke et al. 2009). This technique can be used to distinguish natural ClO_4^- (derived from past application of natural fertilizers or from atmospheric formation) from synthetic ClO_4^- sources and to evaluate the extent of ClO_4^- biodegradation in the environment.

A.1.2 EMD Objectives and Approach

The objective of this study was to determine sources of ClO_4^- in groundwater at multiple locations within Suffolk County on Long Island, NY. The full details of this work are presented in Böhlke et al. (2009).

Groundwater samples were collected from three distinct areas in Suffolk County with ClO_4^- in groundwater (Figure A.1-1). However, historical land use and potential sources of ClO_4^- were distinctly different.

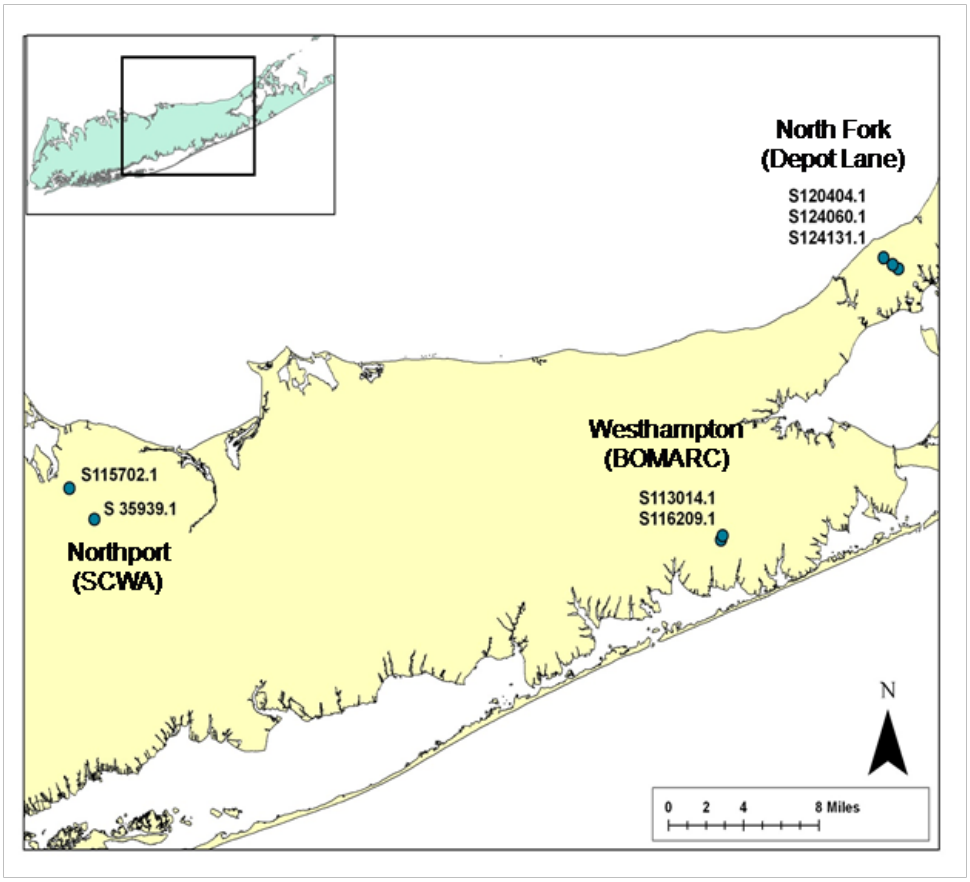

Figure A.1-1. Location map of groundwater wells sampled for perchlorate isotopes on Long Island, NY (North Fork, Northport, and Westhampton).

Source: Adapted with permission from Böhlke, J.K.; Hatzinger, P.B.; Sturchio, N.C.; Gu, B.; Abbene, I.; Mroczkowski, S.J. 2009. Atacama perchlorate as an agricultural contaminant in groundwater: Isotopic and chronologic evidence from Long Island, New York. Environmental Science & Technology 43: 5619-5625. Copyright 2009 American Chemical Society.

The wells are located in the following areas:

- DL Series wells are in a predominantly agricultural area on the North Fork of Long Island, where natural nitrogen fertilizers have been used historically.
- BM Series wells are at the Boeing and Michigan Aerospace Research Center (BOMARC) in Westhampton, where missile were stored between 1957 and 1969. This area was being used a training area for the Suffolk County Police Department at the time of the study and included both a firing range and a fireworks disposal area.

- NP Series wells are large production wells operated by the Suffolk County Water Authority (SCWA) near Northport. This area is currently residential, but was a large scale farming region prior to the 1950s.

CSIA analysis of Cl and O isotopes in ClO_4^- was used to forensically identify the source of ClO_4^- in the wells from each area. Samples also were analyzed for various geochemical parameters, dissolved gases, and atmospheric environmental tracers (^3H and ^3He isotopes, SF_6, and CFCs) to determine the likely timing of the ClO_4^- infiltration to the aquifers.

A.1.3 Results

Perchlorate was present in all three areas, and a number of the wells had concentrations in excess of the New York State Department of Environmental Conservation guidance level of 5 micrograms per liter (µg/l), as follows:

1. DL Series, current agriculture use: 4.6–10 µg/l
2. BM Series, Missile storage and fireworks disposal: 40–4,300 µg/l
3. NP Series, historical agriculture use: 8.4–11.2 µg/l

Stable isotope analyses of Cl ($\delta^{37}Cl$) and O ($\delta^{18}O$, $\Delta^{17}O$) were obtained from groundwater samples in each region as well as data for supporting geochemical and groundwater dating parameters. The $\delta^{37}Cl$, $\delta^{18}O$, and $\Delta^{17}O$ values of the ClO_4^- collected from the BM wells (n=2) were consistent with values typical of synthetic ClO_4^-, while samples from the NP production wells (n=2) and the DL series agricultural wells (n=3) were consistent with natural ClO_4^- from Chilean fertilizers, including the elevated values of $\Delta^{17}O$ that have been reported for this source (Figure A.1-2; Böhlke et al. 2005; Bao and Gu, 2004). There was no indication of isotopic fractionation of ClO_4^- consistent with partial biodegradation in the site groundwater (Hatzinger et al. 2009). In Figure A.1-2, the data from the Long Island wells are plotted as black diamonds, the data from synthetic sources as open red circles, and those from Chilean samples as open blue squares.

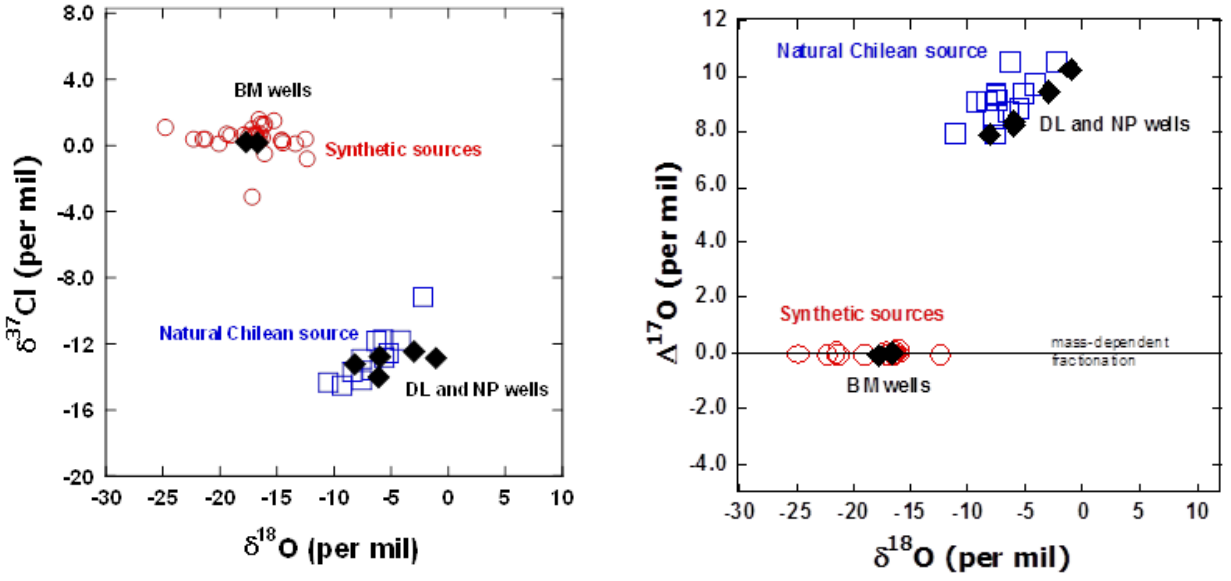

Figure A.1-2. Comparison of $\delta^{37}Cl$ vs. $\delta^{18}O$ (left) and $\Delta^{17}O$ vs $\delta^{18}O$ (right) in ClO_4^- from wells on Long Island with those of synthetic and Chilean source materials.

Source: Adapted with permission from Böhlke, J.K.; Hatzinger, P.B.; Sturchio, N.C.; Gu, B.; Abbene, I.; Mroczkowski, S.J. 2009. Atacama perchlorate as an agricultural contaminant in groundwater: Isotopic and chronologic evidence from Long Island, New York. Environmental Science & Technology 43: 5619-5625. Copyright 2009 American Chemical Society.

The two BM wells, which contained unusually high concentrations of ClO_4^-, were near a fireworks disposal pit used by the Suffolk County Police Department. The groundwater in these wells also had anomalously high concentrations of K, Sr, and Sb, which are present in fireworks to provide various colors (Conklin, 1985). Although a number of local sources of synthetic ClO_4^- may be present at this site, leaching of unexploded fireworks as the cause of groundwater contamination is supported by presence of a fireworks disposal pit in the area, the extremely high ClO_4^- levels in each of the wells, the anomalously high concentrations of trace elements common to many fireworks, and the young ages of the groundwater (1-2 years, based on environmental tracer data).

In contrast to the BM wells, the isotopic characteristics of ClO_4^- from the DL and the NP production wells were consistent with those of the ClO_4^- found in Chilean nitrate deposits and fertilizers. Groundwater in these wells also had relatively high concentrations of NO_3^- and other constituents that are typical of recharge beneath fertilized agricultural land in this region, such as Ca, Mg, and SO_4^{2-}. No other ClO_4^- sources, including the US indigenous sources, are currently known to have the distinctive combination of low $\delta^{37}Cl$, low $d^{18}O$, and high $\Delta^{17}O$ that characterize the Chilean ClO_4^-. Thus, the data indicate that the ClO_4^- in these wells was derived from the historical use of Chilean nitrate fertilizers on Long Island. Age dating of groundwater supports this hypothesis, as much of this water was determined to have recharged decades ago.

A.1.4 Conclusions

The ClO_4^- stable isotope results ($\delta^{37}Cl$, $\delta^{18}O$, and $\Delta^{17}O$) and key supporting chemical and environmental tracer data collected from several wells on Long Island provide strong evidence for the presence of ClO_4^- derived from Chilean nitrate fertilizer as well as that from a synthetic source, presumably fireworks disposal. The groundwater at all locations was aerobic and un-denitrified, and ClO_4^- apparently was not affected isotopically by biodegradation or exchange processes in the subsurface. Stable isotope analysis of ClO_4^- indicates that imported Chilean nitrate fertilizer use on Long Island has led to contamination of some aquifer units, even though this fertilizer may have been applied in relatively small quantities as long as 40 or more years ago. In the absence of CSIA analysis, and key supporting parameters, perchlorate in groundwater could not be attributed to a particular source, making this technique invaluable for forensic investigations. Further information on this study can be found in Böhlke et al. (2009) and Hatzinger et al. (2011).

The CSIA technique described in this case study provided critical information concerning the sources of ClO_4^- in several monitoring and municipal wells on Long Island, NY. The isotope and supporting data clearly showed that multiple sources, including fireworks and imported natural Chilean fertilizers, contribute to ClO_4^- contamination in this region. A recent CSIA study from a site in southern California showed a similar result. In this case, two distinct ClO_4^- plumes were defined, one derived from a synthetic source and the other from past application of natural Chilean fertilizer (Sturchio et al. 2012). In the absence of the CSIA technique used at each site, source discrimination would be difficult, if not impossible.

A.1.5 Costs

The CSIA technique described in this case study is currently available on a commercial basis from the Environmental Isotope Geochemistry Laboratory at the University of Illinois at Chicago. The current cost for the analysis can be obtained from this facility.

A.1.6 Outcomes and Challenges

Compared to many other environmental sampling and analysis techniques, CSIA is relatively new and ClO_4^- stable isotope analysis has only been performed during the past several years. There are currently no USEPA-certified methods for CSIA of organic or inorganic compounds of any type. However, a recent document from the USEPA acknowledges the utility of CSIA for forensics and monitored natural attenuation and provides guidance concerning method application and relevant QA/QC during sampling and analysis (USEPA 2008a). While the USEPA document is primarily focused on (1) carbon isotope analysis in organic compounds and (2) using CSIA to document biodegradation, some of the general principals also apply to ClO_4^- isotope analysis. In addition, a new guidance document specifically focused on perchlorate isotope analysis is now available (Hatzinger et al. 2011).

CSIA for ClO_4^- is a relatively new technique, and methodological development and improvement is ongoing. Some of the current challenges include: (1) the quantity of ClO_4^- required for analysis

(~5 µg is recommended during field collection) which requires sample collection with specialized ion exchange columns (Bohlke et al. 2009; Sturchio et al. 2011; Hatzinger et al. 2011); (2) the requirement for extensive purification of ClO_4^- from other anions and organic compounds prior to analysis; (3) the few laboratories that perform the analysis; and (4) an inability to distinguish different sources of synthetic ClO_4^- from each other due the general similarity between $\delta^{37}Cl$ and $\Delta^{17}O$ among synthetic forms, (although some consistent variability in $\delta^{18}O$ has been reported; Sturchio et al. 2006).

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Adapted with permission from: McHugh, T., T. Kuder, S. Fiorenza, K. Gorder, E. Dettenmaier, and P. Philp. 2011. "Application of CSIA to Distinguish Between Vapor Intrusion and Indoor Sources of VOCs." *Environmental Science & Technology* 45:5952-5958. 2011. Copyright American Chemical Society.

EMD Technology

- Compound Specific Isotope Analysis (CSIA), Air Forensics

Contacts

Thomas McHugh, Ph.D.
GSI Environmental
(713) 522-6300
temchugh@gsi-net.com

R. Paul Philp, Ph.D.
The University of Oklahoma
School of Geology and Geophysics
(405) 325-4469
pphilp@ou.edu

Adria Bodour, Ph.D.
Air Force Civil Engineer Center (AFCEC)
Environmental Center of Excellence (ECoE)

Environmental Restoration Technical Support Branch (CZTE)
(210) 395-8426
adria.bodour.1@us.af.mil

A.2.1 Site Background and Knowledge from Traditional Methods

Hill Air Force Base (AFB), near Ogden, Utah, has been an active military base since before World War II. Historic waste management practices have resulted in contamination of shallow groundwater with trichloroethene (TCE) and other chlorinated volatile organic compounds (VOCs). Impacted groundwater has migrated off site into residential areas and the Air Force has monitored contaminant concentrations in groundwater and indoor air in neighborhoods surrounding the base.

These investigations have identified a number of houses with elevated concentrations of TCE and other chlorinated solvents in indoor air. Subslab depressurization systems have been installed to mitigate vapor intrusion (migration of contaminants from the subsurface into buildings) in homes where detected concentrations of contaminants are above Mitigation Action Levels developed by USEPA Region 8 and the Utah Department of Environmental Quality (UDEQ). While vapor intrusion is a primary source of VOCs in indoor air in these residences, indoor sources of VOCs (such as hobby craft glue and gun cleaning agents) are also suspected sources. Traditional vapor analytical methods have made it difficult to distinguish between these two sources.

A.2.2 EMD Objectives and Approach

This study was performed to determine whether compound specific isotope analysis (CSIA) could be used to differentiate vapor intrusion of VOCs from indoor sources of VOCs (Figure A.2-1). Carbon isotopic ratios and chlorine isotopic ratios for TCE or PCE were measured in indoor air samples, groundwater and soil gas samples, and commercial products containing TCE or PCE. The isotopic ratios in indoor air samples were evaluated by comparing the results to 1) the range of isotopic ratios observed in commercial products and 2) the ratios measured in groundwater and soil gas samples collected near the residences.

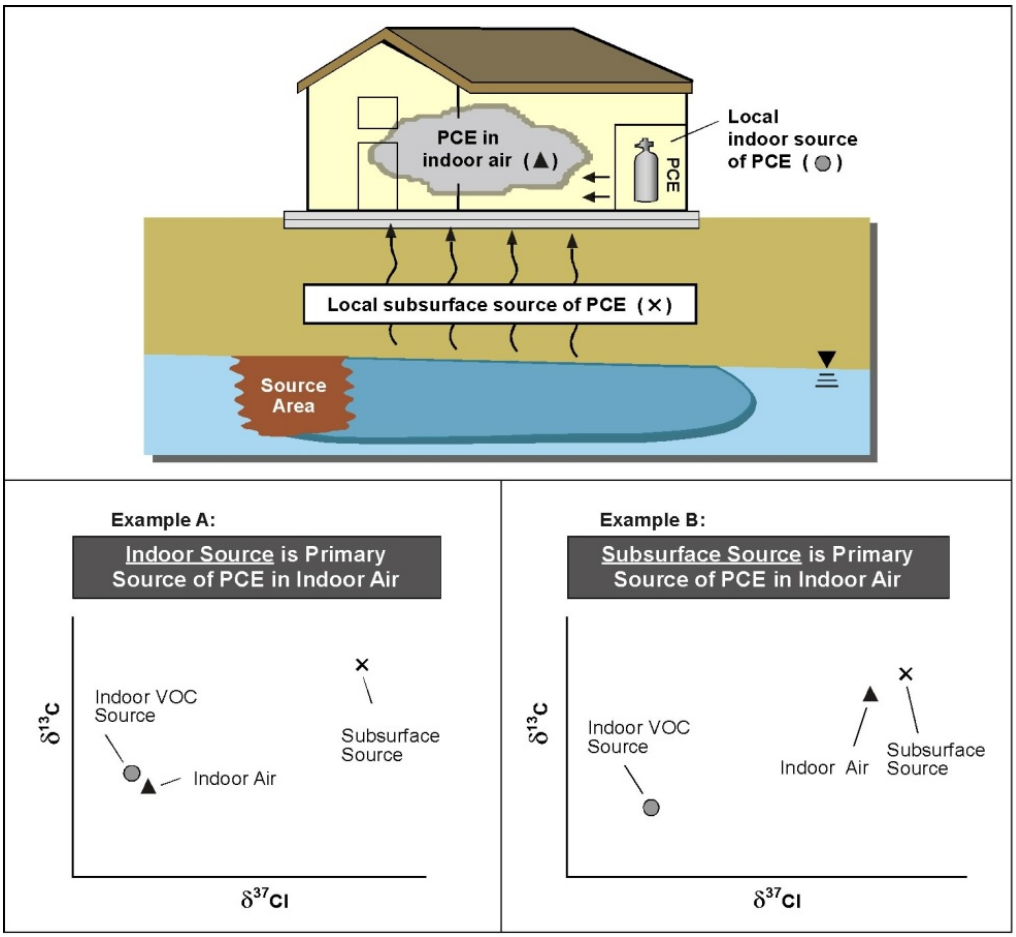


Figure A.2-1 Conceptual graphic illustrating the study approach.

Source: Reprinted with permission from McHugh et al. 2011., T., T. Kuder, S. Fiorenza, K. Gorder, E. Dettenmaier, and P. Philp, 2011, "Application of CSIA to Distinguish Between Vapor Intrusion and Indoor Sources of VOCs," Environmental Science & Technology, 45: 5952-5958. Copyright 2011 American Chemical Society.

Samples were collected from five residences located near Hill AFB (Residences 1 through 5) where TCE or PCE had been detected in indoor air. Figure A.2-2 shows the sampling locations and Table A.2-1 summarizes the contaminant detected in indoor air, the suspected source prior to the CSIA analyses, and the types of samples that were collected near each residence.

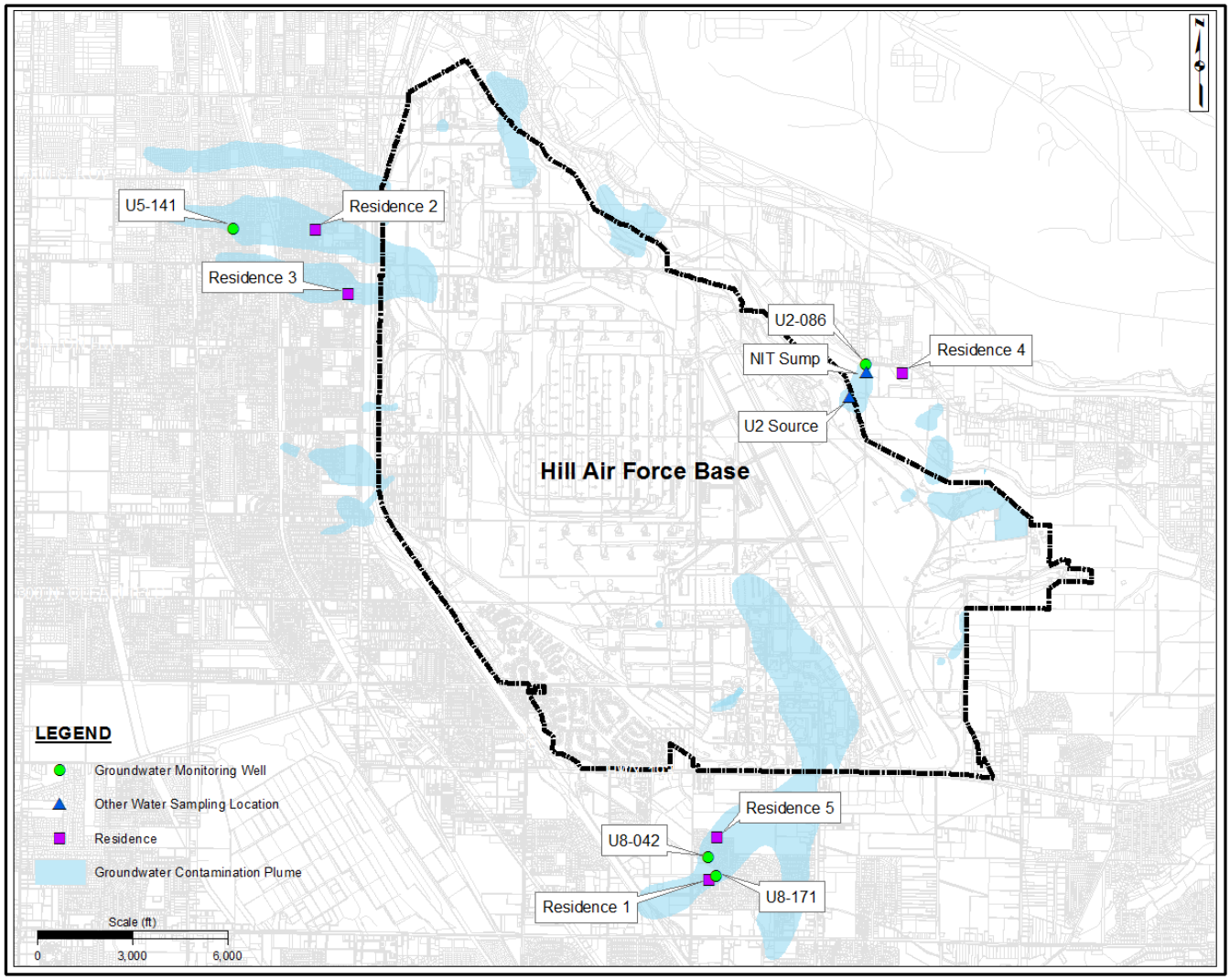


Figure A.2-2. Map of sampling locations.

Source: GSI Environmental, used with permission.

Table A.2-1 Summary of suspected sources and types of samples collected from each residence.

Location	Contaminant Detected in Indoor Air	Suspected Source	Types of Samples Collected
Residence 1	TCE	Vapor intrusion from subsurface	Indoor air, groundwater
Residence 2	PCE	Indoor source	Indoor air, groundwater
Residence 3	PCE	Indoor source	Indoor air, groundwater
Residence 4	TCE	Migration of vapors from sanitary sewer	Indoor air, sewer headspace, groundwater,
Residence 5	TCE	Vapor intrusion from subsurface	Indoor air, groundwater, soil gas

A.2.3 Results

Figure A.2-3 shows the carbon isotopic ratio results for TCE in samples in and around Residence 1 and Figure A.2-4 shows the carbon and chlorine isotope results for TCE and PCE in samples collected in and around Residences 2 through 5.

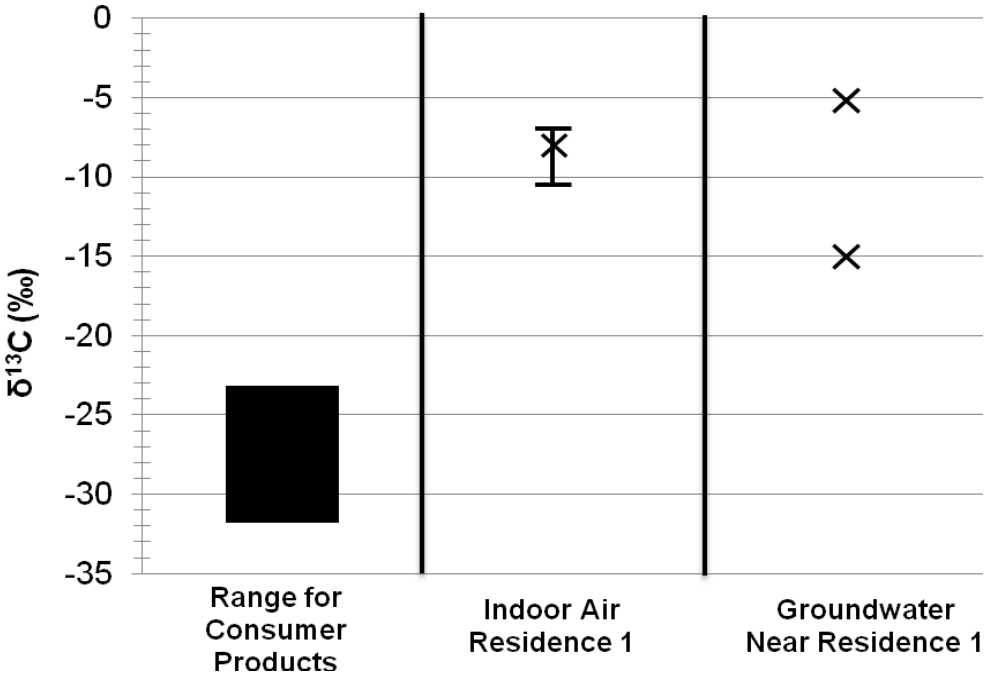


Figure A.2-3. Results for $\delta^{13}C$ for TCE in Residence 1 indoor air and groundwater samples, and the range for consumer products.

Source: Reprinted with permission from McHugh, T., T. Kuder, S. Fiorenza, K. Gorder, E. Dettenmaier, and P. Philp, 2011, "Application of CSIA to Distinguish Between Vapor Intrusion and Indoor Sources of VOCs," Environmental Science & Technology, 45: 5952-5958. Copyright 2011 American Chemical Society.

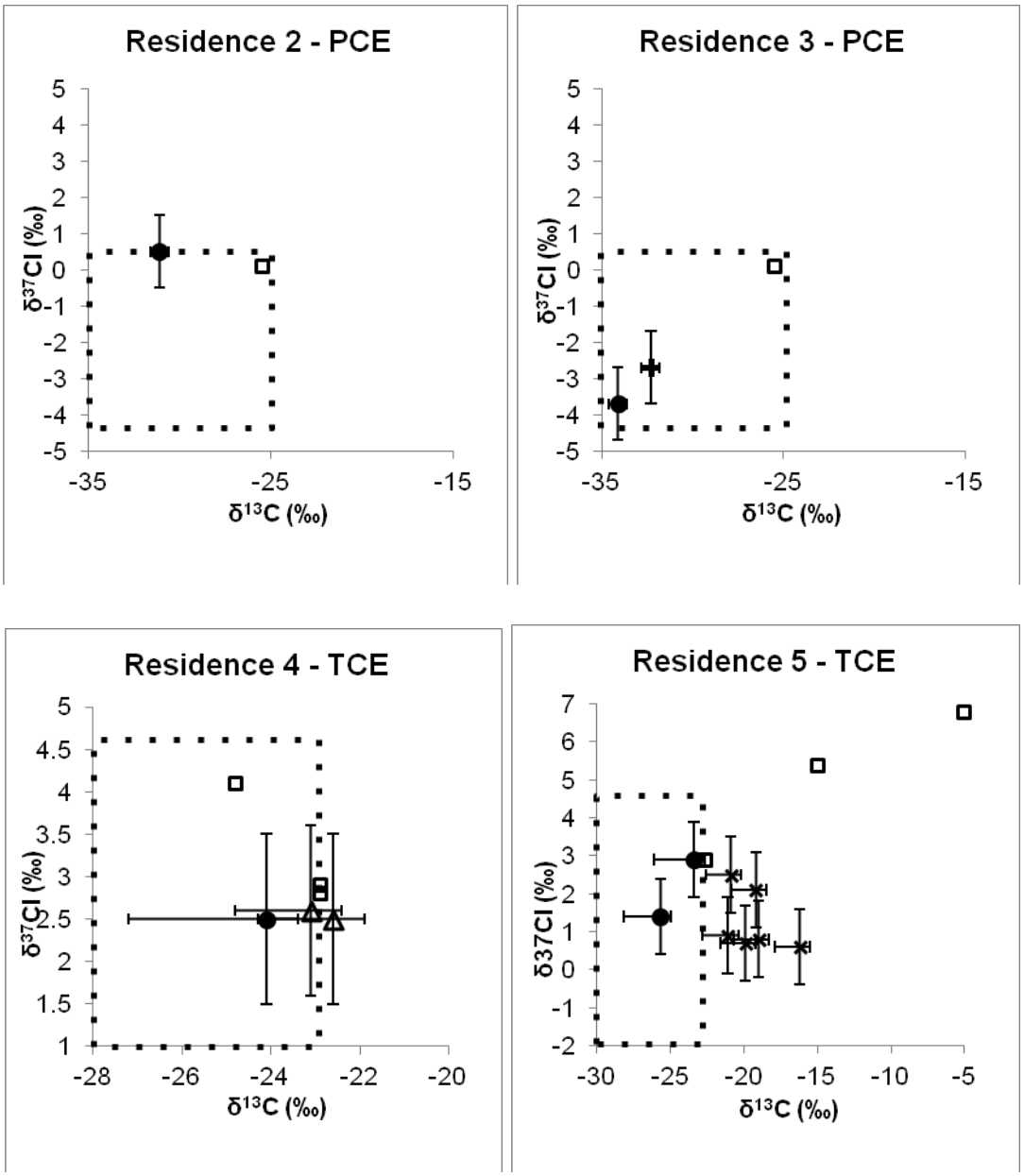


Figure A.2-4. Results for $\delta^{13}C$ and $\delta^{37}Cl$ carbon for PCE or TCE in samples at Residences 2 through 5.

Reprinted with permission from McHugh, T., T. Kuder, S. Fiorenza, K. Gorder, E. Dettenmaier, and P. Philp, 2011, "Application of CSIA to Distinguish Between Vapor Intrusion and Indoor Sources of VOCs," Environmental Science & Technology, 45: 5952-5958. Copyright 2011 American Chemical Society.

The indoor air $\delta^{13}C$ and $\delta^{37}Cl$ values were compared to soil gas sample values, groundwater sample values from sample locations near each residence, and the range of values for commercial products (dashed boxes in Figure A.2-4) that contain PCE or TCE. In Figure A.2-4, the data include groundwater (open squares), indoor air (black circles), and soil gas (x) results.The results support the following observations:

- Residence 1 (Figure A.2-3): The indoor air $\delta^{13}C$ value for TCE was similar to groundwater values for TCE. The indoor air $\delta^{13}C$ value was heavier than the range for commercial products. Although $\delta^{37}Cl$ was not measured, the $\delta^{13}C$ results for TCE in indoor air indicate vapor intrusion as the source. As noted in Table A.2-1, the suspected source of TCE in indoor air, based on the data available prior to the CSIA analyses, was a subsurface source.
- Residence 2 (Figure A.2-4): The indoor air $\delta^{37}Cl$ value for PCE and the groundwater $\delta^{37}Cl$ value were similar. The indoor air $\delta^{13}C$ value was depleted by $6^{0}/_{00}$ relative to that of the groundwater values. The indoor air $\delta^{13}C$ value was within the range measured for commercial products. These results indicated an indoor source of PCE. As noted in Table A.2-1, the suspected source of TCE in indoor air, based on the data available prior to the CSIA analyses, was an indoor source.
- Residence 3 (Figure A.2-4): A PCE-containing glue commonly used for hobby crafts (E6000) was found in the residence. The indoor air $\delta^{13}C$ and $\delta^{37}Cl$ values for PCE closely matched the E6000 values. In addition, the $\delta^{13}C$ and $\delta^{37}Cl$ values were lighter than the groundwater $\delta^{13}C$ and $\delta^{37}Cl$ values. These results are consistent with the source of PCE being E6000 glue in the residence.
- Residence 4 (Figure A.2-4): The indoor air $\delta^{13}C$ and $\delta^{37}Cl$ values for PCE were similar to those for TCE in the sewer headspace and groundwater. Based on site information, the groundwater appears to discharge to the sewer line. These results were consistent with the sewer line as the primary source of TCE in the residence.
- Residence 5 (Figure A.2-4): The groundwater $\delta^{13}C$ and $\delta^{37}Cl$ values exhibited a wide range. For soil gas samples, the $\delta^{13}C$ values were heavier than the values for the closest groundwater sample. Conversely, the indoor air $\delta^{13}C$ values for were lighter than the values for the groundwater samples. A pattern was not evident for the $\delta^{37}Cl$ values at Residence 5. The CSIA results did not identify the source of TCE in indoor air, but indicated a contribution from an indoor source.

A.2.4 Conclusions

Results of this study confirm that CSIA can be useful for differentiating vapor intrusion and indoor sources of VOCs.

- For two residences (Residences 1 and 3), the CSIA results alone provided identification of the VOC source.
- At two residences (Residences 2 and 4), the results were consistent with the likely sources identified with information available before the CSIA analyses were conducted.
- At one residence (Residence 5), the CSIA results were inconclusive with respect to the source of TCE in indoor air.

A.2.5 Costs

For CSIA analyses of groundwater and vapor samples the cost for the isotope analyses for carbon is $350 for the first compound and then $50 for each additional compound that may be present.

The cost for chlorine CSIA is $400 for the first compound and then $50 for each additional compound. If necessary, adsorbent tubes can be rented for $25 per tube.

A.2.6 Outcomes and Challenges

Regulator response to this study was generally positive. For example, the Guidance for the Evaluation and Mitigation of Subsurface Vapor Intrusion to Indoor Air (California DTSC, 2011) mentions: "The use of stable isotopes is a developing technique for vapor intrusion that may merit consideration in some situations."

As discussed in McHugh et al. (2011), the TCE carbon isotopic ratios were affected by the sample handling procedures. Maximum error was estimated and applied as default error bars for the data. In addition, refrigeration of sorbent tubes after collection and shipping samples on ice to maintain a 4°C overnight is recommended.

As part of this study, a lab validation study was performed to evaluate different adsorbents for the sampling tubes to determine which adsorbent would be most appropriate for performing CSIA on vapor samples. The results of the validation study demonstrated that Carboxen 1016 resulted in no fractionation of the compound during sample collection and analysis (McHugh et al. 2011a).

When considering CSIA for the vapor intrusion pathway, a small number of groundwater or soil gas samples located near the buildings of concern should first be evaluated. The isotopic ratios should be measured for the target VOC in the groundwater or soil gas samples. To move forward with a larger-scale investigation, the isotopic ratios measured in these groundwater or soil gas samples should be outside the typical range for commercial products.

A.2.7 References

California Department of Toxic Substances Control (DTSC), 2011. "Guidance for the Evaluation and Mitigation of Subsurface Vapor Intrusion to Indoor Air (Vapor Intrusion Guidance)."

McHugh, T., T. Kuder, S. Fiorenza, K. Gorder, E. Dettenmaier, and P. Philp. 2011. "Application of CSIA to Distinguish Between Vapor Intrusion and Indoor Sources of VOCs." *Environmental Science & Technology* 45:5952-5958.

McHugh, T., T. Kuder, M. Klisch, and P. Philp. 2011a. "Laboratory Validation Report, Use of Compound Specific Isotope Analysis to Distinguish Between Vapor Intrusion and Indoor Sources of VOCs." Prepared for the Department of Defense - Environmental Security Technology Certificate Program (ESTCP), Arlington, Virginia. www.estcp.org

A.3 CSIA for TCE in Groundwater for Site Characterization (NJ)

EMD Technology

- Compound Specific Isotope Analysis (CSIA)

Contacts

Patrick McLoughlin
Microseeps, Inc.
412-826-5245
pmcloughlin@microseeps.com

A.3.1 Site Background and Knowledge from Traditional Methods

At the Aviation plaza site in New Jersey, a release of TCE impacted groundwater. The contamination extended into several layers of the aquifer but at no level was the concentration information conclusive. The information showed two distinct "hot spots" but it could not be proven that there were two separate releases. Determining whether two sources were present was critical for site planning and management decisions.

At the site, the presence of intermediate products *cis*-DCE and VC was evidence of some biodegradation. The intermediates were present in concentrations much lower than that of the parent TCE, and the parent TCE was present in the source zone at concentrations much greater than 1% of solubility. These concentrations indicate that DNAPL is present and that it replaces any dissolved-phase TCE lost to biodegradation by dissolution. Further, it appeared from the concentrations of *cis*-DCE and VC that the biodegradation would not overcome the effects of that DNAPL. This information indicates that the isotopic ratio of the TCE in these wells is not changed by biodegradation. As shown in Figure A.3-1, the TCE concentration data did not clearly show which locations were sources and which locations were areas impacted by the up-gradient sources.

A.3.2 EMD Objectives and Approach

The concentration contour maps indicated several "hot spots," but it was unclear if they were sources or the result of heterogeneous contaminant dissolution, heterogeneous contaminant flow, and heterogeneous biodegradation. Further, it was unclear if intermediate points were a mixing of the contributions from multiple sources. In addition, there are two vertical zones and it was unclear if the contamination in the upper zone originated from the same source as the contamination in the lower zone.

Resolving the unclear issues was crucial to site management. Because robust biodegradation was not occurring at the site, CSIA data could be more clearly evaluated for this purpose. Initially, it was an open question whether both carbon and chlorine CSIA were to be performed to understand the site. In the interest of cost control, it was decided to perform the carbon analyses only first, complementing it with chlorine analyses in a future sampling event based on the results of the carbon CSIA. The carbon CSIA proved to be sufficiently definitive.

Groundwater samples were collected from six site wells as shown in Figure A.3-1.

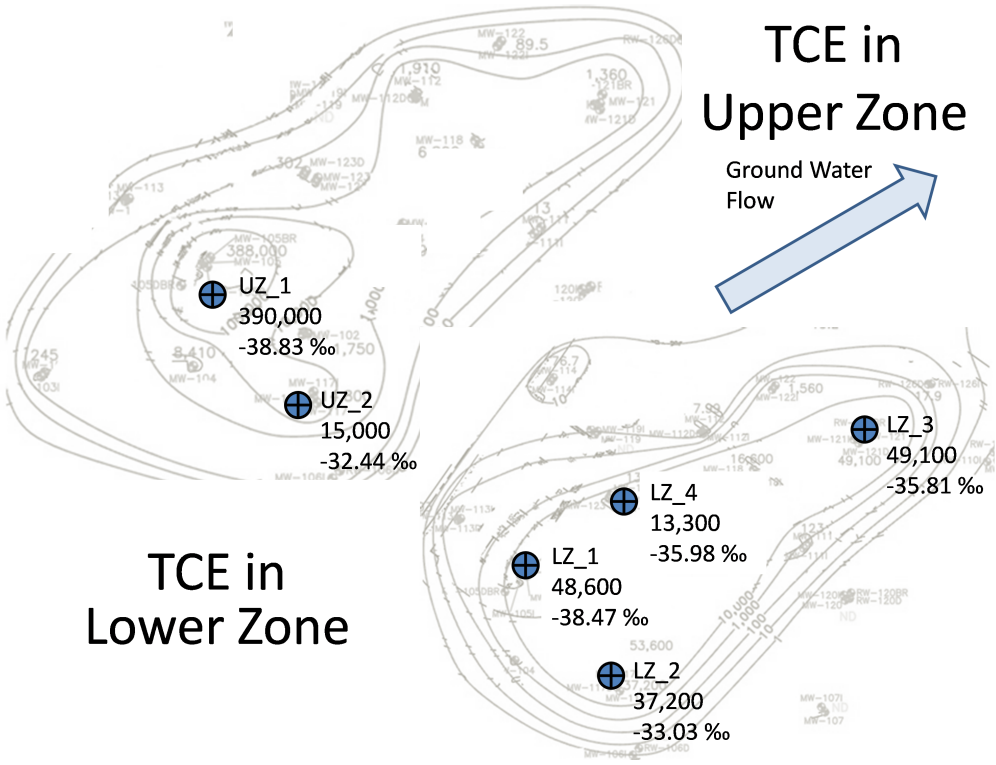


Figure A.3-1. The TCE concentrations and $\delta^{13}C$ values for the Aviation Plaza site. The upper zone is portrayed on the top, the lower zone is portrayed on the bottom.

Source: Microseeps, Inc. 2012. Used with permission.

A.3.3 Results

As shown in Table A3-1, the $\delta^{13}C$ of the TCE in UZ_1 and LZ_1 are very similar in both the upper and lower zones. It can also be seen that the $\delta^{13}C$ of the TCE in UZ_2 and LZ_2 is very similar in both the upper and lower zones but very different from that in UZ-1 and LZ-1. This suggests that the TCE in UZ-1/LZ-1 is from a source that is different from that of the TCE in UZ-2/LZ-2. Further, because the $\delta^{13}C$ of the TCE in LZ_3 and LZ_4 are between the results in UZ_1/LZ_1 and UZ_2/LZ_2, it is suspected those wells do not represent a unique source but are impacted by both sources.

Table A.3-1 Results from CSIA

Upper Zone			Lower Zone		
Well ID	δ (‰)	% saturation	Well ID	δ (‰)	% saturation
UZ_1	-38.83	34	LZ_1	-38.47	4.5
UZ_2	-32.44	1.4	LZ_2	-33.03	3.4
-	-	-	LZ_4	-35.98	0.3
-	-	-	LZ_3	-35.81	4.5

A.3.4 Conclusions

The conclusions from this part of the site work include:

- CSIA showed a significant difference in the $\delta^{13}C$ of the TCE at the two different "hot spots" which indicates there are two different sources.
- Carbon only CSIA was enough to differentiate these sources and further investigation of the chlorine isotopic ratio was not necessary. This provided a cost savings at this site. At other sites, it may be necessary to analyze multiple elements in order to develop a clear understanding of the site.
- The downgradient wells appeared to be influenced by both sources.
- The contamination in the lower zone appears to be from the same source as the contamination in that same geographic location in the upper zone.
- A membrane interface probe (MIP) investigation was performed to confirm the presence of two different sources. Although not typically needed to support the CSIA results, the MIP investigation was performed at this site because the two-source determination had significant site management consequences. The MIP investigation both confirmed the CSIA data and provided other useful information for the conceptual site model (CSM).

A.3.5 Costs

The cost of the CSIA for these six samples was approximately $3,000.

A.3.6 Outcomes and Challenges

CSIA proved to be a powerful tool for forensic purposes. The information gathered through the carbon CSIA results was sufficient to document the existence of two different sources of TCE at this site. Prior to conducting the CSIA study the existing traditional data did not provide sufficient resolution of the two sources. No significant challenges were encountered during the CSIA study at this site.

A.4 Application of qPCR for Chlorinated Solvents in Groundwater for Remediation (NY)

Adapted with permission from: Davis, G., B.R. Baldwin, A.D. Peacock, D. Ogles, G.M. White, S.L. Boyle, E. Raes, S.S. Koenigsberg, and K.L. Sublette. 2008. "Integrated approach to PCE-impacted site characterization, site management and enhanced bioremediation." *Remediation*. 18 (4):5-17.

EMD Technology

- Primary: Quantitative Polymerase Chain Reaction (qPCR)
- Complementary: EMD Sampling Methods

Contacts

Todd M. Caffoe, P.E.
NYSDEC - Region 8
6274 East Avon-Lima Road
Avon, New York 14414
(585) 226-5350
tmcaffoe@gw.dec.state.ny.us

Aaron Peacock, Ph.D.
Haley and Aldrich
(865) 356-0498
APeacock@haleyaldrich.com

A.4.1 Site Background and Knowledge from Traditional Methods

The site is located in upstate New York and industrial use contaminated the area with chlorinated solvents. Several remedial actions were implemented over a period of six years, including pump and treatment remediation followed by multi-phase high vacuum extraction. An estimated 9,600 pounds of volatile organic compounds were removed using these processes before deactivation once asymptotic conditions were achieved. Identification of an in situ remedial approach was sought to obtain site closure.

Additional details are as follows:

- The shallow aquifer was impacted by the chlorinated solvents tetrachloroethene (PCE) and trichloroethene (TCE).
- Examination of groundwater geochemical parameters (dissolved oxygen, nitrate, ferrous iron, sulfate and others) suggested mildly anaerobic conditions. Furthermore, elevated levels of sulfate were observed at the site.
- Under anaerobic conditions, PCE and TCE can undergo sequential reductive dechlorination through the degradation products *cis*-dichloroethene (DCE) and vinyl chloride to ethene.
- Detection of degradation products in groundwater samples suggested that reductive dechlorination was occurring at least to a limited degree under existing site conditions.
- High DCE concentrations combined with relatively low vinyl chloride and ethene concentrations suggested that DCE was accumulating (commonly referred to as “DCE stall”).

A.4.2 EMD Objectives and Approach

The objectives of this study were to 1) quantify the types of microorganisms present under baseline conditions, 2) confirm the presence/absence of microorganisms capable of complete or partial reductive dechlorination of PCE to ethene, and 3) quantify the changes induced in the indigenous microbial community due to injection of different electron donors (biostimulation). Design criteria for the study included the following:

- At the time of site characterization, *Dehalococcoides mccartyi* (*Dhc*) populations were below the laboratory detection limit indicating that the complete reductive dechlorination of PCE to ethene was unlikely.
- qPCR results during the baited Bio-Trap® study and subsequent pilot study demonstrated that addition of electron donor B (two different electron donors were tested and are referred to here as A and B) would stimulate growth of *Dhc* and promote reductive dechlorination.
- During performance monitoring, qPCR data revealed that the observed lag prior to the onset of enhanced reductive dechlorination was due to a temporary increase in methanogens and decrease in *Dhc* abundance following electron donor addition.
- Continued qPCR monitoring demonstrated the rebound in the *Dhc* abundance and most importantly the increase in vinyl chloride reductase genes. Thus, continued reductive dechlorination of vinyl chloride to ethene could be expected.

Site characterization and remedy selection focused on answering the following questions:

- Under existing site conditions, are microorganisms (*Dhc*) present that are capable of complete reductive dechlorination of PCE to ethene?
- Will adding an electron donor (biostimulation) promote growth of *Dhc* and enhance reductive dechlorination of chlorinated ethenes?

To address these questions, a preliminary study was conducted in which sets of three passive microbial sampling devices (specifically, Bio-Traps®) were deployed in select monitoring wells located within the dissolved plume:

- The control Bio-Trap® represented existing subsurface conditions and therefore contained no addition electron donors (Control).
- The second Bio-Trap® contained a commercial electron donor A (BioStim A).
- The third Bio-Trap® contained an alternative commercial electron donor B (BioStim B).

Following a 60 day in-well deployment period, the passive microbial sampling devices were recovered for Quantitative Polymerase Chain Reaction (qPCR) analysis to quantify:

- *Dhc* – the only known species of microorganisms capable of complete reductive dechlorination of PCE to ethene.
- Vinyl chloride reductase gene (*bvcA*) – functional gene encoding the enzymes responsible for reductive dechlorination of vinyl chloride to ethene.

A.4.3 Results

The results of the studies are presented here. Figure A.4-1 includes the results of the qPCR analyses across the three wells where the Bio-Traps® were deployed.

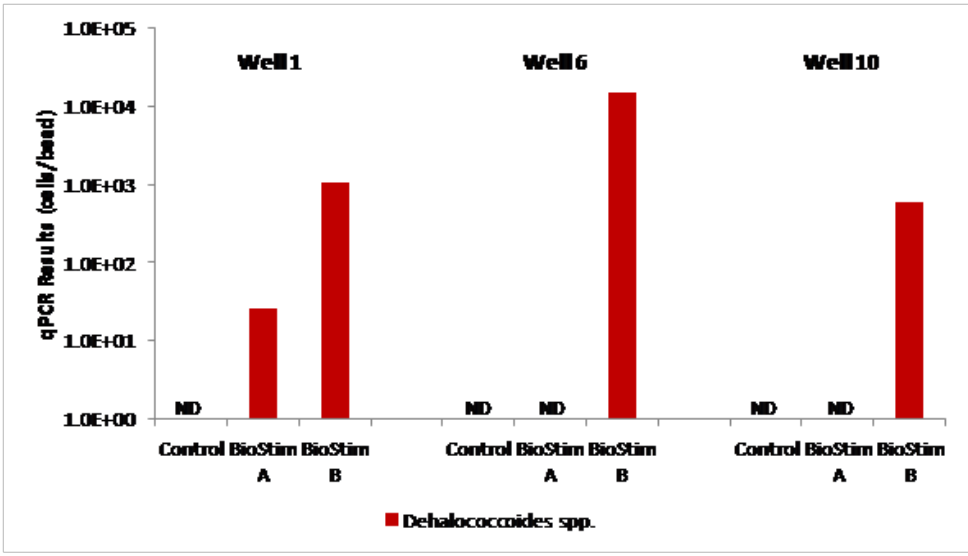


Figure A.4-1: Results of qPCR quantification of *Dhc* following recovery of Bio-Traps® from select monitoring wells following a 60 day deployment period.

Source: Adapted from Davis et al. 2008. Used with permission.

Observations and implications for the qPCR results (Figure A.4-1):

- In the Control Bio-Traps®, *Dhc* populations were below the laboratory detection limit indicating that complete reductive dechlorination of PCE to ethene was unlikely under existing site conditions.
- With BioStim A Bio-Traps®, *Dhc* were only detected at Well 1 and at a low concentration suggesting that the addition of electron donor A did not promote growth of these key halorespiring bacteria, at least within the deployment period.
- Conversely, *Dhc* were detected in each of the BioStim B samplers and at concentrations up to 10^4 cells/bead.

The results suggested the following for the site remedy selection:

- Monitored natural attenuation (MNA) was eliminated as a potential remedy based upon *Dhc* populations being below laboratory detection limits under existing site conditions and historical groundwater monitoring data suggesting DCE stall.
- Biostimulation with electron donor A was eliminated as a potential remedy based upon the fact that *Dhc* populations in the BioStim A samplers were not substantially greater than in the Control sampler.
- Biostimulation with electron donor B was selected for subsequent pilot and full scale corrective actions based in part upon qPCR evidence demonstrating growth of *Dhc* in the preliminary Bio-Trap® study.

In the pilot scale test, electron donor B was injected in the vicinity of Well 12. Groundwater samples were obtained for VOC analysis. Standard, un-amended Bio-Traps® deployed in the injection zone wells were recovered quarterly for qPCR analysis of:

- *Dhc* – the only known group of microorganisms capable of complete reductive dechlorination of PCE to ethene.
- Methanogens – methanogens can compete with *Dhc* and other reductive dechlorinating bacteria for available electron donors.
- Vinyl chloride reductase gene (*bvcA*) – functional gene encoding the enzymes responsible for reductive dechlorination of vinyl chloride to ethene.
- The results of the monitoring during the pilot scale testing are shown in Figure A.4-2 (Days 0 through 200):

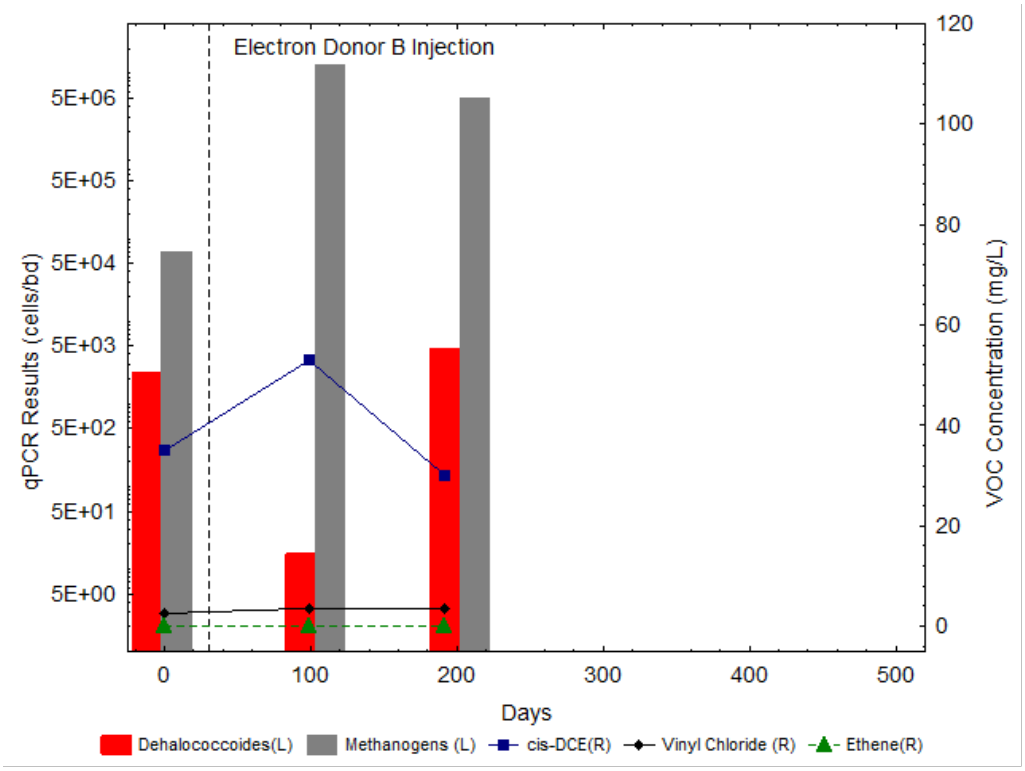


Figure A.4-2. Performance monitoring results through day 200.

Source: Adapted from Davis et al. 2008. Used with permission.

- Prior to the electron donor injection, *Dhc* were detected on the order of 10^3 cells/bead. However, methanogens, who can compete with *Dhc* for available electron donors were present on the order of 10^4 cells/bead.
- After approximately 100 days, the DCE concentration had increased while production of vinyl chloride and ethene was not evident suggesting that electron donor addition had not enhanced continued reductive dechlorination of DCE.

- The qPCR results revealed that electron donor addition had initially promoted growth of methanogens while *Dhc* populations decreased substantially. A temporary increase in competing microorganisms combined with a decrease in *Dhc* is not uncommon following an electron donor addition.
- By day 200, the *Dhc* population had at least rebounded to levels detected prior to injection while the methanogen population decreased. Substantial reductive dechlorination of DCE to vinyl chloride and ethene, however, was still not observed.

The results of the monitoring during the pilot scale testing are shown in Figure A.4-3 (Days 300 through 400):

- By day 300, the *Dhc* population had increased by three orders of magnitude to over 10^6 cells/bead.
- By day 400, the DCE concentration had decreased from 30 to 5.8 mg/L with corresponding production of vinyl chloride.

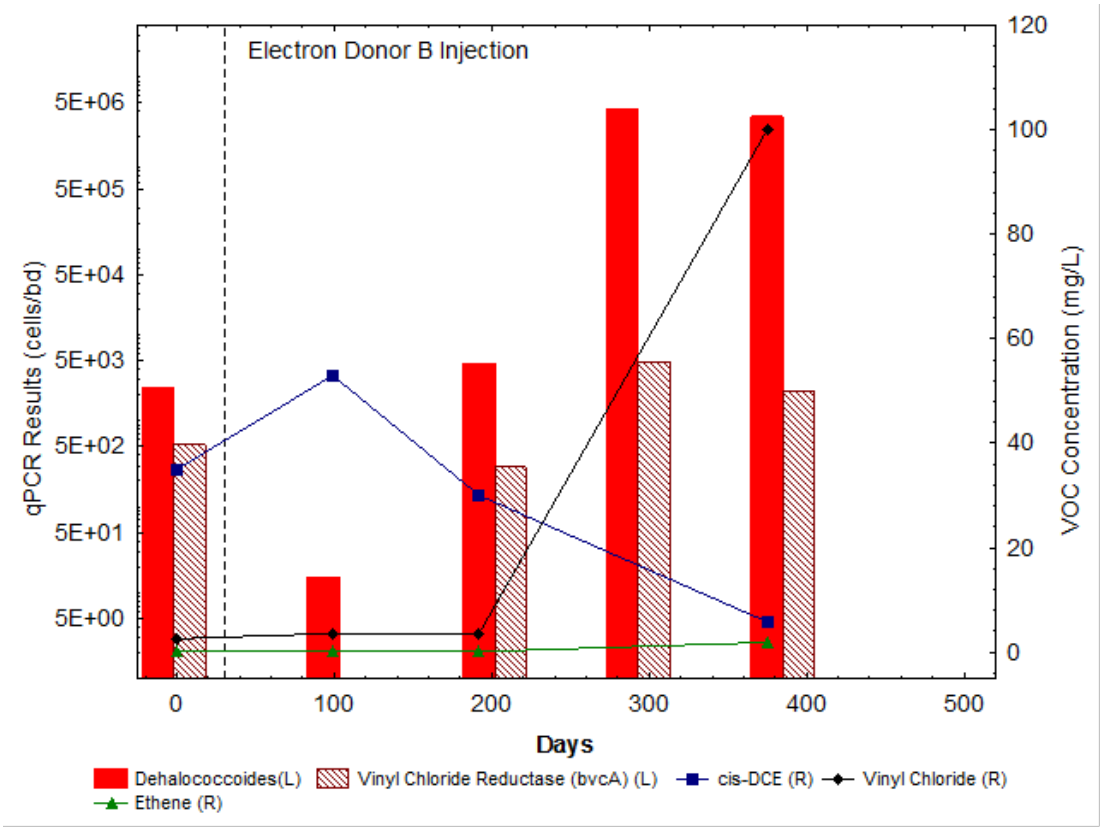


Figure A.4-3. Performance monitoring results through day 400.

Source: Adapted from Davis et al. 2008. Used with permission.

- Production of vinyl chloride, which is documented to be more hazardous than DCE, TCE or PCE, occurred.
- The qPCR quantification of the *bvcA* vinyl chloride reductase gene indicated the presence of microorganisms capable of reductive dechlorination of vinyl chloride to ethene. Thus, at day 400, the qPCR results provided stakeholders with reassurance that the increase in vinyl chloride concentration would be temporary, and complete reductive dechlorination to ethene could be expected.

The results of the monitoring during the pilot scale testing are shown in Figure A.4-4 (Days 400 through 500):

- The *Dhc* population and abundance of vinyl chloride reductase genes were even greater at day 500 than at day 400.
- Consistent with the qPCR results, the vinyl chloride concentration decreased, with a corresponding increase in ethene production.

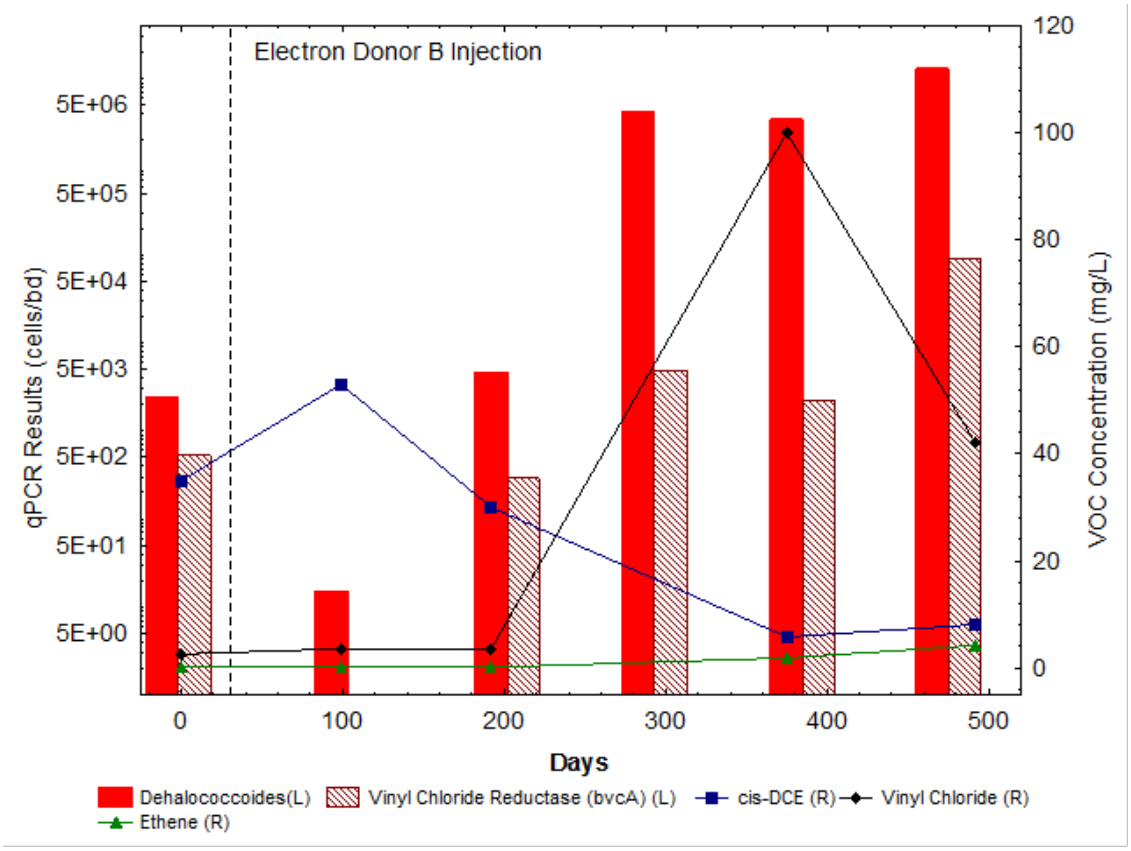


Figure A.4-4. Performance monitoring results through day 500.

Source: Adapted from Davis et al. 2008. Used with permission.

A full scale biostimulation project was implemented at the site with similar results. More information about the project is reported in Davis et al. 2008.

A.4.4 Conclusions

Site characterization and remedy selection resulted in the following conclusions:

- qPCR results demonstrated that the observed DCE stall was due to the lack of microorganisms capable of continued reductive dechlorination of DCE to vinyl chloride and ethene.
- MNA was eliminated as a potential remedy. *Dhc* and the *bvcA* vinyl chloride reductase gene were not detected under baseline conditions, indicating that complete reductive dechlorination of PCE to ethene was unlikely.
- Biostimulation with electron donor B was selected as the site remedy. qPCR results demonstrated that electron donor B, but not electron donor A, stimulated growth of *Dhc* species capable of complete reductive dechlorination of PCE to ethene.

Performance monitoring resulted in the following conclusions:

- qPCR analysis revealed that the initial lag in reductive dechlorination (through day 200) was due to stimulation of competing microorganisms (methanogens) and a decrease in chlorinated ethene degrading bacteria (*Dhc*) following electron donor injection.
- During the pilot study, qPCR results conclusively demonstrated that electron donor B stimulated growth of bacteria capable of complete reductive dechlorination of PCE.
- Increases in *Dhc* and the vinyl chloride reductase gene *bvcA*corresponded to decreases in DCE concentration and production of the degradation products vinyl chloride and ethene, linking the changes in contaminant concentrations to growth of known halorespiring bacteria.
- When vinyl chloride concentrations increased, qPCR quantification of the vinyl chloride reductase gene provided stakeholders with reassurance that the increase in vinyl chloride concentration would be temporary and that complete reductive dechlorination to ethene could be expected.
- Maintenance of elevated populations of *Dhc* and *bvcA* vinyl chloride reductase gene strongly suggested that enhanced reductive dechlorination would continue and that a second electron donor injection was not necessary.

A.4.5 Costs

The cost for a study as described above is around $4,500 per well. This cost includes using a control and two different biostimulations per monitoring well, along with monitoring for qPCR analysis (*Dhc*, vinyl chloride reductase, iron/sulfate reducing bacteria, methanogens), geochemistry, and contaminant concentrations.

Continuing with quarterly qPCR monitoring of *Dhc*, vinyl chloride reductase (*bvcA*), iron/sulfate reducing bacteria, and methanogens was approximately $500 per sample, per event.

A.4.6 Outcomes and Challenges

- The qPCR results were accepted as a valuable line of evidence and the site was reclassified to "No Further Action Required" status.
- Integrating qPCR results with traditional chemical and geochemical analyses provided the converging lines of evidence required to direct site management activities.
- In addition to qPCR, quantification of contaminant degrading microorganisms and quantification of competing microorganisms (such as methanogens and sulfate reducing bacteria) can provide valuable insight when biostimulation is not performing as well as anticipated.

A.4.7 References

Davis, G., B.R. Baldwin, A.D. Peacock, D. Ogles, G.M. White, S.L. Boyle, E. Raes, S.S. Koenigsberg, and K.L. Sublette. 2008. "Integrated approach to PCE-impacted site characterization, site management and enhanced bioremediation." *Remediation* 18(4): 5-17.

A.5 qPCR for Remediation (CA)

Adapted with permission from: ESTCP, 2010, "A Low Cost Passive Approach for Bacterial Growth and Distribution for Large -Scale Implementation of Bioaugmentation," Project ER-200513, Washington, DC. www.serdp.org.

EMD Technology

- Primary: Quantitative Polymerase Chain Reaction (qPCR)
- Complementary: Compound Specific Isotope Analysis (CSIA)

Contacts

Joey Trotsky
Naval Facilities Engineering and Expeditionary Warfare Center
1100 23rd Ave
Port Hueneme, CA 93043
805-982-1258
joey.trotsky@navy.mil

CDM Smith
Ryan A. Wymore
720-264-1110
wymorera@cdmsmith.com

A.5.1 Background and Knowledge from Traditional Methods

Naval Weapons Station Site 70 is the former National Aeronautics and Space Administration (NASA) Research Testing and Evaluation Area, which was a rocket engine test facility in Seal Beach, California. Past operations at the facilities reportedly included the use of trichloroethene (TCE) along with other contaminants. Currently groundwater is contaminated with TCE in the area of interest.

Sequential reductive dechlorination of TCE under anaerobic conditions is a well documented pathway to remediate TCE in groundwater. However, certain conditions are required for complete dechlorination to ethene. These conditions include groundwater geochemistry, presence of dechlorinating bacteria *Dehalococcoides* (*Dhc*), and pH. Because very little dechlorination of TCE to degradation products *cis*-1,2-DCE (DCE), vinyl chloride, and ethene had occurred, it was apparent that the conditions required for complete dechlorination were not present. Geochemical data suggest that high sulfate concentrations may be limiting full anaerobic dechlorination of TCE to ethene, but there were also concerns that the *Dhc* presence was not strong enough for complete dechlorination.

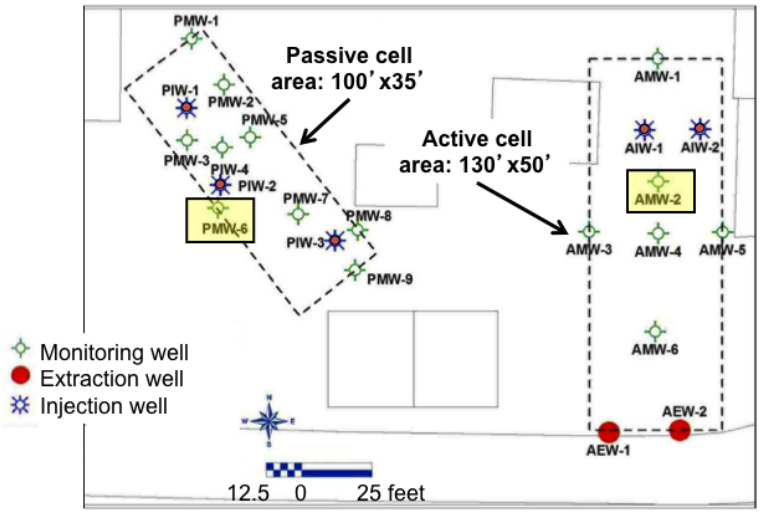


Figure A.5-1. Site location showing TCE concentration contours and passive/active treatment cells.

Source: Adapted from ESTCP 2010.

A.5.2 EMD Objectives and Approach

The Environmental Security Technology Certification Program (ESTCP) recently funded a study to evaluate passive and active approaches to bioaugmentation to clean up the TCE contaminated groundwater. Analytical techniques were used to assist in the evaluation of the study included

quantitative polymerase chain reaction (qPCR) and carbon specific isotope analysis (CSIA). More details are available in ESTCP 2010.

The overall objective of this study was to compare bioaugmentation strategies using passive and active distribution approaches for chlorinated solvent contaminated groundwater. To support this objective, the following objectives required qPCR and CSIA to evaluate performance:

- Use qPCR methods to demonstrate that at least one commercially available bioaugmentation culture can carry out complete dechlorination in the presence of high sulfate concentrations.
- Use qPCR to determine if *Dhc* are present on site; if so, use qPCR to select a culture that contains a *Dhc* strain or functional gene not present naturally at site.
- Use qPCR to determine bacterial growth and distribution throughout the treatment cells using both bioaugmentation approaches.
- Use CSIA as a supplemental tool to help determine extent of dechlorination in both treatment cells during the test period.

A.5.3 Results

qPCR was used during three key phases of the demonstration: baseline monitoring, pre-conditioning, and post-bioaugmentation performance assessment. The use of qPCR during each phase is discussed further below.

A.5.3.1 Baseline monitoring

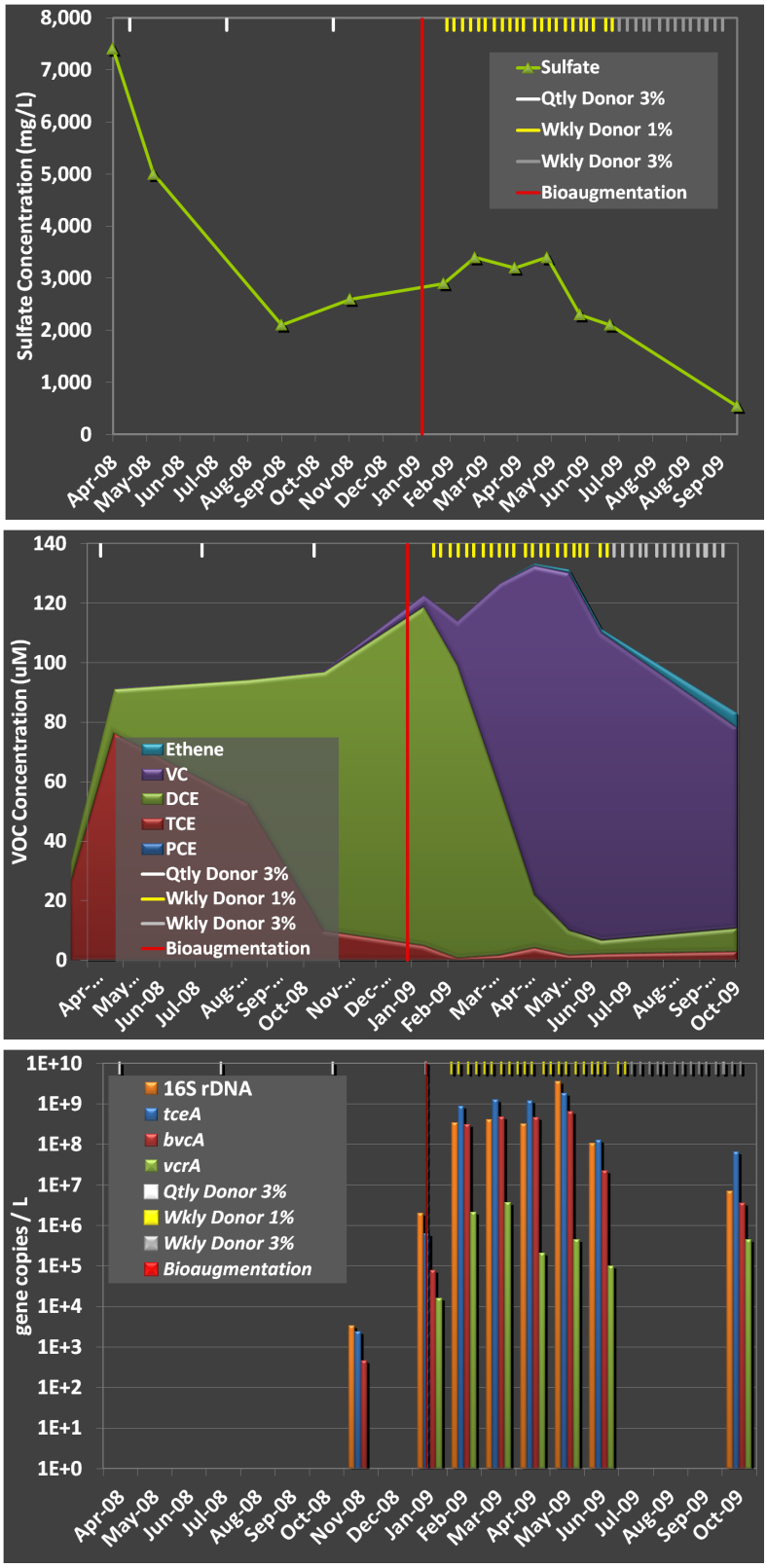
During baseline monitoring activities, TCE was detected at concentrations up to 60,000 micrograms per liter (µg/L). However, intermediate products DCE, vinyl chloride, and ethene were either not detected or were less than 5% of the TCE concentrations. Additionally, although the groundwater geochemistry appeared relatively anaerobic, sulfate was detected between 1,000 and 8,000 milligrams per liter (mg/L). Lastly, qPCR analysis indicated that *Dhc* was not present in the study area in the majority of the samples collected. In the few areas where *Dhc* was present, only *tceA* was present at levels above detection but below reporting limits, while *bvcA* and *vcrA* were not observed.

These results indicated that high sulfate concentrations were likely limiting complete anaerobic dechlorination. Additionally, because the *Dhc* only contained *tceA* (which encodes enzymes to degrade TCE to vinyl chloride) and not *bvcA* or *vcrA* (which both encode enzymes to degrade vinyl chloride to ethene), the naturally occurring *Dhc* was not likely to perform complete dechlorination.

A.5.3.2 Pre-conditioning

Based on these data, a “pre-conditioning” step was performed by adding sodium lactate to the study area to decrease sulfate concentrations and to create reducing conditions suitable for bioaugmentation. Figures A.5-2a-c show results of the pre-conditioning phase (prior to the bioaugmentation event shown by the vertical orange line). Although the pre-conditioning step was

successful at creating highly reducing conditions and reducing sulfate concentrations (Figure A.5.2a), complete dechlorination was still not occurring (Figure A.5.2b). This result was expected, as qPCR results indicated that even after pre-conditioning, *Dhc* populations remained undetected or below 10^4 gene copies per liter (Figure A.5-2c). Additionally, functional gene analysis indicated that even with the slight increase in *Dhc* populations after pre-conditioning, the functional gene *vcrA* was still not detected throughout the study area.



Figures A.5-2a-c from active cell well AMW-2 show decreased sulfate following initiation of pre-conditioning in April 2008. However, "DCE-stall" was observed until bioaugmentation

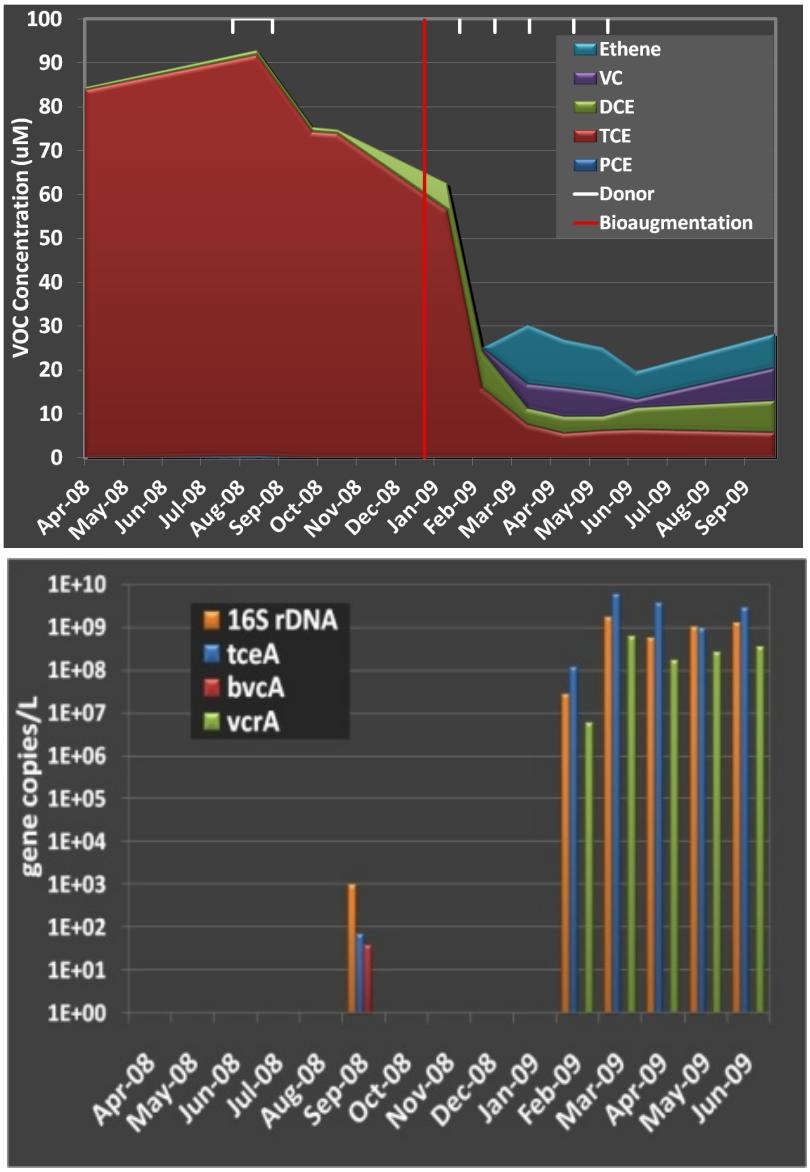
in January 2009. qPCR results show that although Dhc started to appear by November 2008, populations remained low and *vcrA* was not present.

Source: ESTCP 2010.

A.5.3.3 Bioaugmentation

Following pre-conditioning, injection wells were inoculated in January 2009 with commercially available *Dhc* culture SDC-9™. The use of qPCR and standard analytical techniques were used to evaluate the function of the bioaugmented culture in dechlorination.

qPCR was used to evaluate distribution of the bioaugmentation culture, including "first arrival," as well as growth of Dhc over time. Results showed that the bioaugmentation culture had transport times similar to that of a conservative tracer, with the first detection of *Dhc* at monitoring wells two weeks following bioaugmentation. As shown in Figures A.7.2b-c (active cell) above and A.7.3 a, b below (passive cell), enhanced dechlorination and sustained elevated Dhc (with *vcrA*) populations were observed almost immediately following bioaugmentation. In both the active and passive cells, *Dhc* and functional gene populations increased 4-7 orders of magnitude, indicating that bioaugmentation using both approaches was successful to introduce a more effective culture with increased abilities to fully dechlorinate TCE.



Figures A.5-3 a, b from passive cell well PMW-6 show minimal dechlorination until bioaugmentation in January 2009. PMW-6 was 8 feet downgradient from injection wells and showed almost immediate *Dhc* presence. Additionally, since culture used had functional genes *tceA* and *vcrA* but no *bvcA*, these functional genes increased with *Dhc* but *bvcA* was no longer detected.

Source: ESTCP 2010.

A.5.3.4 CSIA to Verify Dechlorination

CSIA was used along with the qPCR and dechlorination data to verify that dechlorination was occurring during the study and the plume was not being diluted or displaced by injection activities. CSIA data were consistent with the dechlorination data, in that they suggested degradation to vinyl chloride and ethene was occurring where VOC data suggest active dechlorination was occurring.

An example CSIA chart is included as A.5.4 for PMW-6. This chart shows that TCE, c-DCE, and VC become enriched in the heavier isotope (^{13}C) during the course of the demonstration, indicating degradation is occurring.

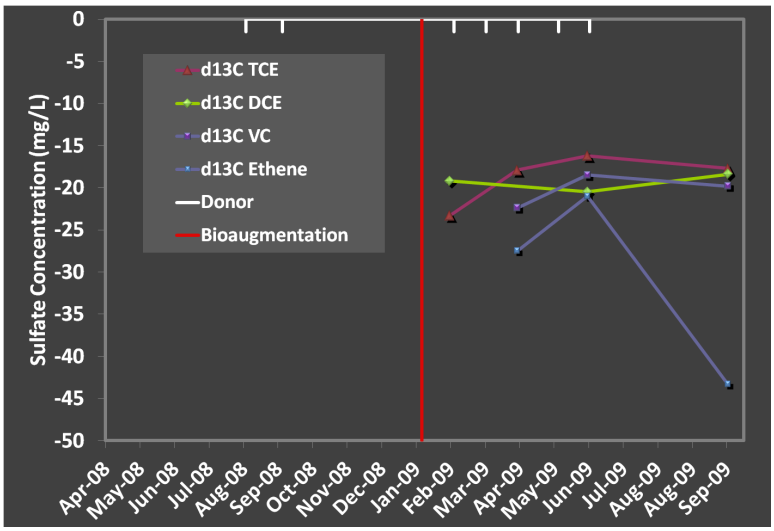


Figure A.5-4 CSIA Data

Source: ESTCP 2010.

A.5.4 Conclusions

The conclusions from this part of the site work include:

- qPCR showed that dechlorinating bacteria were not present at adequate levels prior to addition of electron donor.
- The functional gene analysis using qPCR showed that even after electron donor addition, vinyl chloride reductase gene *vcrA* was not present in the indigenous community, and thus could be used as a "biomarker" for the bioaugmentation culture.
- qPCR was used to track bacterial distribution following bioaugmentation, with results indicating that *Dhc* transport occurred nearly as fast as groundwater velocity.
- qPCR was used to assess growth of *Dhc* over time in response to bioaugmentation and repeated electron donor injections. Results showed a strong correlation to presence of *Dhc* (and specifically *vcrA*) at or above 10^6 gene copies/L to complete dechlorination.
- Overall, similar electron donor distribution and dechlorination performance was achieved in both passive and active cells; however, more donor was required and more operational issues were encountered with active approach.
- CSIA was used as a secondary line of evidence to demonstrate that complete dechlorination was occurring at the site.

A.5.5 Costs

Costs for analysis of qPCR samples for *Dhc* and the three functional genes *bvcA*, *vcrA*, and *tceA* were $300 per sample for this demonstration project. However, this cost was based on the large number of samples required for this project. Costs for this analysis for a nonresearch-based project may range from $350-$400/sample.

A.5.6 Outcomes and Challenges

- The regulatory agencies understood that the purpose of the work at this facility was for an applied research project. Therefore, no issues were encountered with regulatory acceptance.
- In addition, bioremediation with bioaugmentation is the final CERCLA remedy for this site. qPCR is being used to track growth and distribution of *Dhc* as a part of the performance monitoring program for the final remedy.
- Integrating qPCR results with traditional chemical and geochemical analyses provided the converging lines of evidence required to optimize the demonstration activities.
- One challenge at the site was the high sulfate concentrations (up to 9,000 mg/L), which created uncertainty regarding whether complete dechlorination could be stimulated at the site, even with bioaugmentation. The qPCR results provided the earliest indication that *Dhc* could be distributed and could grow at this site, well before the chemistry data indicated that dechlorination was occurring.

A.5.7 References

ESTCP, 2010, "A Low Cost Passive Approach for Bacterial Growth and Distribution for Large - Scale Implementation of Bioaugmentation," Project ER-200513, Washington, DC.

A.6 RT-qPCR for BTEX and MTBE in Groundwater for Remediation (CA)

Adapted with permission from: Baldwin, B.R., A. Biernacki, J. Blair, M.P. Purchase, J.M. Baker, K. Sublette, G. Davis, and D. Ogles. 2010. "Monitoring gene expression to evaluate oxygen infusion at a gasoline-contaminated site." *Environmental Science & Technology* 44(17): 6829-6834.

EMD Technology

- Primary: Reverse Transcriptase-Quantitative Polymerase Chain Reaction (RT-qPCR)
- Complementary: Quantitative Polymerase Chain Reaction (qPCR), EMD Sampling Methods

Contacts

Dora Ogles
Microbial Insights, Inc.
(865) 573-8188
Dogles@microbe.com

A.6.1 Site Background and Knowledge from Traditional Methods

The site is an operating gasoline station located in northern California.The shallow aquifer is impacted by petroleum hydrocarbons, including benzene, toluene, ethylbenzene, and xylenes (BTEX) along with the fuel oxygenate methyl tertiary-butyl ether (MTBE).

Examination of groundwater geochemical parameters (such as dissolved oxygen, nitrate, ferrous iron, sulfate) indicated highly anaerobic conditions. Although biodegradation of BTEX and MTBE has been well documented under anaerobic conditions, historical trends in contaminant concentrations led stakeholders to believe that monitored natural attenuation (MNA) would not provide site closure within an acceptable timeframe.

An oxygen infusion system was installed in the vicinity of the dispenser islands to promote aerobic biodegradation of BTEX and MTBE. The oxygen infusion system consisted of oxygen cylinders, 2-stage regulators, manifolds, and in-well emitters (iSOC, inVenture Technologies, ON Canada; and Waterloo, Solonist, ON, Canada). For the original system, a three oxygen infusion wells (IP-1 through IP-3) and two downgradient monitoring points (MP-1 and MP-2) were installed at the site.

A.6.2 EMD Objectives and Approach

RT-qPCR was performed to quantify the expression of toluene dioxygenase (TOD) and phenol hydroxylase (PHE) genes as well as *Methylibium petroleiphilum* PM1 16S rRNA to address the following question: Will oxygen infusion stimulate activity of benzene and MTBE degrading microorganisms at the infusion point and downgradient locations?

Pure oxygen was infused through emitters installed in wells IP-1 through IP-3. Dissolved oxygen (DO) concentrations were measured periodically at the injection points and downgradient monitoring points MP-1 and MP-2 throughout system operation. Standard, unamended Bio-Traps® deployed in the injection point IP-3 and downgradient wells MP-1 and MP-2 were recovered for RT-qPCR quantification of the following:

- Toluene dioxygenase (TOD) – functional gene encoding a key enzyme in one of the pathways for aerobic biodegradation of benzene and toluene. TOD expression demonstrates that aerobic benzene and toluene utilizing bacteria are active.
- Phenol hydroxylase (PHE) – functional gene encoding a monooxygenase enzyme in a different pathway for aerobic BTEX biodegradation. Like TOD, expression of PHE genes indicates that aerobic BTEX degrading bacteria are active.

- *Methylibium petroleiphilum* PM1 16S rRNA (PM1) – quantifies 16S rRNA from one of the few known bacteria capable of aerobic metabolism of MTBE and TBA.

A.6.3 Results

As discussed in detail in Baldwin et al. 2010, the impact of system operation at the oxygen infusion point can be summarized as described below (see Figure A.6-1):

- Prior to system activation, PHE and TOD expression was not detected at IP-3 (Figure A.6-1), MP-1 (Figure A6.2A), or MP-2 (Figure A.6-2B) indicating that these pathways for aerobic BTEX biodegradation were not active under existing site conditions. Likewise, PM1-like 16S rRNA was not detected prior to system activation indicating that one of the few known MTBE metabolizing microorganisms was not active.
- After system startup and during operation, DO levels at the infusion point IP-3 rapidly increased and remained on the order of 30 to 40 mg/L.
- After about 200 days of operation, PHE transcripts and PM1 rRNA were detected on the order of 10^3 and 10^5 copies/bead respectively at IP-3. Thus, oxygen infusion had stimulated aerobic BTEX degrader activity at least at the infusion point.
- When the system was deactivated for maintenance and upgrades (days 225-300 and days 550-650), DO levels at the infusion points decreased rapidly. However, once the system was reactivated, PHE and TOD expression as well as PM1 16S rRNA were again detected at IP-3 demonstrating the activity of aerobic BTEX and MTBE degraders in response to system operation.

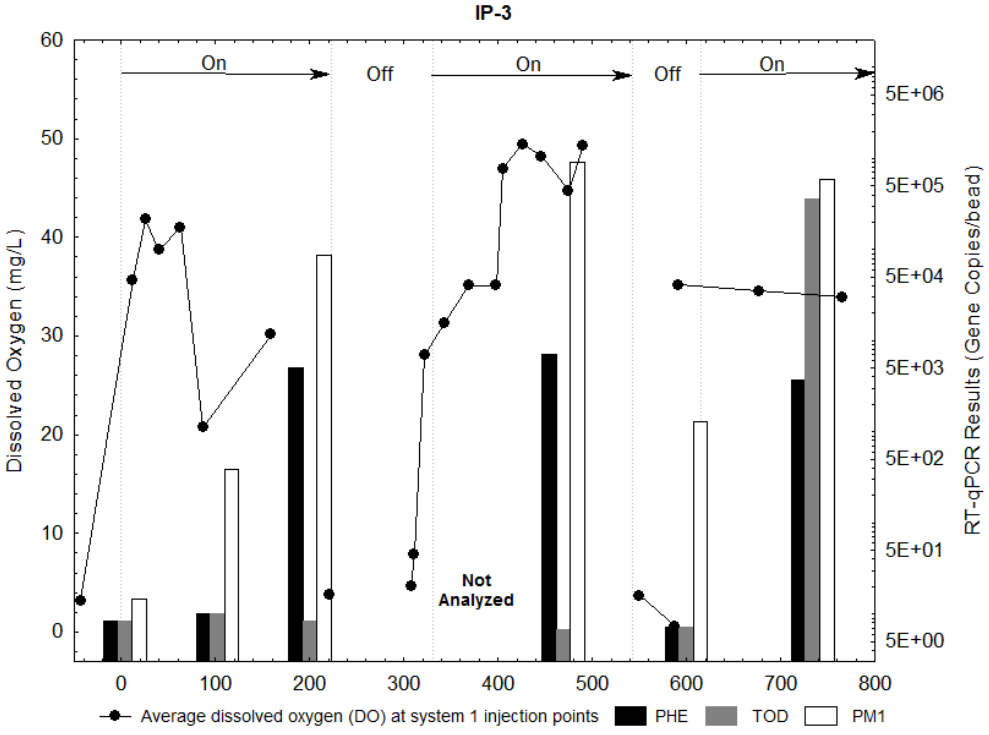


Figure A.6-1. RT-qPCR results for quantification of M. petroleiphlilum 16S rRNA and expression of phenol hydroxylase and toluene dioxygenase genes at the oxygen infusion point IP-3.

Source: Adapted with permission from Baldwin, B.R., A. Biernacki, J. Blair, M.P. Purchase, J.M. Baker, K. Sublette, G. Davis, and D. Ogles. 2010. Monitoring gene expression to evaluate oxygen infusion at a gasoline-contaminated site. Environmental Science & Technology 44(17): 6829-6834. Copyright 2010 American Chemical Society.

- While the system was deactivated (day 600), PHE and TOD expression decreased to below detectable levels at IP-3 thus linking the aerobic BTEX degrader activity to system operation. PM1 16S rRNA abundance also decreased dramatically during system shutdown.

While RT-qPCR demonstrated stimulation of aerobic BTEX and MTBE degraders at the infusion point, questions remained regarding the radius of influence of the system. As discussed in detail in Baldwin et al. 2010, RT-qPCR results also demonstrated that system operation stimulated activity of BTEX and MTBE degrading bacteria at the downgradient wells MP-1 and MP-2 even though DO levels remained low (Figure A.6-2A and B).

- At the downgradient monitoring points MP-1 and MP-2, DO levels never increased even during periods of consistent system operation. Thus, by conventional measures, MP-1 and MP-2 were beyond the radius of influence of the system.
- During the first 200 days of system operation, PHE and TOD expression was not detected at either downgradient well.
- By day 450 however, RT-qPCR analysis revealed expression of two pathways for aerobic BTEX biodegradation (PHE and TOD) and activity of MTBE utilizing strain PM1 at downgradient monitoring point MP-1 (Figure A.6-2A). Although at a lower concentration, PM1 16S rRNA was also detected further downgradient (Figure A.6-2B).
- While the system was deactivated for maintenance around Day 550, PHE and TOD expression decreased to below detectable levels MP-1 and PM1 16S rRNA was no longer detected at MP-2.
- Once the system was reactivated, PHE and TOD expression as well as PM1 16S rRNA were again detected at the downgradient wells.
- Therefore, the RT-qPCR results demonstrated that, after an initial lag period, system operation stimulated aerobic BTEX and MTBE degrader activity at the downgradient locations which would not have been predicted based on geochemical monitoring alone.

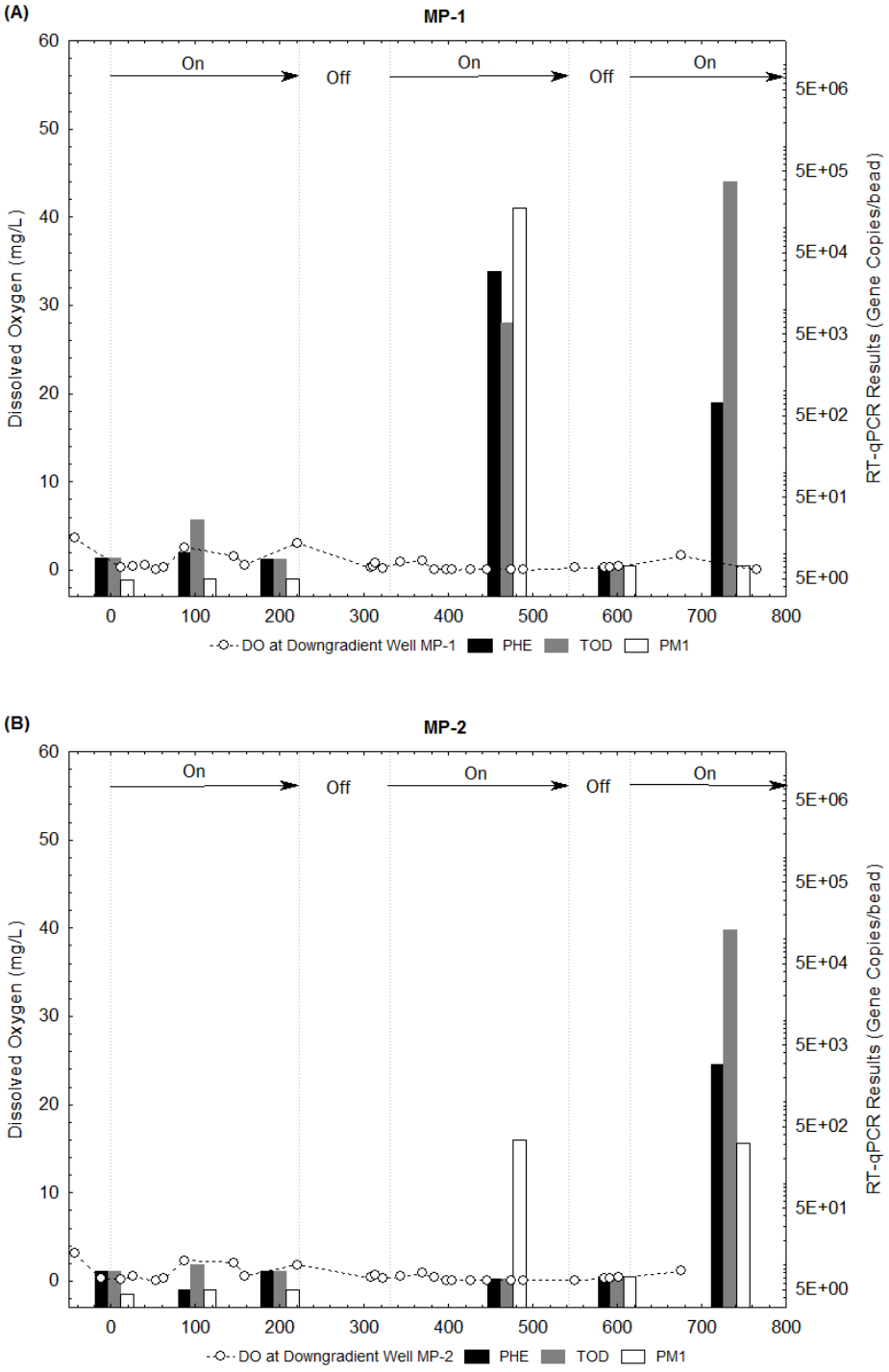


Figure A.6-2. RT-qPCR results for quantification of *M. petroleiphlilum* 16S rRNA and expression of phenol hydroxylase and toluene dioxygenase genes at downgradient monitoring points MP-1 (A) and MP-2 (B).

Source: Adapted with permission from Baldwin, B.R., A. Biernacki, J. Blair, M.P. Purchase, J.M. Baker, K. Sublette, G. Davis, and D. Ogles. 2010. Monitoring gene expression to evaluate oxygen infusion at a gasoline-con-

taminated site. Environmental Science & Technology 44(17): 6829-6834. Copyright 2010 American Chemical Society.

RT-qPCR analysis provided site managers with rapid feedback (7-10 day turnaround time) on the effect of system operation on BTEX and MTBE degrader activity before trends in contaminant concentration would have been evident. Ultimately, the changes in contaminant concentrations were consistent with the RT-qPCR results demonstrating aerobic BTEX degrader activity.

- At the infusion point IP-3, system operation and PHE expression corresponded with decreases in benzene concentrations (Figure A.6-3A).
- Note the spike in the benzene concentration in IP-3 at Day 600. When the system was deactivated, PHE and TOD were no longer expressed and the benzene concentration increased substantially. When the system was reactivated, PHE and TOD expression was evident and benzene concentrations again decreased.
- Despite the consistently low DO levels at MP-1 and MP-2, RT-qPCR demonstrated that aerobic BTEX and MTBE degraders became active after initial lag periods.
- At MP-2 however, benzene concentrations were stable or increasing through much of the study. The observed lag but eventual expression of PHE and TOD at MP1 by day 500 (Figure A.6-2A) improved stakeholder confidence that system operation would eventually enhance benzene biodegradation at MP-2 at a time when benzene concentrations were actually increasing (Figure A.6-3B).
- By day 750, when PHE and TOD expression was evident, benzene concentrations had begun to decrease.
- RT-qPCR analysis provided the critical link between system operation, expression of functional genes involved in aerobic BTEX, BTEX degrader activity, and ultimately observed decreases in contaminant concentrations (Figure A.6-3).

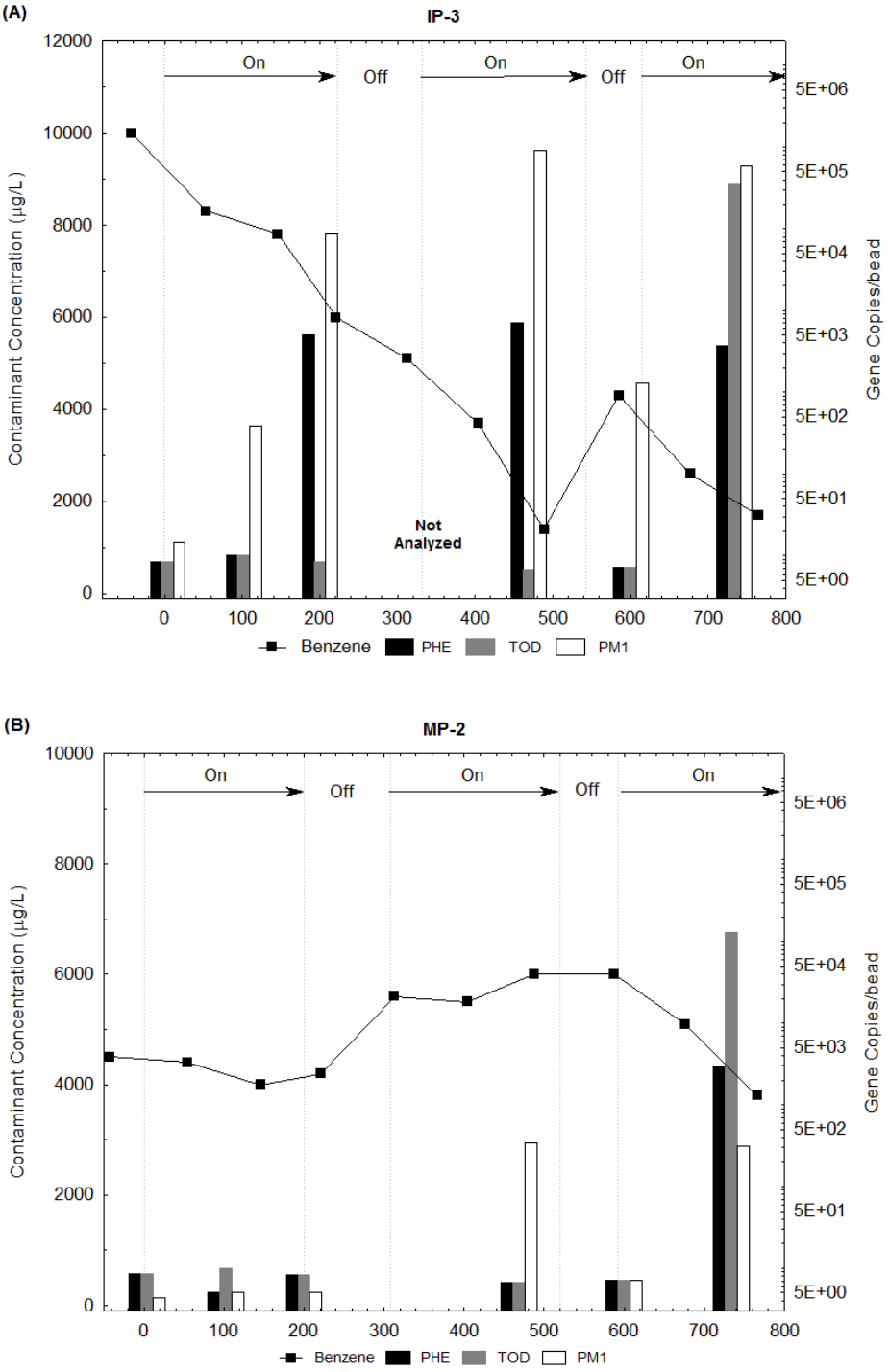


Figure A.6-3. RT-qPCR results and dissolved benzene concentrations at the oxygen infusion point IP-3 (A) and downgradient monitoring point MP-2 (B).

Source: Adapted with permission from Baldwin, B.R., A. Biernacki, J. Blair, M.P. Purchase, J.M. Baker, K. Sublette, G. Davis, and D. Ogles. 2010. Monitoring gene expression to evaluate oxygen infusion at a gasoline-contaminated site. Environmental Science & Technology 44(17): 6829-6834. Copyright 2010 American Chemical Society.

A.6.4 Conclusions

The following conclusions can be drawn based on the results for this site:

- Documenting PHE expression at IP-3 during the first six months of operation provided rapid evidence that the infusion system would achieve its primary goal of enhancing BTEX biodegradation in the source area before a clear trend in contaminant concentrations would have been evident.
- RT-qPCR analysis demonstrated that system activation stimulated expression of a known pathway for aerobic BTEX biodegradation and activity of a known MTBE utilizing strain.
- RT-qPCR results demonstrating stimulation of aerobic BTEX and MTBE degrader activity was a critical line of evidence in the decision to install a second oxygen infusion system side-gradient of the original system.
- Overall, RT-qPCR analysis provided direct evidence of enhanced biodegradation at times not evident in chemical or geochemical results and provided the basis for greater stakeholder confidence in the remediation strategy.

A.6.5 Costs

Continued RT-qPCR monitoring of TOD and PHE expression along with PM1 16S rRNA (three target genes) would cost approximately $575 per sample.

A.6.6 Outcomes and Challenges

- The RT-qPCR results were accepted as a valuable line of evidence and the system was ultimately expanded to a total of 11 oxygen infusion points.
- As described in the qPCR section, RT-qPCR that quantifies gene expression is often more appropriate than qPCR when evaluating biodegradation of petroleum hydrocarbons (see Section 4.0).
- RT-qPCR can provide a more accurate delineation of the zone of influence of an aerobic treatment system than DO monitoring.

A.6.7 References

Baldwin, B.R., A. Biernacki, J. Blair, M.P. Purchase, J.M. Baker, K. Sublette, G. Davis, and D. Ogles. 2010. "Monitoring gene expression to evaluate oxygen infusion at a gasoline-contaminated site." *Environmental Science & Technology* 44(17):6829-6834.

A.7 EAP for TCE in Groundwater for Remediation (KY).

Adapted with permission from: Lee, Hope M., Looney, Brian B., Hampson, S.K. 2008. "Enzyme Activity Probe and Geochemical Assessment for Potential Aerobic Cometabolism of Tri-

chloroethene in Groundwater of the Northwest Plume, Paducah Gaseous Diffusion Plant, Kentucky." WSRC-STI-2008-00309.

EMD Technology

- Primary: Enzyme Activity Probes (EAP)
- Complementary: Compound Specific Isotope Analysis (CSIA)

Contacts

Blaine Rowley
U.S. Department of Energy
(301) 903-2777
blaine.rowley@em.doe.gov

M. Hope Lee
Pacific Northwest National Laboratory
(240) 818-2987
Hope.Lee@pnnl.gov

A.7.1 Site Background and Knowledge from Traditional Methods

Groundwater below a portion of Paducah Gaseous Diffusion Plant in Paducah Kentucky is contaminated with chlorinated solvents, primarily trichloroethene, TCE. TCE was first released to the subsurface as the result of site operations, which included disposal and burial of hazardous and radioactive materials across the site, from 1952 to the mid 90s (Lee et al. 2008b, p. 11). The dominant source of contamination at the site is associated with continued TCE and ^{99}Tc releases in the vicinity of building 400. The site is the location of an old river bed and as such groundwater moves quickly, approximately 3 feet per day; site operations, and the local geology have resulted in two defined groundwater contaminant plumes, northwest and northeast, which originate near building 400 and end in tributaries off site. Other defined contaminant plumes (not considered in this study) exist on site and are associated with waste and burial grounds; all of the mentioned groundwater plumes share similar geochemistry and thus potential for attenuation of the contaminants under intrinsic conditions.

In 1997 Clausen et al. documented biological attenuation of chlorinated solvents in groundwater at the Paducah site; however the observed half-life degradation rates were estimated to be between 9.4 and 26.7 years. Based on the chemical and geochemical sampling results, the relatively slow rates of degradation, aerobic co-metabolism of TCE was suspected as the dominant mechanism for attenuation but was not confirmed in the study. At that time, the estimated rates were considered much too slow to accomplish site goals and no further studies were proposed.

Unlike reductive dechlorination of TCE under anaerobic conditions, where biological break-down products, such as 1-2, *cis*-dichloroethene or vinyl chloride are produced, degradation of TCE under

aerobic conditions can only be inferred by indirect measures of carbon dioxide, disappearance of the contaminants, geochemical conditions (elevated dissolved oxygen and an appropriate carbon substrate), and EMDs.

A.7.2 EMD Objectives/Approach

Enzyme Activity Probes (EAPs) were used to directly measure enzyme activity of target enzymes known to be produced during co-metabolic degradation of chlorinated solvents such as TCE.

EAPs directly measure if methane and/or aromatic (substrates such as toluene, benzene, phenol) enzyme production is occurring. These enzymes are documented to be produced for the degradation of methane and aromatic compounds and also break down TCE in a process referred to as co-metabolism (Lee et al. 2005, 2008a; Keener et al. 1998, 2001; Clingenpeel et al. 2005; Wymore et al. 2007; Miller et al. 2002).

For this study, the EAP results were compared to the following additional lines of evidence: (1) quantitative and traditional PCR for the genes of interest (oxygenases), (2) carbon stable isotopic analysis, (3) geochemical conditions, (4) contaminants trends, and (5) conservative tracers (^{99}Tc).

Collectively, these additional lines of evidence were used to validate the EAPs as a useful tool for confirming the presence of co-metabolic processes and more importantly to confirm co-metabolic degradation of TCE was occurring in situ and at measurable rates at the Paducah site (Lee et al. 2008a).

A.7.3 Results

An assessment was conducted to determine if EAPs could be used to confirm co-metabolic destruction of TCE. In this study, 12 wells were selected along the centerline of the northwest plume as well as two control wells outside the contaminant plume at the site. The wells are shown in Figure A.7-1.

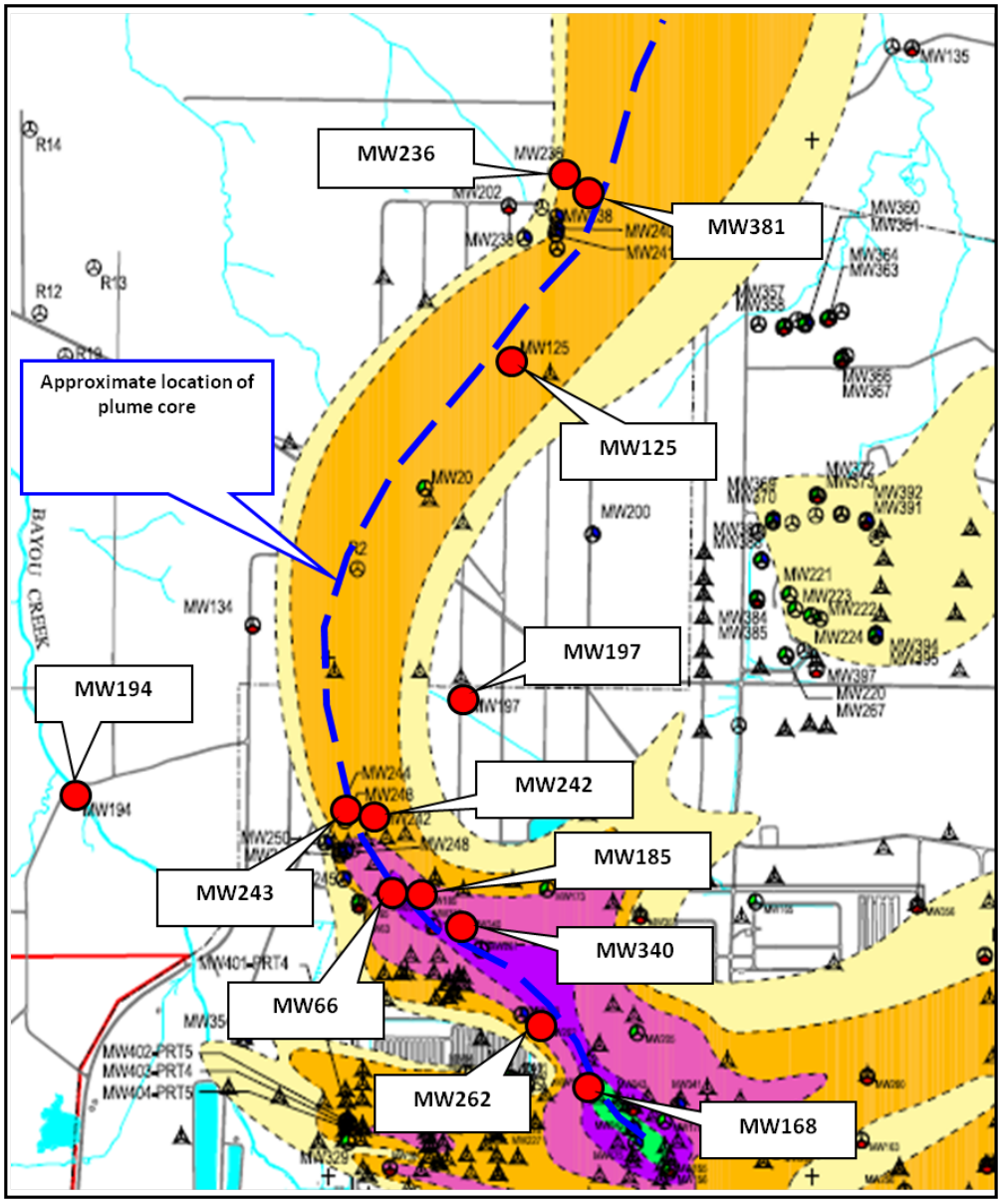


Figure A.7-1: Map displaying wells where either sMMO enzyme-activity probe, and/or one or more toluene enzyme-activity probes were used.

Source: PRS, 2007; reprinted in Lee et al. 2008b.

As shown in Table A.7-1, positive results were established for both toluene oxygenases (9 out of 12 wells) and the soluble methane monooxygenase (sMMO) enzymes (7 out of 12 wells). Inhibitor studies supported these findings (data not shown). Quantitative toluene probe results represent the percentage of the total biomass determined to be active; values greater than 3% are considered significant.

Table A.7-1. Comparison of qualitative and quantitative enzyme-activity probe results to contaminant concentrations in groundwater from monitoring wells at the Northwest Plume at PGDP. Source: Table 3, Lee et al. 2008b.

Well	Aquifer	Screened interval depth (ft)	TCE * (µg/L)	Tc-99 (pCi/L)	Qualitative pre-liminary data (6/4/7)		Toluene probes quantitative data (% of Total DAPI)			Total – DAPI cells/mL
					sMMO probe Coumarin	Toluene probes	3HPA	PA	Cinnamo-nitrile	
1MW16-8	URGA	63 - 68	10,000 to 100,000	3260	-	-	0.00	1.2-7	0.00	1.90E+0-5
MW66		55 - 60	1,000 to 10,000	3670	+	+++	3.90	5.7-3	2.49	3.67E+0-5
MW194		47 - 52	<MCL	17	+	+++	1.78	5.4-1	6.80	1.76E+0-5
MW197		58 - 63	<MCL	283	-	+	1.09	3.9-5	0.14	1.59E+0-6
MW185	MRGA	68 - 73	1,000 to 10,000	1260	-	++	1.84	1.4-1	0.20	9.75E+0-5
MW242		65 - 75	100 to 1,000	341	-	-	0.46	0.1-6	1.14	7.76E+0-5
MW243		65 - 75	100 to 1,000	3860	-	-	0.77	1.0-8	0.31	4.27E+0-5
MW381		66 - 76	100 to 1,000	329	-	++	6.36	3.6-4	0.57	9.66E+0-5
MW262	LRGA	90 - 95	1,000 to 10,000	4178	+	+++	3.83	3.8-6	7.92	3.52E+0-5
MW340		85.5 - 95.3	1,000 to 10,000	747	+	+	0.05	1.3-2	0.00	7.25E+0-5
MW236		69.5 - 79.5	100 to 1,000	936	+	+++	3.66	5.9-5	1.05	8.84E+0-5
MW125		78 - 88	100 to 1,000	273	+	++	1.74	7.9-7	2.54	7.99E+0-5

- URGA: Upper Regional Gravel Aquifer
- MRGA: Middle Regional Gravel Aquifer
- LRGA: Lower Regional Gravel Aquifer
- TCE: Trichloroethene
- Tc-99: Technicium 99
- 3HPA: 3-hydroxy-phenylacetylene
- PA: Phenylacetylene
- DAPI: 4',6-Diamidino-2-Phenylindole (double stranded DNA staining)
- MCL: Maximum Contaminant Level

ft bgs– feet below ground surface
µg/L – micrograms per liter
pCi/L – picocuries per liter
cells/mL – per mil-liliter

- DNA Control Study: A DNA control study was performed to determine if key genes of interest were present in the groundwater samples collected from the northwest plume, corresponding to groundwater samples taken for EAP analyses. Positive responses were observed for the PCR and qPCR analyses, providing a secondary line of evidence that the oxygenase genes of interest, various oxygenases, were present in situ and correlated well with the positive results observed with EAPs.
- Geochemical Data: Although the groundwater geochemistry varied spatially within the plume, the results were generally conducive to aerobic co-metabolism (neutral pH, dissolved oxygen from 0.6 to 5.8 mg/L).
- CSIA Study: The CSIA data showed that isotopic fractionation from paired groundwater wells within the northwest plume was indicative of aerobic degradation of the contaminant TCE. In this case, differences between $\delta^{13}C$ values (even small, > -0.9), in groundwater monitoring wells and 'fresh solvent' or current source zone values, is indicative of slow aerobic degradation. The $\delta^{13}C$ values obtained in this evaluation are similar to other published data (-0.8 to -1.3), but differ from those focused on reductive dechlorination, which typically result in a decreasing trend over the length of the contaminant plume. These data support the conclusion that degradation of the TCE occurs along the centerline of this plume. These data serve as another line of evidence for natural attenuation of chlorinated solvents at the Paducah site and validate the EAP results which confirmed the presence and activity of key enzymes involved in the co-metabolic degradation of TCE.

Table A.7-2: Summary of CSIA Results (Source: Table 7, Lee et al, 2008b)

Sample ID	$\delta^{13}C$ (per mil)	Comments
Northwest plume wells along the flow path		
MW-168	-24.8	Near source
MW-262	-25.8	
MW-340	-25.9	
MW-185	-25.9	
MW-242	-24.6	
MW-243	-25.3	
MW-125	-25.6	
MW-381	-25.4	
MW-236	-25.3	Distal portion of plume
MW-66	-25.3	Near downgradient source
MW-197	-23.1	Control well, outside of plume

A.7.4 Conclusions

- Through EAP, the key enzymes produced in the aerobic co-metabolic degradation of TCE were confirmed present and active in situ.
- CSIA determined that aerobic biodegradation of TCE was likely occurring within the north-west plume, based on isotopic fractionation; these data correlated well with the EAP data.
- DNA analyses also supported the EAP data, providing evidence that the genes of interest, oxygenases, were present throughout the groundwater locations sampled.
- EAPs provided key data in support of natural attenuation processes occurring within the northwest plume at the Paducah site.

A.7.5 Costs

Analytical costs associated with EMDs are included in Table A.7-3 below.

Table A.7-3. Summary of analytical costs associated with the EMDs during the study.

EMD	No. of Samples	Cost per Sample	Total Cost
EAP and PCR	12	2700	$32,400
CSIA	12	500	$ 6,000
Total			$38,400

A.7.6 Outcomes and Challenges

The technologies, CSIA, enzyme activity probes, and microcosm studies, were still in demonstration phase when applied at the Paducah site. A collaborative effort was undertaken to demonstrate the appropriate knowledge and understanding was in place prior to applying the technology which proved very successful for this site. Specific criteria were defined before a groundwater sample was taken and included the definition for success for any one of the technologies. As a result, the contractor, technical staff, and regulators all agreed prior to data collection, what would be deemed sufficient evidence for aerobic co-metabolism to be a significant process within the sampled groundwater plume.

While there was a large amount of historical and current groundwater monitoring data including contaminant concentrations and a range of geochemical parameters, many of the aerobic biological indicators were not known or previously measured. A large effort was undertaken by the site in collaboration with the state and federal regulators to determine the appropriate geochemical parameters to be measured in support of aerobic degradation of chlorinated solvents. During the investigation it was also determined that the method for determining oxygen concentrations in groundwater had been changed several times over the past decade, and more importantly the regulators were not confident in the methods used. Working with the site contractor, a more robust method (time and expense) was used to monitor oxygen concentrations throughout several groundwater plumes at the site. It was also concluded that there were insufficient developed wells within

the predominant groundwater plume and the DOE along with the site, installed 75 new MW locations across the site in order to better monitor groundwater and in order to develop a more complete conceptual site model.

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A.8 SIP for Chlorinated Solvents in Groundwater for Remediation (AZ)

Adapted with permission from: Chiang, S. D., R. Mora, W. H. Diguiseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. 2012. “Characterizing the intrinsic bioremediation potential of

1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." *Journal of Environmental Monitoring* 14: 2317-2326. Reproduced by permission of The Royal Society of Chemistry (RSC). http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b.

EMD Technology

- Primary: Stable Isotope Probing (SIP)
- Complementary: Enzyme Activity Probes (EAPs), qPCR

Contacts

Rebecca Mora
AECOM
(714) 689-7254
Rebecca.Mora@aecom.com

Air Force Civil Engineer Center (AFCEC)
Environmental Center of Excellence (ECoE)
Environmental Restoration Technical Support Branch (CZTE)
Adria Bodour, Ph.D.
(210) 395-8426
adria.bodour.1@us.af.mil

A.8.1 Site Background and Knowledge from Traditional Methods

Air Force Plant 44 is a missile assembly plant that historically used trichloroethene (TCE) and 1,1,1-trichloroethane (1,1,1-TCA) as solvents. 1,4-dioxane was a stabilizer in 1,1,1-TCA and consequently, was also released to the environment. Currently, primary contaminants at the site are TCE, 1,4-dioxane, and 1,1-dichloroethene (1,1-DCE).

A groundwater extraction, treatment (air stripping), and reinjection system has been operating since 1987. Treatment was upgraded to advanced oxidation in 2009 to treat 1,4-dioxane, in addition to volatile organic compounds (VOCs). Monitored natural attenuation (MNA) is being considered as part of the final remedy to reduce the operational timeframe of the pump and treat system. TCE and 1,4-dioxane contaminant trend analysis indicates concentrations are declining steadily over time. Examination of groundwater geochemical parameters (dissolved oxygen, nitrate, ferrous iron, sulfate, methane, and oxidation reduction potential) indicated conditions were aerobic.

It has been established that TCE can be biodegraded to carbon dioxide under aerobic conditions through co-metabolism without accumulation of toxic intermediate products. Biodegradation of 1,4-dioxane, which historically was thought to be insignificant, has been confirmed in recent years and can occur through co-metabolism as well as where 1,4-dioxane is used as a growth-supporting substrate (Zenker et al. 2000; Fam 2005; and Mahendra and Alvarez-Cohen 2006). The 1,4-dioxane biodegradation pathway, which also results in mineralization to carbon dioxide, was

documented in Mahendra et al. (2007), and is the same for co-metabolic and growth-supporting processes.

A.8.2 EMD Objectives and Approach

The study was designed to evaluate intrinsic aerobic biodegradation (via co-metabolism and/or growth-supporting processes) of TCE and 1,4-dioxane to determine whether MNA could be considered as a component of the site remedial strategy. Four EMDs were used to evaluate site-specific biodegradation and confirm degradation mechanisms. The EMDs were applied using a stepwise approach which involved separate sequential sampling events. This approach allowed for optimization of sampling location selection for the more expensive analyses as they were based on results of previous steps.

The study involved answering the following questions using specific EMDs, which were applied in the order they are presented:

1. Are bacteria and enzymes capable of aerobically degrading TCE and/or 1,4-dioxane present at the site?
2. Are TCE and/or 1,4-dioxane being aerobically degraded at the site?
3. Are enzymes capable of degrading TCE and/or 1,4-dioxane metabolically active at the site?

A.8.3 Methods and Results

To address Question 1, Bio-Trap® and groundwater samples were collected from wells throughout the TCE and 1,4-dioxane plume (source area, mid-plume, and downgradient) and analyzed by qPCR for available qPCR targets related to TCE and/or 1,4-dioxane aerobic degradation. Table A.8-1 includes the qPCR targets.

Table A.8-1. Biomarkers related to biodegradation of TCE and 1,4-dioxane

Biomarker Code	Bacteria or Enzymes	TCE	1,4-Dioxane
MOB	Methane oxidizing bacteria (Methanotrophs)	Yes	Yes
sMMO	Soluble methane monooxygenase	Yes	Yes
PHE	Phenol hydroxylase/ Toluene 2-,3-,4-monooxygenase	Yes	Yes
RMO	Toluene 3-,4-monooxygenase	Yes	Yes
TOD	Toluene 2,3-dioxygenase	Yes	No

Figure A.8-1 shows the results for qPCR quantification of bacteria and enzymes capable of degrading TCE and 1,4-dioxane from the Bio-Trap® samplers.

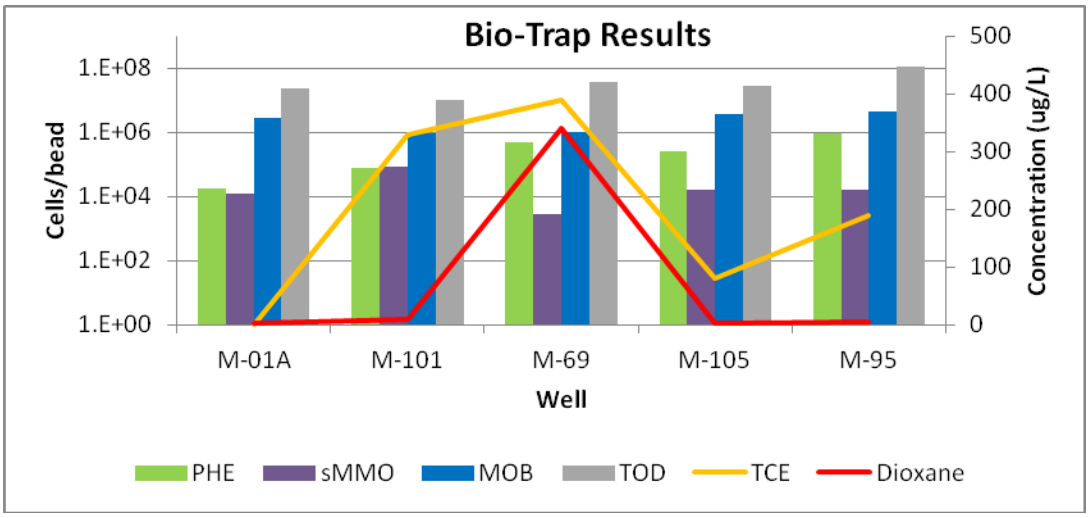

Figure A.8-1: Results for qPCR quantification of bacteria and enzymes capable of degrading TCE and 1,4-dioxane in Bio-Trap® samples from select monitoring wells.

Source: Adapted from Chiang , S.D., R. Mora, W. H. Diguiseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. 2012. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." Journal of Environmental Monitoring 14: 2317-2326. Reproduced by permission of The Royal Society of Chemistry (RSC). http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b.

Figure A.8-2 shows the results for qPCR quantification for groundwater samples.

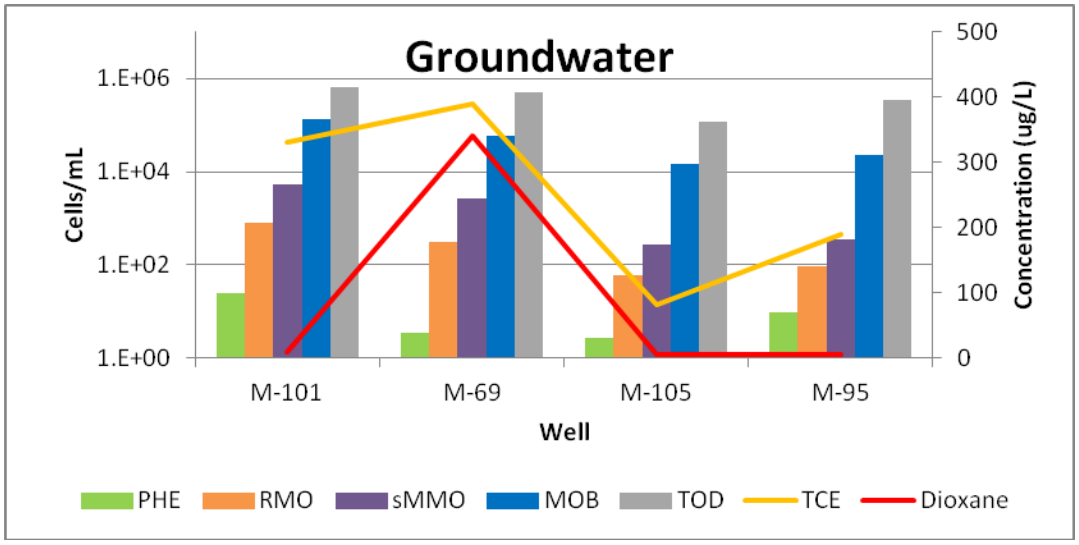

Figure A.8-2: Results for qPCR quantification of bacteria and enzymes capable of degrading TCE and 1,4-dioxane in groundwater samples from select monitoring wells.

Source: Adapted from Chiang, S.D., R. Mora, W. H. Diguiseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. 2012. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." Journal of Environmental Monitoring 14: 2317-2326. Repro-

duced by permission of The Royal Society of Chemistry (RSC). http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b.

The observations based on qPCR results include:

- Bacteria and enzymes capable of degrading TCE and 1,4-dioxane were present and abundant at the site.
- No correlation exists between the abundance of each target and contaminant concentrations (that is, targets were not more abundant at locations with high contaminant concentrations).
- Some differences were noted between Bio-Trap® and groundwater samples, especially with regard to the RMO and PHE biomarkers.

To address Question 2, SIP was performed. While the qPCR step revealed the potential for biodegradation of TCE and 1,4-dioxane, SIP provides direct proof of contaminant biodegradation. Bio-Traps® baited with specially- synthesized TCE and 1,4-dioxane that were approximately 15% ^{13}C (as compared to the typical 1% ^{13}C present in organic compounds) were deployed in select wells. Once SIP Bio-Traps® were retrieved after approximately 60 days of incubation, the BioSep beads were analyzed for:

- ^{13}C incorporation into phospholipid fatty acids (PLFA), an essential component of cell membranes, indicating the contaminant supports bacterial growth;
- Dissolved inorganic carbon (DIC) (equivalent to carbon dioxide) indicating complete mineralization of the contaminant; and
- Percent loss of the ^{13}C baited compound off of the Bio-Trap® during deployment.

Figure A.8-3 includes the results for ^{13}C incorporation into PLFA for ^{13}C TCE and ^{13}C 1,4-dioxane.

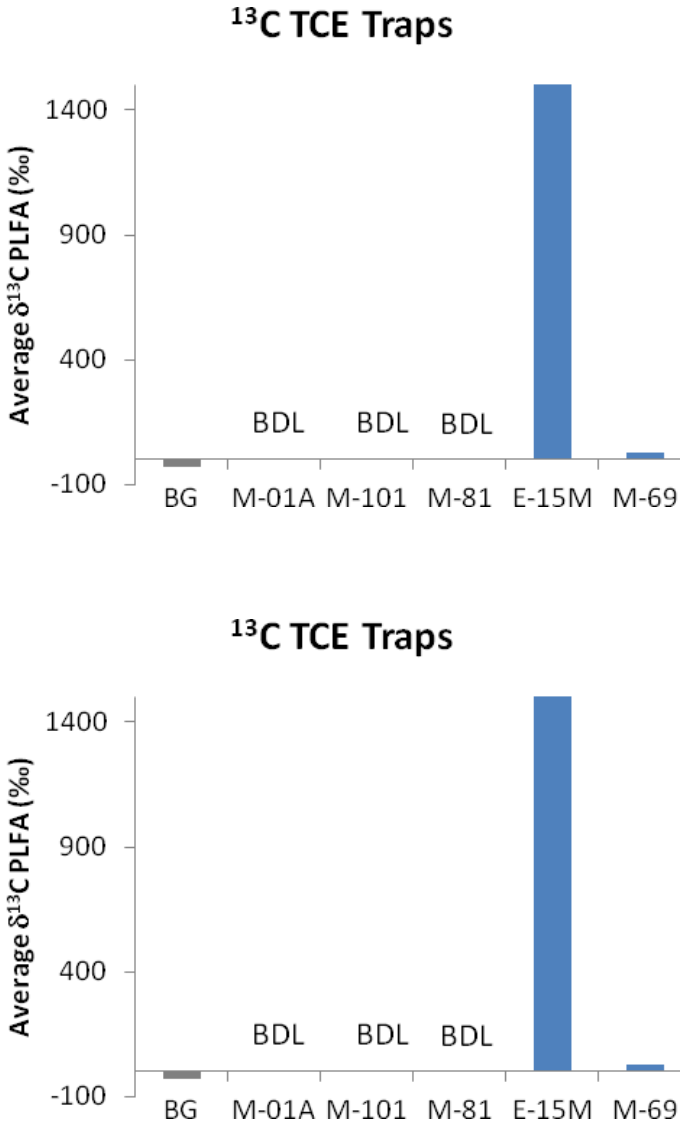

Figure A.8-3: SIP results for ^{13}C incorporation into PLFA for ^{13}C TCE and ^{13}C 1,4-dioxane baited Bio-Traps® from select monitoring wells.

Source: Chiang, S.D., R. Mora, W. H. Diguiseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. 2012. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." Journal of Environmental Monitoring 14: 2317-2326. Reproduced by permission of The Royal Society of Chemistry (RSC). http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b.

Figure A.8-4 shows the results for ^{13}C incorporation into DIC (carbon dioxide) for ^{13}C TCE and ^{13}C 1,4-dioxane.

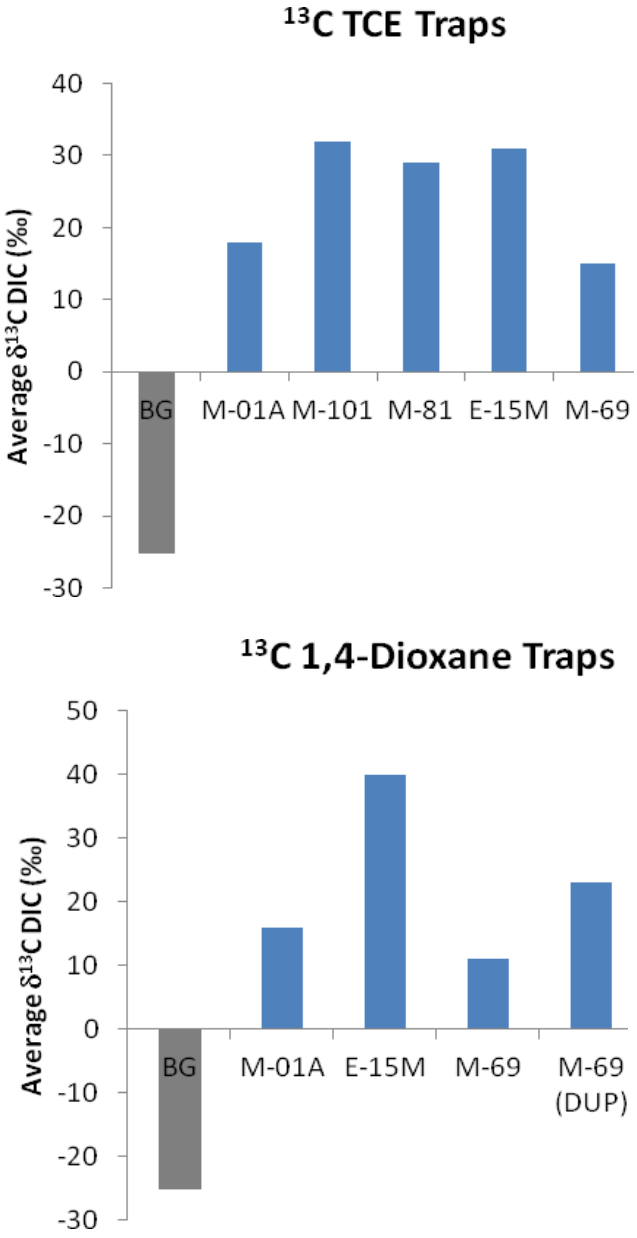

Figure A.8-4: SIP results for ^{13}C incorporation into DIC (carbon dioxide) for ^{13}C TCE and ^{13}C 1,4-dioxane baited Bio-Traps® from select monitoring wells.

Source: Chiang, S.D., R. Mora, W. H. Diguiseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. 2012. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." Journal of Environmental Monitoring 14: 2317-2326. Reproduced by permission of The Royal Society of Chemistry (RSC). http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b.

Figure A.8-5 shows the results for percent loss of ^{13}C TCE and ^{13}C 1,4-dioxane.

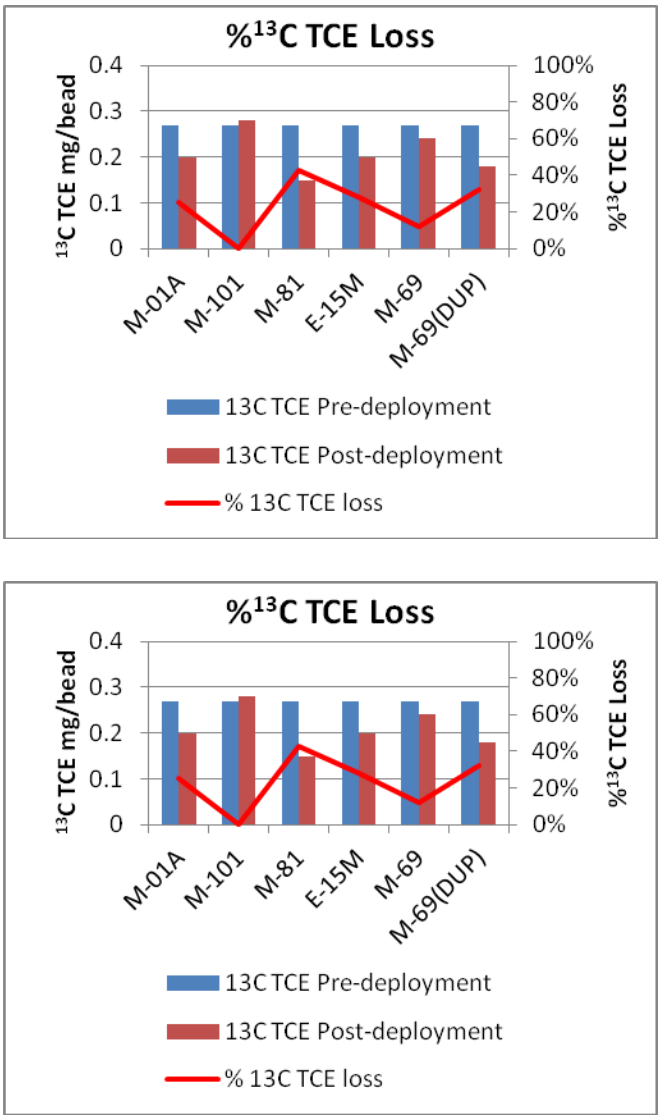

Figure A.8-5: SIP results for percent loss of ^{13}C TCE and ^{13}C 1,4-dioxane from baited Bio-Traps® from select monitoring wells.

Source: Chiang, S.D., R. Mora, W. H. Diguiseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. 2012. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." Journal of Environmental Monitoring 14: 2317-2326. Reproduced by permission of The Royal Society of Chemistry (RSC). http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b.

The observations based on SIP results include:

- ^{13}C incorporation into PLFA for the ^{13}C TCE baited Bio-Traps® was observed in two wells (E-15M and M-69). Because TCE is not directly metabolized under aerobic conditions (only co-metabolically metabolized), the enrichment is likely due to direct metabolism of co-metabolic TCE intermediate products such as formic and glyoxylic acids.

- ^{13}C incorporation into PLFA for the ^{13}C 1,4-dioxane baited Bio-Traps® was observed in three out of four wells with significant incorporation in Well E-15M. These data indicate that 1,4-dioxane is supporting bacterial growth at the site.
- ^{13}C incorporation into DIC was detected in all of the ^{13}C TCE and 1,4-dioxane baited Bio-Traps® indicating at least some conversion of both contaminants to carbon dioxide.
- ^{13}C TCE loss ranged from variable ranging from 0% to 43% while ^{13}C 1,4-dioxane loss was consistent and significant ranging from 82% to 90%. The loss of ^{13}C 1,4-dioxane was likely due to physical leaching of 1,4-dioxane off the BioSep beads into the aquifer.

To address question number 3, groundwater samples were collected and analyzed using enzyme activity probes (EAPs). Four probes, phenylacetylene (PA), 3-hydroxyphenylacetylene (3-HPA), trans-cinnamonitrile (CINN), and 3-ethylnyl benzoate (3EB), were used to measure the activity of toluene monooxygenase and/or dioxygenase enzymes (PHE, RMO, TOL, and TOD). One probe, coumarin, was used to measure the activity of sMMO. Table A.8-2 includes the EAP results for toluene oxygenases and soluble methane monooxygenase enzymes.

Table A.8-2. EAP results for toluene oxygenases and soluble methane monooxygenase enzymes from selected monitoring wells in cells per milliliter.

	Probes for toluene oxygenases (PHE, RMO, TOL, TOD)				Probe for sMMO
Well	PA	3-HPA	CINN	3EB	Coumarin
M-69	-	$1.05x10^4$	-	-	15.22
M-69	$8.21x10^3$	$1.25x10^4$	-	-	-
M-01A	$2.54x10^4$	-	$2.14x10^4$	$8.12x10^3$	-
M-81	$2.15x10^4$	$2.04x10^4$	-	-	42.11
M-105	$2.68x10^4$	$2.21x10^4$	$1.12x10^4$	-	-
M-101	$3.54x10^4$	-	-	-	-
M-95	$2.45x10^4$	-	$1.42x10^4$	-	-

The observations based on EAP results include:

- There is widespread enzyme activity in the wells that were sampled, with each well showing at least one positive result with one EAP and five out of six wells showing activity for more than one EAP.
- These results are evidence that intrinsic aerobic biodegradation is occurring at the site.

A.8.4 Conclusions

- qPCR results showed that bacteria and enzymes capable of degrading TCE and 1,4-dioxane under aerobic conditions are present and abundant at the site.

- SIP results showed that ^{13}C from 1,4-dioxane was incorporated into bacterial PLFAs, indicating the contaminant may serve as a growth-supporting substrate at the site.
- SIP results demonstrated TCE and 1,4-dioxane mineralization to carbon dioxide is occurring at the site.
- EAP results confirmed that enzymes capable of degrading TCE and 1,4-dioxane under aerobic conditions are not just present, but metabolically active at the site.
- EMD results confirmed that aerobic degradation of TCE and 1,4-dioxane is occurring and may be responsible for decreasing contaminant trends at the site.
- MNA can be considered as a component of the site remedy.
- This was the first study confirming intrinsic biodegradation of 1,4-dioxane under field conditions.

A.8.5 Costs

Table A.8-3 summarizes the analytical costs associated with the EMDs used in this study.

Table A.8-3: Summary of analytical costs associated with the EMDs during the study.

EMD	No. of Samples	Cost per Sample	Total Cost
qPCR (5 biomarkers)	25	$425	$10,625
CSIA (TCE)	5	$350	$1,750
SIP (TCE)	6	$1,650	$9,900
SIP (1,4-Dioxane)	4	$2,070	$8,280
EAP (5 probes)	7	$2,375	$16,625
Total			$47,180

A.8.6 Outcomes and challenges

The significant outcomes and challenges were as follows:

- A significant physical loss of ^{13}C 1,4-dioxane from the SIP Bio-Traps® likely occurred during deployment. However, valuable data regarding aerobic biodegradation of 1,4-dioxane at the site was still obtained.
- SIP and EAP are expensive EMDs which limited their broad application throughout the plume.
- Using multiple EMDs provided multiple lines of evidence to support the conclusion that intrinsic aerobic biodegradation of both TCE and 1,4-dioxane is occurring at the site.
- qPCR and EAP confirmed the presence and activity of desired enzymes but SIP provided unambiguous results confirming TCE and 1,4-dioxane biodegradation at the site.
- SIP can be used to prove that degradation is occurring but it cannot provide information on the rate at which it is occurring. A microcosm study using groundwater and soil from the site is currently being conducted by Dr. Shaily Mahendra, at UCLA, to evaluate the intrinsic aerobic biodegradation rate for TCE and 1,4-dioxane at the site as well as any increases in the rate through biostimulation (e.g., addition of a primary substrate such as methane or propane)

or bioaugmentation with Pseudonocardia dioxanivorans, a bacteria known to grow on and degrade 1,4-dioxane.
- Using a stepwise approach allowed evaluation of the results of each EMD before selecting sampling locations for the next EMD. This resulted in a more optimized and cost-effective approach.

A.8.7 References

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A.9 SIP for Fuel Oil in Groundwater for Remediation (NJ)

Adapted with permission from: K. Key, K.L. Sublette, T. Johnnes, E. Raes, E. Sullivan, D. Ogles, B.R. Baldwin, and A, Biernacki. 2013. An in situ bioreactor for the treatment of petroleum hydrocarbons in groundwater. *Remediation*. Spring. (Publication Pending).

EMD Technology

- Primary: Stable Isotope Probing (SIP)
- Complementary: Quantitative Polymerase Chain Reaction (qPCR)

Contacts

Mr. Jon Malkin
New Jersey Department of Environmental Protection
(609) 633-1201
Jon.Malkin@dep.state.nj.us

Mr. Eric J. Raes, PE, PP
Engineering and Land Planning Associates, Inc.
(908) 238-0544, ext. 10
Eric@elp-inc.com

A.9.1 Site Background and Knowledge from Traditional Methods

In 1994, a release of No. 2 fuel oil occurred beneath a historic house constructed in 1839. Fuel oil compounds persist beneath the structure. To date, several remedial efforts have been completed including soil removal, oxygen release compound injections, and a small-scale chemical oxidation remediation. Biostimulation was observed after the chemical oxidation, but was not sustained. Chemical testing indicated the following:

- Low levels of fuel oil related compounds persist in groundwater above regulatory levels.
- Two oxygen release product injections failed to achieve and sustain acceptable levels.
- Chemical oxidation injection failed to achieve and sustain acceptable levels due to low permeability soils and the source location.

A.9.2 EMD Objectives and Approach

SIP was used for this site to evaluate the impacts of in situ chemical oxidation on the indigenous microbial organisms. Once biostimulation was observed, SIP and qPCR were used to confirm that biostimulation processes could be sustained through the use of an in situ bio-reactor (ISBR).

The initial testing included the following activities.

- SIP was used prior to, during, and subsequent to the chemical oxidation (persulfate-based chemical oxidation) program to monitor the effects of the remedial action on the indigenous microbial community.
- Each EMD sampling device (Bio-Trap®) was pre-loaded (baited) with a known, small quantity (97+/- ug/bead) of ^{13}C labeled naphthalene. Mass loss by chemical oxidation and mass loss and mineralization of naphthalene through microbial degradation was monitored. Mineralization was quantified through dissolved inorganic carbon readings and gene expression by mRNA/qPCR analysis (Geyer et al. 2005).
- Background groundwater samples collected away from the release revealed almost no microbial activity. This result explained the failure of the two previous, oxygen release compound remedial efforts at the site.

The use of EMDs during chemical oxidation revealed that the site was suitable for biostimulation of the petroleum compounds. The full-scale effort included sustainable aerobic biostimulation and microbial analyses

Sustainable aerobic biostimulation was achieved through the installation of a novel ISBR (see Figure A.9-1). The central portion of the ISBR is filled with Bio-Sep beads; the sparge stone resides on the bottom of bead bed and serves two functions:

- Dissolved oxygen is provided to hydrocarbon-degrading microbes that populated the Bio-Sep medium.
- The tiny air bubbles create a circulation element within the ISBR, where contaminated groundwater is pulled in from the bottom of the ISBR, passes through the Bio-Sep medium and exits the top, as illustrated in the dye test study below. This configuration allows for healthy microbes from within the ISBR to migrate into the well column and into the formation beyond the well to further promote the biodegradation of the residual petroleum hydrocarbons in the aquifer.

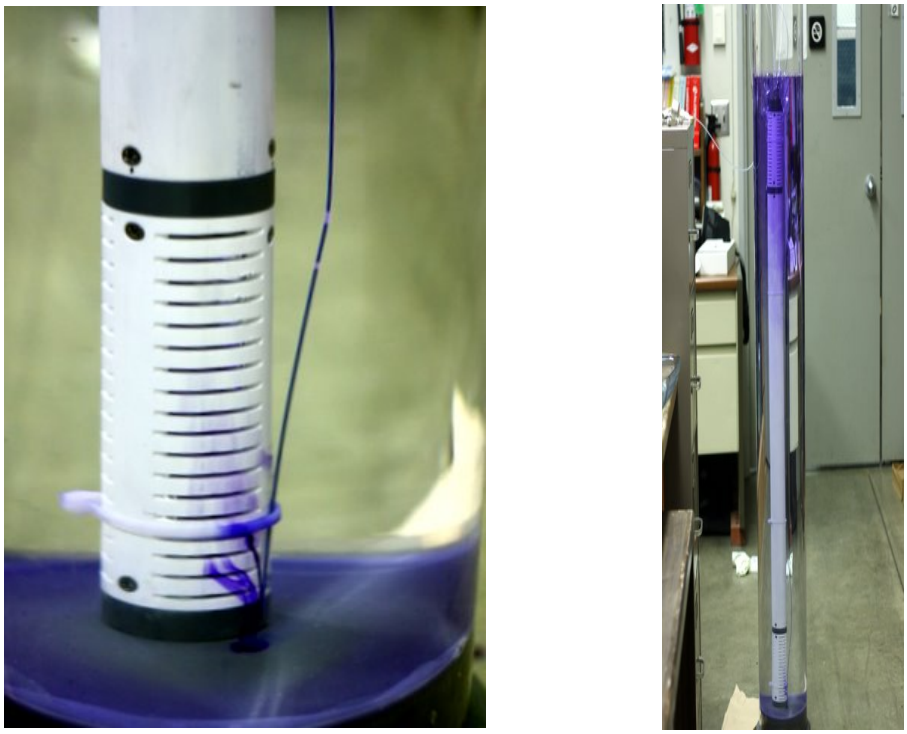

Figure A.9-1. Photographs of the ISBR; photographs show dye released at base of ISBR is "uplifted" through the beads and exits the top into the well.

Source: K. Sublette 2012. Used with permission.

Microbial analyses using qPCR of the EMD sampling device (Bio-Trap® Sampler) three months after the ISBR was installed in the well confirmed that microbial gene NAH expression was occurring, supporting the conclusion that biodegradation of the petroleum hydrocarbons was occurring.

A.9.3 Results

The results from sampling during and after chemical oxidation events demonstrated:

- The persulfate-based injection, while removing mass from the baited EMD sampling device (54%), had little impact on the microbial community, which was predominantly dormant for aerobic processes prior to and during the chemical oxidation remedial action.
- Subsequent to the injection, biostimulation of petroleum-degrading microorganisms was inadvertently promoted. Significant ^{13}C was incorporated in the dissolved inorganic carbon (DIC) indicating that naphthalene losses were the result of biodegradation with mineralization of the hydrocarbon. In addition, mRNA analyses of naphthalene dioxygenase (NAH) and phenol hydroxylase (PHE) genes were conducted to investigate the potential for and the microbial gene expression of metabolic pathways responsible for aerobic biodegradation of naphthalene (Baldwin et al. 2003). NAH expression, which had not been noted previously, was detected after chemical oxidation treatment was completed indicating activity of aerobic naphthalene-utilizing bacteria (Baldwin et al. 2010). The biostimulation could have been predicted, as the chemical oxidation process generates partially-oxidized materials that are more readily consumed by bacteria. In addition, the chemical oxidation included ozone (O_3) injection, which left behind dissolved oxygen in the groundwater. This was used by petroleum-degrading bacteria, which were previously detected but were not functional under background conditions.
- The biostimulation was not sustainable, as the partially-oxidized materials and dissolved oxygen were not maintained, as observed in the microbial analysis (lack of NAH expression) collected four months after the chemical oxidation events. Figure A.9-2 includes the data from the SIP results.
- The use of SIP defined a transition point in the remedial strategy, whereas a less costly and more effective sustainable biostimulation remedy was pursued in lieu of the continuation of the more costly chemical oxidation strategy.

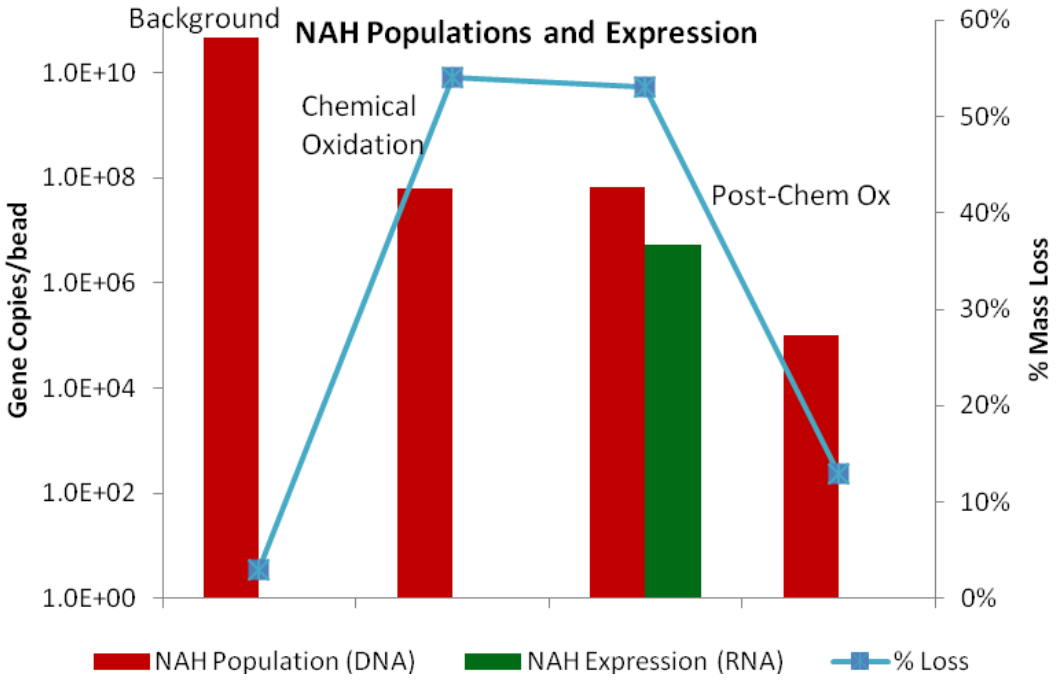

Figure A.9-2. SIP results before, during, and after ISCO.

Source: E. Raes 2012. Used with permission.

After six months of operations, a groundwater sample was collected from well WP-1R; the results were below the NJDEP GWQS for the first time in 17 years (see Figure A.9-3). In fact, the results were reported as nondetect for all targeted volatile organic and base neutral compounds, and non-detect for VOCs TICs. Base neutral TICs were reported as 135 mg/l.

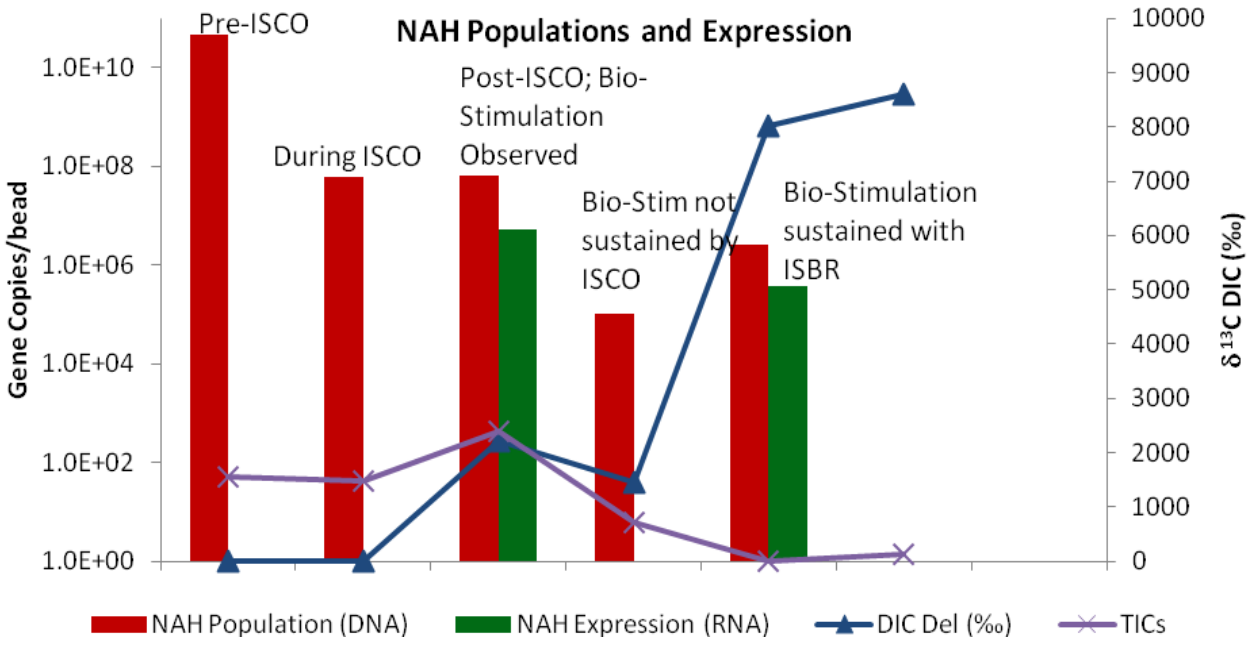

Figure A.9-3. Comparison of SIP results after ISCO and ISBR operations.

Source: E. Raes 2012. Used with permission.

A.9.4 Conclusions

- Through SIP, aerobic biodegradation processes under background conditions were confirmed to be insufficient.
- A more effective and less costly remedial strategy was determined through the use of EMDs, specifically the combination of SIP and qPCR analyses (Suzuki et al. 2000).
- Using EMD sampling devices coupled with EMD laboratory techniques, sustainable biostimulation was confirmed (Johnson et al. 2005).

A.9.5 Costs

Table A.9-1 summarizes the analytical costs associated with the EMDs used in this study.

Table A.9-1 Summary of analytical costs associated with the EMDs during the study

EMD	No. of Samples	Cost per Sample	Total Cost
Bio-Trap®	9	$75	$675
SIP (Mass loss)	9	$300	$2,700
qPCR (DNA/mRNA)	9	$550	$4,950
DIC	9	$250	$2,250
TOTAL		-	$10,575.0-0

A.9.6 Outcomes and Challenges

The most significant challenges were as follows:

- The ISBR was installed in the compliance well. The regulatory agency required the ISBR be removed, and for groundwater levels to remain below their regulatory standard for 90 days after biostimulation ceased at the site.
- Determining what, if any, permits were required, was a challenge because the SIP used EMD sampling devices that were a relatively new technology to the regulatory agency. In the end, the agency decided no permits were required.
- Sample handling became a significant issue, when the field technician failed to properly store and ship the Bio-Traps®. While mass loss and DIC analysis were still possible, the mRNA data was compromised and invalidated since the holding times for microbial analyses were exceeded.

A.9.7 References

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A.10 Microarrays for U(VI) Bioremediation Monitoring (CO)

Adapted with permission from: Chandler, D.P., A. Kukhtin, R. Mokhiber, C. Kinckerbocker, D. Ogles, G. Rudy, J. Golova, P. Long and A. D. Peacock. 2010. Monitoring Microbial Community Structure and Dynamics during in situ U(VI) Bioremediation with a Field-Portable Microarray Analysis System, *Environmental Science & Technology*. 44:5516-5522. Copyright 2010 American Chemical Society.

The Rifle IFRC is funded by the U.S. Department of Energy, Office of Science, Biological and Environmental Research, Climate and Environmental Science Division, Subsurface Biogeochemistry Program. The Rifle IFRC web site includes a list of publications based on research at the Rifle IFRC.

EMD Technologies

- Primary: DNA Microarray Analysis
- Complementary: Quantitative Polymerase Chain Reaction (qPCR)

Contact

Dr. Aaron D. Peacock
Haley & Aldrich, Inc.
(865) 356-0498
apeacock@haleyaldrich.com

A.10.1 Background and Knowledge from Traditional Methods

The Old Rifle Uranium Mill Tailings Remedial Action (UMTRA) Project site is a former ore processing facility located east of the city of Rifle in Garfield County, Colorado. Figure A.10.1 is a site location map. The site is located adjacent to the Colorado River. The site is bounded to the north, west and east by steep upward slopes of sedimentary rocks belonging to the Wasatch Formation and to the south by a steep downward slope to the river. Remediation was required for the uranium mill tailings and other radioactive material associated with the former operations. The U.S. Department of Energy (DOE) completed surface remediation at the site and the contaminated materials were taken to the Estes Gulch disposal cell. The surface remediation was conducted from

1992 to 1996. The cleanup level used for the remediation was 15 pico curies/gram Radium 226 + 228. The National Regulatory Commission concurred that the site was cleaned up and no supplemental standards exist. Groundwater at the site is still above the action level for uranium (DOE 1999). The Rifle IFRC is managed for the U.S. Department of Energy by Lawrence Berkeley National Laboratory, Earth Sciences Division. Additional information for the site can be found on the DOE Legacy Management web site (http://www.lm.doe.gov/Rifle/Old_Processing/Sites.aspx?view=1).

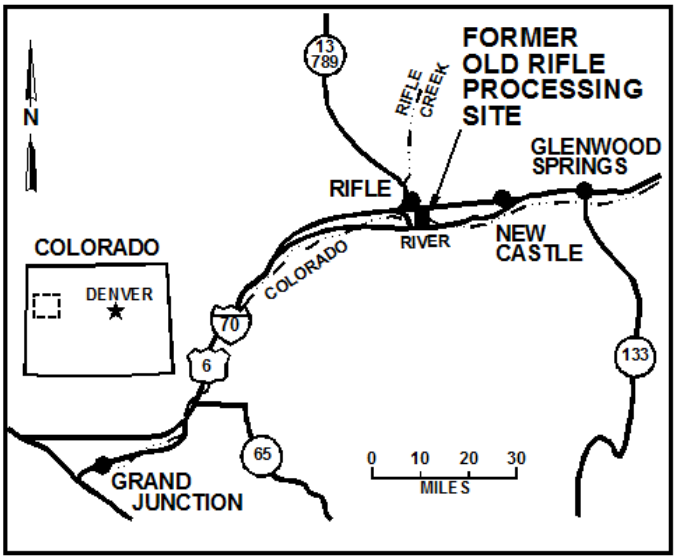

Figure A.10-1. Location map of the Old Rifle Site.

Source: DOE 1999.

Groundwater contamination by uranium is a localized but worldwide problem. While uranium contamination can derive from natural sources, most groundwater is contaminated by uranium leaching from mining waste and mill tailings (Wall and Krumholtz 2006). There is currently no proven cost effective remediation strategy for uranium-contaminated aquifers (DOE 1999). Strategies such as soil washing, solidification, chemical immobilization, chemical reduction, and phytoremediation are being explored (El-Sabour 2007). Uranium can be removed from potable waters by ion-exchange or reverse osmosis, but these technologies are too expensive to be applied to a large subsurface aquifer. An additional background reference to experiments at the site is Williams et al. 2011.

Bio-immobilization has emerged as an attractive approach to controlling uranium groundwater contamination. The soluble form of uranium is U(VI), usually complexed with carbonate (Wall & Krumholtz 2006). Some metal-reducing bacteria, in particular *Geobacter*, can reduce U(VI) to U (IV), which forms insoluble uranite, UO_2. There is also evidence that U(VI) is adsorbed by metal sulfides formed by sulfate-reducing bacteria. Acetate, ethanol, and glucose are the subsurface amendments most commonly used to drive bio-immobilization in situ. Issues to be resolved include the timing and amount of subsurface amendment to maximize bio-immobilization, and the long-

term stability of bio-immobilized uranium. Microorganisms in the subsurface have a direct impact on the nature, extent, and fate of many contaminants. Microorganisms can create conditions that decrease contaminant mobility or directly transform contaminants into innocuous or immobile forms. However, there are presently very few readily available methods for assessing in situ microbial community structure, activity or remediation potential within a time frame that impacts treatment or remediation decisions.

A.10.2 EMD Objectives and Approach

The objective of this effort was to develop and validate a simple-to-use, field-portable, microarray-based system for monitoring microbial community structure and dynamics in groundwater and subsurface environments. The full details of this work are presented in Chandler et al. (2010).

The field-portable microarray study using the TruArray® was part of a series of biostimulation experiments designed to investigate the use of adding carbon substrates to the subsurface aquifer in order to reduce soluble U (VI) to insoluble U (IV) via microbial enzymatic mechanisms. Groundwater samples were acquired from a multi-level sampling transect of U02-D02-D06-D10 at three depth intervals (12 feet, 15 feet and 20 feet) and four phases of the field experiment (pre-injection, iron-reduction, iron-sulfate transition and sulfate-reduction). Figure A.10-2 shows these sampling locations. Background samples were also acquired for other boreholes and areas in the site. Nucleic acids were extracted and split for microarray and matched qPCR analyses. Akonni Biosystems processed each sample in triplicate (300 total arrays) according to the optimized procedures at an equivalent sample volume used for qPCR tests.

A.10.3 Results

Replicate (n=33) negative control reactions were all negative by microarray analysis, indicating that if there were any contaminating DNA that found its way into the samples it did not affect the microarray signatures. Analysis of the background samples supported the hypothesis of a residual shift in microbial community structure as a consequence of previous donor injections in the same gallery, especially with respect to the *Dechloromonas* and nitrate-reducer signatures that were much more pronounced [that is Signal/Noise (S/N) ratios] in all of the current samples than in any previous sample. However, there were also many more dechlorinator and fermenter signals in the current background than in previous background samples, which may simply reflect an overall improvement in the assay performance.

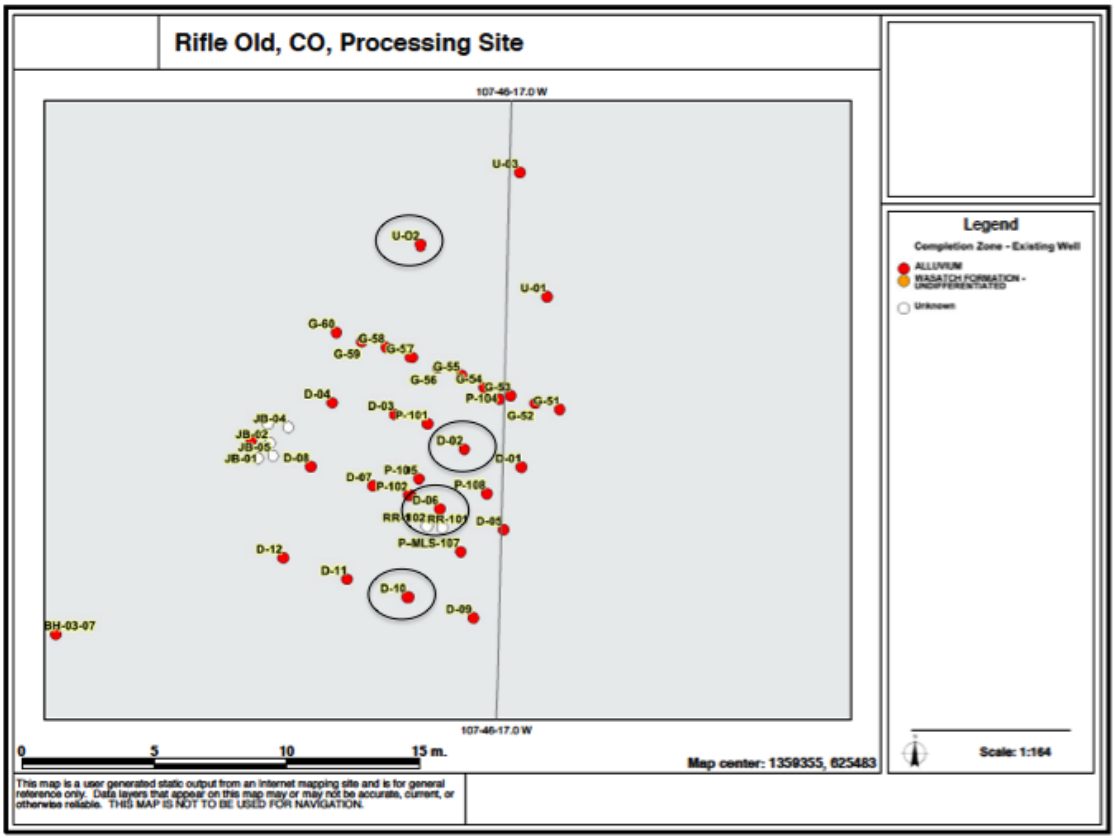

Figure A.10-2. Rifle monitoring well gallery.

Source: Data from http://gem-s.lm.doe.gov/imf/imf.jsp?site=rifleoldprocessing&title=Rifle%20Old,%20CO,%20Processing%20Site, map created 2013.

The heat map (Figure A.10-3) is divided into four panels that display array results for each of the geochemical conditions during the experiment. Each panel is further divided into 12-foot, 15-foot and 20-foot intervals that display array results with depth. The heat map showed the expected progression of microbial signatures from iron- to sulfate -reducers with changes in acetate amendment and in situ geochemical field conditions. Once acetate addition started there was an increase in both the nitrate-reducers and iron-reducing microbes. The microarray response for *Geobacter* (a known uranium reducer) was highly correlated with qPCR (Figure A.10-3 panel B) for the same target gene ($R^2 = 0.84$). Probes targeting *Desulfobacter* and Desulfitobacterium were the most reactive during the iron- to sulfate-reducing transition and into sulfate-reduction, with a consistent Desulfotomaculum signature throughout the field experiment and a general decrease in Geobacter signal to noise ratios during the onset of sulfate-reducing conditions. Nitrate reducers represented by *Dechloromonas* and *Dechlorosoma* signatures were consistently detected throughout the field experiment. The intensity of the microarray signatures were also correlated with depth (Figure A.10-3, panel C), where the 12- and 15-foot intervals showed a stronger response than the 20-foot interval. Microarray results and S/N ratios were in concordance with quantitative PCR data sets

with the array data providing a more in depth community profile and the qPCR a more quantitative result of specific community constituents.

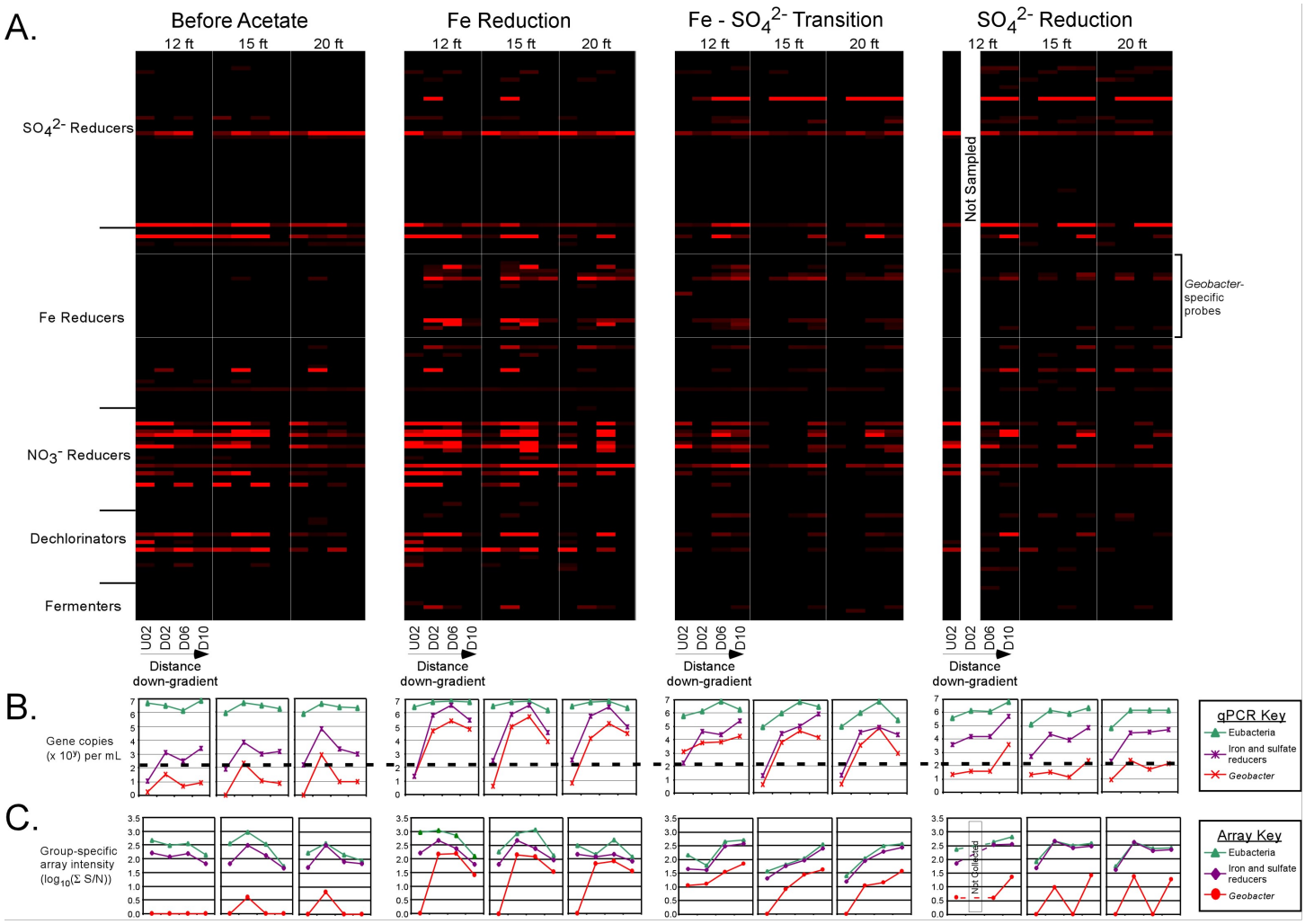

Figure A.10-3. (A) Heat map, (B) TaqMan® qPCR data, (C) microarray signal intensity.

Reprinted with permission from Chandler, D.P., A. Kukhtin, R. Mokhiber, C. Kinckerbocker, D. Ogles, G. Rudy, J. Golova, P. Long and A. Peacock. 2010. Monitoring Microbial Community Structure and Dynamics during in situ U(VI) Bioremediation with a Field-Portable Microarray Analysis System, Environmental Science & Technology. 44:5516-5522. Copyright 2010 American Chemical Society.

A.10.4 Conclusions

This study has established some fundamental technology milestones that are generally applicable to environmental science, in that this was the first successful deployment of a low-cost, low-complexity, portable, array-based environmental monitoring system that can generate a community profile on site, within four hours of sample receipt. Method complexity, logistical burden and analysis time have been reduced so that field deployment of microarray technology and real-time monitoring of microbial community response to environmental conditions is possible. Results from the

validation study showed that interpreting microarray field data probably requires several levels of detail, from fine-scale analysis of individual probe responses, to summed intensities over genera, to integrated intensities over boreholes and/or the entire site. Translating these analyses and results into simple, intelligible outputs for site engineers and decision makers can now be accomplished through relatively straightforward analysis macros and software upgrades. That the TruArray® Geobacter response was quantitatively and strongly correlated with qPCR data provide evidence that the asymmetric PCR portion of the protocol is relatively unbiased, and provide hope that S/N ratio values may someday be used as a proxy for in situ microbial abundance. The combined body of evidence presented here demonstrates that the field portable TruArray® is capable of monitoring real, ecologically significant changes in microbial community composition during in situ bioremediation.

A.10.5 Costs

There are several considerations when assessing costs for array technologies, however one of the most critical is volume. Because of the way arrays are manufactured the more arrays produced the less expensive (all other parameters equal). It is estimated that the arrays would cost from $200 to $750 each depending on the type of array required for the analyses.

A.10.6 Outcomes and Challenges

Currently most array applications in the environmental remediation field have been for investigation or research use only. A significant challenge for microarrays from an Environmental Molecular Diagnostics (EMD) regulatory perspective would or will most likely consist of issues with the following:

- Sensitivity
- Specificity
- Repeatability
- Cost per test
- Preferred platform and approach for generating the information

At this point the methods, manufacture and use of array technologies can have a direct impact on the quality and reliability of the results. While it may not be critical for remediation per se the continued development of array technology for other environmental applications with direct impact on human health (such as food safety, drinking water, and biosecurity) will be dependent on manufacturing quality assurance (QA) and quality control (QC) and adequate uniform laboratory procedures.

A.10.7 References

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APPENDIX B. SURVEY OF REGULATORS, CONSULTANTS, AND STAKEHOLDERS

B.1 Introduction

The web-based survey was conducted using Survey Monkey and opened on November 30, 2010 and remained open until August 14, 2011. It was first circulated among ITRC State Points of Contact (POCs) who are state regulators ITRC Board members from the Dept. of Energy, Dept. of Defense, USEPA, Industry Affiliate Members, and public and tribal stakeholders. A request was included with the circulation that the survey be distributed within these organizations to anyone with an interest in or experience with EMDs. The target audience for the survey included regulators (e.g., project managers, executive staff, and administrators of state/agency-funded cleanup programs), regulatory agency consultants, and ITRC industry affiliates program members.

The survey contained four parts: Introduction, Respondent Information, Technical Questions, Training Interest and Needs. The goals for this survey were to identify the following:

- Current use and level of interest in various EMDs
- Regulatory and other constraints or barriers to using EMDs
- Training needs of potential EMD end users
- Respondents who have experience with EMDs and may be willing to share case studies with a wider audience

The survey results revealed the following:

- Genuine potential exists for EMDs to contribute towards regulatory decision.
- Most EMDs are new to many regulators.
- Although a new area, most regulators expressed a willingness to be trained on EMDs.
- The state of the art is evolving; a need exists for a resource for people to learn more about EMDs.
- Some regulatory issues are unresolved regarding implementation at sites.

B.2 Evaluation of Survey Results

- 90 people started the survey; 78 completed it.
- As seen in Figure B-1, 60% were regulators, and 21% were consultants.

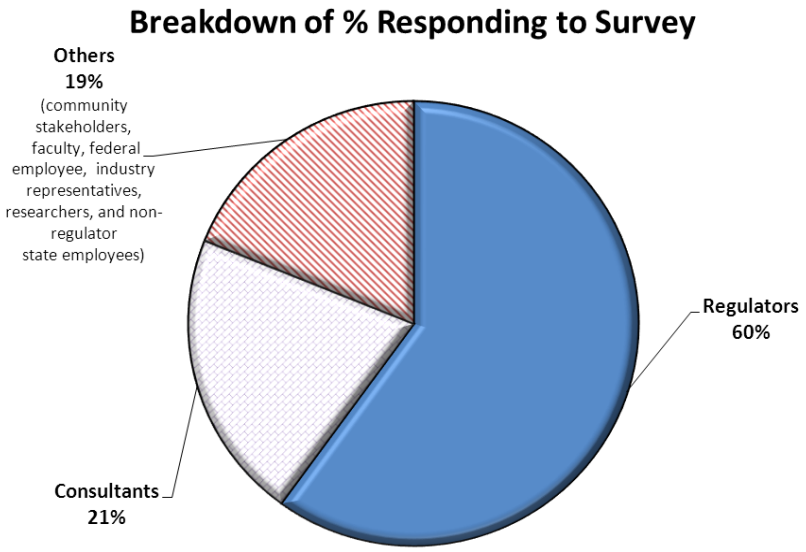

Figure B-1. Survey participants by profession.

- 29 states were represented (see Figure B-2): AK, AL,CA, CO, DE, FL, GA, IL KS, MA, MD, MI MO NC, NE, NH, NV, NY, OH, OK, OR, PA, RI, SC, SD, TX, VA, WV, WY.

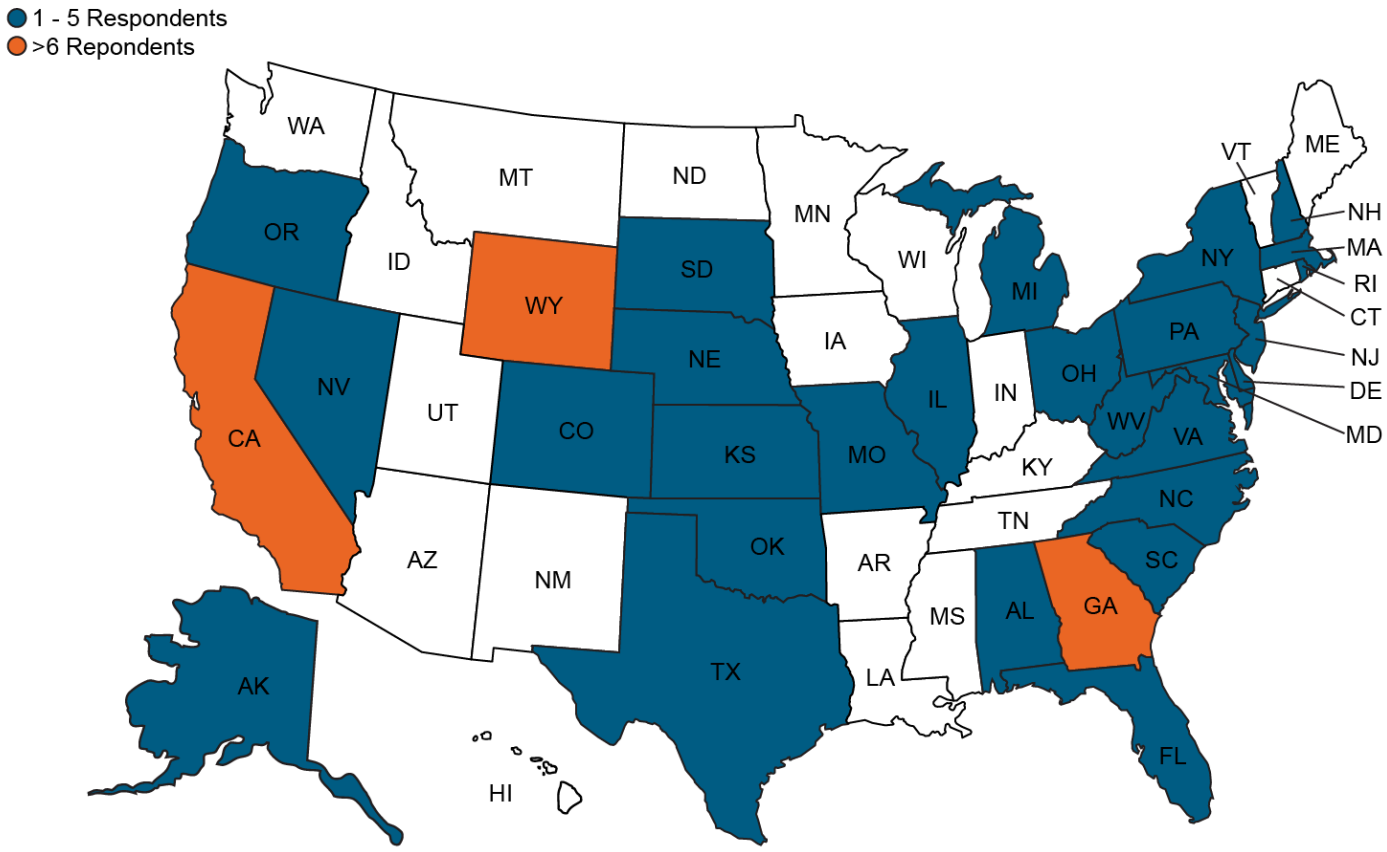

Figure B-2. States with participants in the EMD Survey.

- Regulator participation
  - 2 federal staff; neither reported having EMD experience.
  - 54 state staff; 4 reported having EMD experience.
- Familiarity with EMDs (see Figure B-3)
  - 34.5% familiar with CSIA
  - 34.5% familiar with PCR
  - 24.1% familiar with Fingerprinting Methods
  - 18.4% SIP
  - 14.9% FISH
  - 11.5% Microarray analysis

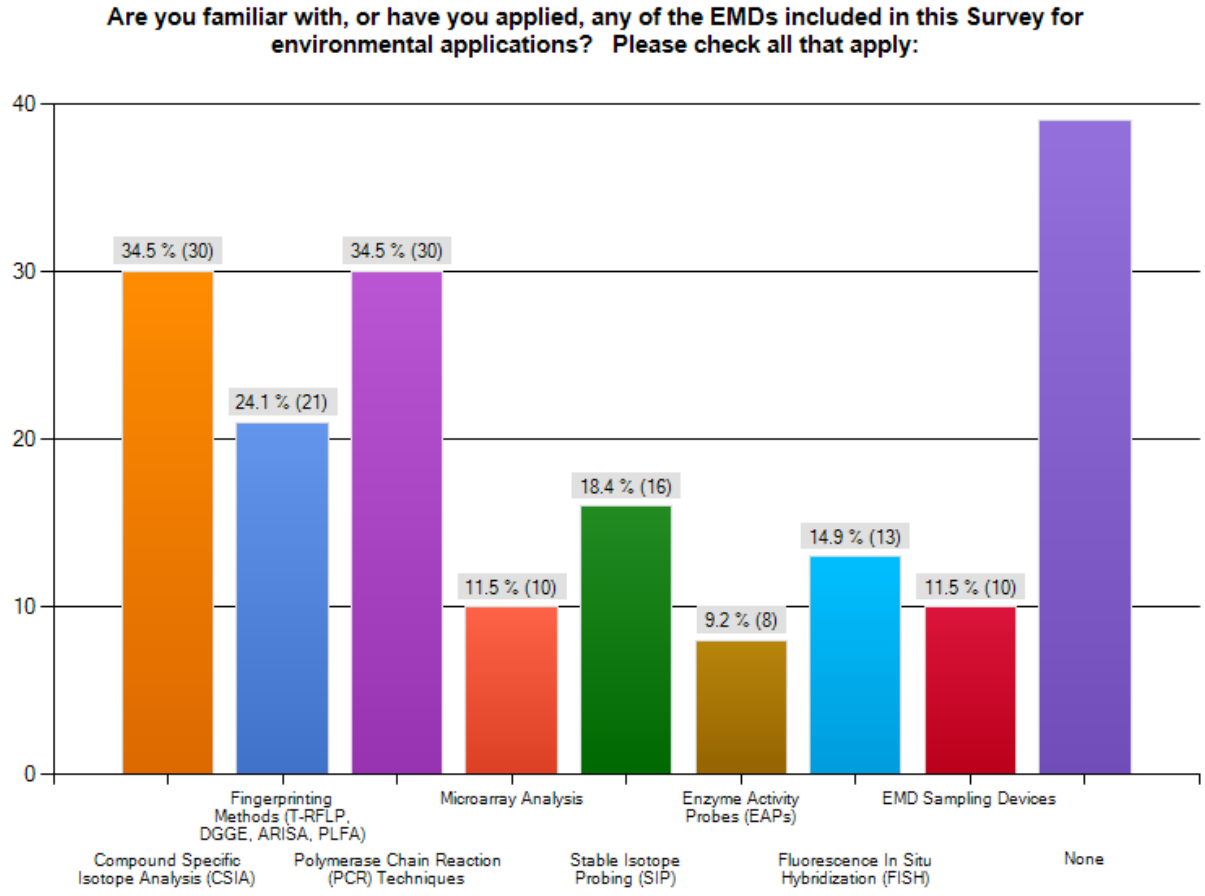

Figure B-3. Percentage of respondents reporting familiarity with EMDs (figure generated from Survey Monkey results).

- EMD Application ─ almost 66% of survey participants reported having no experience with EMDs (see Figure B-4).

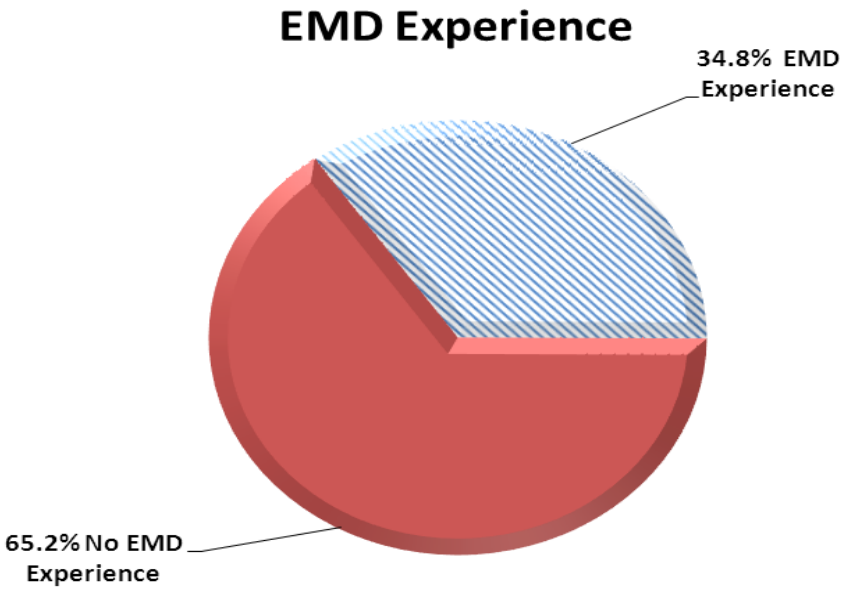

Figure B-4. Percentage of respondents with EMD experience.

- Potential barriers, ways to increase confidence and the number of people/number of sites where EMDs have been used.

Table B-1. Summary information from survey results

Method	Barriers that prevent use of EMDs	Ways to increase confidence	Number of sites where EMDs have been used by survey respondents
Compound Specific Isotope Analysis (CSIA)	• cost • availability of qualified laboratory	• guide to data interpretation • method standardization	• 1 to 4 sites,15 respondents • 5 to 9 sites, 2 respondents
Fingerprinting Methods (T-RFLP, DGGE, ARISA, PLFA)	• knowledge of EMD • availability of qualified laboratory	• data validation process • guide to data interpretation • method standardization	• 1 to 4 sites, 14 respondents • >15 sites, 1 respondent • 5 to 9 sites, 2 respondents
Polymerase Chain Reaction (PCR) Techniques	• cost	• method standardization • data validation process • guide to data interpretation	• 1 to 4 sites, 15 respondents • 5 to 9 sites, 4 respondents • 10 to 14 sites, 3 respondents • >15 sites, 1 respondent

Table B-1. Summary information from survey results (continued)

Method	Barriers that prevent use of EMDs	Ways to increase confidence	Number of sites where EMDs have been used by survey respondents
Microarray Analysis	• knowledge of EMD • availability of qualified laboratory • cost • availability of qualified technical personnel	• guide to data interpretation • method standardization	• 1 to 4 sites, 4 respondents • 5 to 9 sites, 1 respondent
Stable Isotope Probing (SIP)	• cost • availability of qualified laboratory	• guide to data interpretation • method standardization	• 1 to 4 sites, 9 respondents • 10 to 14 sites, 1 respondent • > than 15 sites, 1 respondent
Enzyme Activity Probes (EAPs)	• availability of qualified laboratories • knowledge of EMD • availability of qualified technical personnel • cost	• guide to data interpretation • method standardization • data validation process	• 1 to 4 sites, 3 respondents • 5 to 9 sites, 1 respondent • > 15 sites, 1 respondent
Fluorescence In Situ Hybridization (FISH)	• availability of qualified laboratories • knowledge of EMD • cost • availability of qualified technical personnel	• guide to data interpretation • method standardization	• 1 to 4 sites, 7 respondents • 5 to 9 sites, 1 respondent
EMD Sampling Devices	• availability of qualified laboratories • availability of qualified technical personnel • cost	• standardized QA/QC procedures • a guide for field sampling	• 1 to 4 sites, 7 respondents • 5 to 9 sites, 3 respondents • 10 to 14 sites, 1 respondent • > than 15 sites, 2 respondents

Participants listed knowledge of EMDs, cost, availability of qualified laboratories, and availability of qualified technical personnel as barriers to using EMDs. Standardized QA/QC procedures, a guide to data interpretation, method standardization, a data validation process, and a guide to field sampling were all listed as ways to increase confidence in using EMDs. A summary of the potential barriers, ways to increase confidence and the number of people/number of sites where EMDs have been used are listed by each specific EMD in Table B-1.

B.3 Training Needs

The highlights of the results for the training needs questions on the survey include:

- 80.6% described EMD training needs at their agency or organization from moderate to very extensive. Only 19.5% of the survey respondents expressed training needs as "not very extensive".
- The majority of those that participated thought that basic sciences information should be a part of the EMD training program.
- Most thought that their agency or organization would benefit from training on specific EMDs. See Figure B-5 for details.

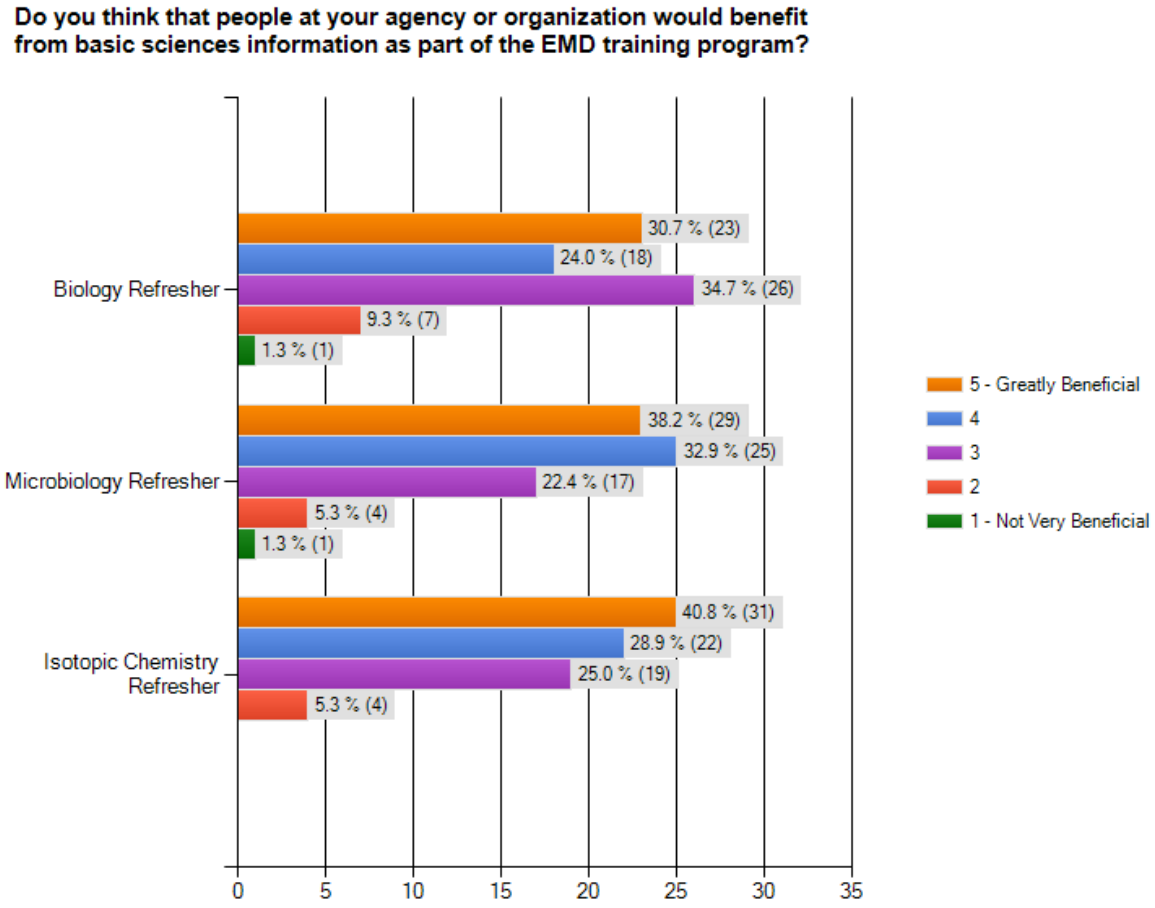

Figure B-5. Summary of training needs (figure generated from Survey Monkey results).

APPENDIX C. ISOTOPIC CHEMISTRY

This appendix provides a basic overview of stable isotope chemistry. Common questions are addressed and examples of applications to environmental problems are presented.

C.1 Review of the Atom

The atom consists of protons (positive charge, mass of 1 atomic mass unit, amu), neutrons (no charge, mass of 1 amu), and electrons (negative charge [-1], mass much smaller than 1 amu). The number of protons in an atom determines the element and determines the behavior of the atom in chemical reactions. A neutral atom has the same number of electrons and protons.

Ions

Atoms with a charge are called ions. If an atom has fewer electrons than protons, then the atom has a positive charge and is a positive ion. If an atom has more electrons than protons then the atom has a negative charge and is negative ion.

Isotopes

Finally, the number of neutrons can vary within atoms of the same element. The charge is not affected and the chemical behavior is not affected. Two atoms with the same number of protons but a different number of neutrons are called isotopes. At a basic level, isotopes of an element behave the same chemically (with an important caveat that will be discussed for stable isotopes). Isotopes have the same number of protons, and are therefore the same element with the same atomic number. Isotopes have a different number of neutrons, however, and therefore a different atomic mass.

While many isotopes are possible for a given element, the nucleus will only be stable for a few configurations of protons and neutrons (roughly equivalent numbers of each). For example, carbon-12 (6 protons and 6 neutrons) is stable. Carbon-13 (6 protons, 7 neutrons) is stable, but carbon-14 (6 protons, 8 neutrons) is unstable, and will undergo radioactive decay. The extra neutron in the case of carbon-14 will become a proton, leaving an atom with 7 protons and 7 neutrons. By changing the number of protons, the atom becomes a new element (in this case nitrogen-14).

C.2 How does CSIA compare to radioisotope chemistry?

CSIA as discussed in this document is concerned only with stable isotopes of individual compounds. Stable isotope compositions of individual compounds are a result of their original source feedstock, and then they undergo isotopic changes as a result of biodegradation and other processes. The changes in the stable isotopes compositions are relatively small during these processes but can be measured with great precision and accuracy.

Radioactive isotopes are different, since radioactive isotopes such as carbon-14 undergo

radioactive decay; over time, carbon-14 will be converted into nitrogen-14. Carbon-14 has a half-life of 5,739 years and can be used for age dating of recent carbon. Carbon with ages up to about 60,000 years can be dated with carbon-14, but compounds or material older than that will not contain any carbon-14.

Tritium is the radioactive isotope of hydrogen and has a half-life of 12.32 years. Radioactive decay of tritium produces helium-3. Tritium has been used as a tool to examine ocean circulation, since large amounts of tritium were introduced into the stratosphere during the nuclear tests of the 1960's. Before the nuclear tests, the Earth's surface contained only about 3 to 4 kilograms of tritium, but these amounts rose by two or three orders of magnitude during the post-test period. This spike provides a valuable age-dating marker.

At this time, methods are becoming available to measure the carbon-14 composition of individual compounds. In the future these methods could have important applications in environmental studies, particularly where there is a need to differentiate recent carbon sources from fossil fuel sources (for example, biofuels).

C.3 What are stable isotopes?

Stable isotopes do not undergo radioactive decay. For example, deuterium (a hydrogen atom with one neutron) is stable; however, tritium (another isotope of hydrogen) will transform over time to a different element (helium) with a change in the number of protons, and is therefore a radioactive isotope. While isotopes behave identically in chemical reactions, a very small difference in bond energy exists between heavy and light isotopes. In general, a compound containing the lighter isotope will react faster than one with the heavier isotope, leading to a fractionation effect during reactions.

In the field, various factors affect isotopic composition. Individual compounds have different isotopic compositions as a result of various fractionation processes occurring during formation and degradation of the compounds. The stable isotope composition of an individual compound at a site reflects the natural abundance of the isotopes and may or may not have been affected by a number of biological or nonbiological processes.

C.4 Which stable isotopes are most commonly used in environmental studies?

At present, the most commonly used stable isotopes in environmental studies are carbon and hydrogen. However, in addition to the widely applicable carbon and hydrogen isotopes, several other stable isotopes apply for some sites, especially isotopes of chlorine, nitrogen, and oxygen. In the past several years, an increasing number of studies have used chlorine isotopes, primarily as a result of online techniques becoming available for analyzing chlorine isotopes. Nitrogen isotopes have been used in a number of studies, particularly those involving explosive residues at military sites. Oxygen isotopes have been used in studies of inorganic contaminants, primarily perchlorate.

C.5 What methods are available for the determination of stable isotopic ratios?

Stable isotopes can be determined in two ways: bulk (offline) and gas chromatography-isotope ratio mass spectrometry (GC-IRMS). The traditional bulk or offline method has been used for the past 60 or 70 years and converts the compound of interest to the measured species (for instance, CO_2 for carbon). The converted sample is then introduced into a dual inlet mass spectrometer simultaneously with the relevant isotope standard and the relative isotope fractionation is measured. This method can be used for pure compounds or for complex mixtures such as crude oils without pre-separation of individual compounds. However in the case of mixtures, only one isotope value is obtained (bulk isotope value), which generates a weighted average of the isotopic composition of all the individual compounds in the mixture.

Recently, GC-IRMS has become available and has led to an increase in applications in many different areas, including the environmental area. In the GC-IRMS approach, compounds are separated on the GC and then pass directly into a reactor that converts the individual compounds into the species that are measured in the IRMS to determine their isotopic composition. isotopic ratios can be determined for any compound that is visible and resolved on the GC chromatogram. Contaminants in groundwater samples can be introduced into the GC using purge and trap, as in conventional analyses. Common contaminants such as MTBE, PCE, and BTEX can be characterized isotopically at concentration levels of 1 ppb.

With this detection limit, the compound identification capability of a GC-IRMS is no greater than that of a GC with a nonspecific detector such as a flame induction detector (GC-FID). To further complicate matters, with the current technology retention times shift gradually, but measurably, over the time scale of weeks and once again whenever instrument maintenance occurs. Consequently, recent standard runs can be used for analyte identification, but the standard GC practices for setting retention time windows are not appropriate.

Further, GC-IRMS requires complete (that is, baseline) resolution of target analyte peaks on a noise-free, flat background. Proper identification and compound resolution are critical to the reliability of GC-IRMS results. Accordingly GC-IRMS data should be accompanied by positive identification and quantification from scanning mass spectral data such as that recommended by SW846-8260. If the analyte elutes with any other compound, it will be evident in the GC-IRMS peak shape and the ratio of the GC-IRMS peak area to the (dilution corrected) GCMS concentration. GC-IRMS measures isotopic ratios and not contaminant concentrations, so this relationship is expected to be somewhat loose, but still should be roughly proportional. Additionally, operator expertise is needed to evaluate peak shapes. Given these two criteria, co-elution reporting should be pass/fail rather than quantitative.

C.6 What about oxygen, which has three stable isotopes?

The isotopic ratios for oxygen are of particular interest because there are three stable isotopes of oxygen, ^{16}O, ^{17}O, and ^{18}O, which are of interest in environmental applications.

$\Delta^{17}O$ = is a deviation from the expected mass-dependent fractionation process for O ($^{18}O/^{16}O$ and $^{17}O/^{16}O$). The three stable isotopes of oxygen, ^{16}O, ^{17}O, and ^{18}O, have natural abundances of ~ 99.76%, 0.04%, and 0.2%, respectively. Typical variations in oxygen isotopic ratios are reported as $\delta^{18}O$ and $\delta^{17}O$ (see previous δ definition) in parts per thousand (‰). When isotopic fractionation is strictly mass-dependent, the following relationship occurs:

$$\text{Equation 1:} \quad \delta^{17}O \cong 0.52 \cdot \delta^{18}O$$

Because of this relationship, $\delta^{17}O$ is typically not reported. However, a number of different compounds, including ozone, and atmospherically generated perchlorate, sulfate and nitrate among others, have been observed to have values of $\delta^{17}O$ and $\delta^{18}O$ values that do not conform to the above mass-dependent relationship (Eq. 1). In this case a value termed "$\Delta^{17}O$" is often reported. $\Delta^{17}O$ represents the deviation from the abundance expected for mass-dependent fractionation, according to the approximation in Eq 2:

$$\text{Equation 2:} \quad \Delta^{17}O = \delta^{17}O - 0.52 \cdot \delta^{18}O$$

or, alternatively:

$$\text{Equation 3:} \quad \Delta^{17}O = [(1 + \delta^{17}O) / (1 + \delta^{18}O)^{0.525}] - 1$$

The $\Delta^{17}O$ value is typically reported in parts per thousand (‰) following multiplication by 1,000 of both sides of Equation 2 or Equation 3.

C.7 What is actually measured?

What is actually measured is technique dependent. For carbon in VOCs, hydrogen in VOCs and both chlorine and oxygen in perchlorate, as well as many other applications, the chemical conversion technique is used. For chlorine in VOCs, either the direct GC-IRMS or the GC-MS technique is used.

C.7.1 Chemical conversion technique

For many applications, the specific compound is chemically converted to a small target molecule such as carbon dioxide for carbon or molecular hydrogen for hydrogen. These target molecules are then measured by an IRMS. Some exceptions exist for chlorine isotopes; these are presented after the IRMS applications.

The possible configurations that involve the two most common isotopes are listed in Table C-1. The mass (in atomic mass units) is also listed for each. Using the data in Table C-1 the abundance relative to $^{1}H^{1}H$ is also calculated based on Table C-2.

Table C-1. Molecular Isotopes, molecular masses and relative abundances for H_2.

Molecular Isotope	Molecular Mass (in amu)	Relative abundance in standard
$^{1}H^{1}H$	2	1
$^{1}H^{2}H$	3	3.11×10^{-4}
$^{2}H^{2}H$	4	2.42×10^{-8}

Current IRMS can measure the signal at m/z=2 and m/z = 3, but the relative abundance of m/z=4 makes it impractical to measure m/z=4. Accordingly, $^{2}H^{2}H$ is ignored and the isotopic ratio $^{2}H/^{1}H$ is simply the ratio of the signals at m/z = 3 and m/z = 2.

Absolute measurements are very hard to make. Many of the errors encountered are systematic, and they would affect a standard just as they affect an unknown sample. For that reason CSIA measurements are performed with an internal reference standard, and the mathematical computations used to account for that referencing is beyond the scope of the current document. In addition, for hydrogen there is a slight correction for the ionic reaction $H_2^+ + H_2 \rightarrow H_3^+ + H$ contributing to the m/z=3 signal. Details are available in Sessions et al. (2001).

This same approach can be applied to the calculation of atomic carbon isotopic ratios from the measured IRMS signals. However, to a very good approximation the isotopes of oxygen can be ignored and the carbon isotopic ratio is the ratio of the signal at m/z=45 over the signal at m/z = 44. In practice the signal at m/z = 46 is also measured and it is used to correct for the contributions of the isotopes of oxygen. The details of that correction are beyond the scope of this work but can be found in the study by Santrock, Studley, and Hayes (1985). Also, as with hydrogen, in carbon CSIA, measurements are relative so each unknown is measured against a standard.

For elements such as oxygen or nitrogen the process is similar: the target molecule was chosen to give a robust and simple calculation of the atomic isotopic ratio based on a measurement of a single m/z signal normalized by the most common m/z signal, with slight corrections made by measurement of one additional m/z signal. Further, for all but hydrogen, three signals are always measured, with the lowest being the m/z with the molecular mass of the target with the lightest isotopes (for practical reasons, only m/z=2 and m/z =3 are measured for hydrogen).

C.7.2 Direct GC-IRMS

In this technique the specific compounds are not chemically converted but pass directly into the IRMS where they undergo electron bombardment. The electron bombardment breaks up or "fragments" the target analyte in a predictable fashion. The relatively large isotopic ratio of chlorine allows contributions from much less abundant isotopes to be ignored; so specific m/z ratios are monitored in the IRMS to give the isotopic ratio of the chlorine. In a GC-IRMS the signal is

measured in "cups" that are positioned according to the mass (actually, m/z mass to charge ratio) being measured. Since these cups monitor masses heavier than the mass of the typical IRMS analytes CO_2 (carbon), H_2 (hydrogen) CO (oxygen) or CH_3Cl (chlorine) and since they cannot be reassigned during the course of a single run, it is often required to have the IRMS specially engineered. This requirement is a significant limitation of this technique. For further details, see Shouakar-Stash et al. (2006).

C.7.3 GC-MS

This process is very similar to that used in direct GC-IRMS. However, since a regular quadrupole mass spectrometer is used rather than an IRMS, the precision of the monitored m/z ratios is not nearly as good. To make up for this several m/z ratios are monitored and the calculations are a bit more intensive. For more details see Jin et al. (2011).

C.8 What is the basic notation for expressing isotopic ratios?

Isotopic ratios are expressed using the delta notation (δ), and the same formula is used regardless of the isotope being determined, although the international standards used are different for the different isotopes (see Section C.9, Table C-2). The δ notation is expressed in the following manner, shown for carbon-13:

$$\delta\left({}^{13}C\right)_x = 1000 \times \frac{R_x - R_{Std}}{R_{Std}}$$

R_x corresponds to the ratio of the intensity of the heavy to light isotope in the sample and in the case of R_{Std}, it is the ratio of the heavy to light isotope in the international standard. In the case of carbon, the species actually measured by the IRMS are mass 45 and 44 which correspond to the masses of the $^{13}CO_2$ and the $^{12}CO_2$ produced by combustion of the sample. For other isotopes, the appropriate species are again measured and compared to the appropriate standards for each isotope.

C.9 What are the international standards used in the isotopic measurements?

Table C-2 presents the reference standards for some of the most commonly applied stable isotopes.

Table C-2. The most commonly applied stable isotope ratios for environmental applications. Source: Data from Coplen et al. 2002.

Element	Isotope of interest	Ratio measured	Reference standard	Isotopic ratio in standard
Hydrogen	^{2}H	$^{2}H/^{1}H$	Vienna Standard Mean Ocean Water (VSMOW)	1.558×10^{-4}
Carbon	^{13}C	$^{13}C/^{12}C$	Vienna Pee Dee Belemnite (VPDB)	1.118×10^{-2}
Nitrogen	^{15}N	$^{15}N/^{14}N$	N_2 in air	3.676×10^{-3}

Element	Isotope of interest	Ratio measured	Reference standard	Isotopic ratio in standard
Oxygen	^{18}O	$^{18}O/^{16}O$	VSMOW	2.005×10^{-3}
			VPDB	2.065×10^{-3}
	^{17}O	$^{17}O/^{16}O$	VSMOW	3.799×10^{-4}
Sulfur	^{34}S	$^{34}S/^{32}S$	Vienna Canyon Diablo Troilite (VCDT)	4.416×10^{-2}
Chlorine	^{37}Cl	$^{37}Cl/^{35}Cl$	Standard Mean Ocean Chlorine (SMOC)	3.196×10^{-1}

C.10 Natural Abundance of Stable Isotopes and Isotopic Fractionation

Elements have multiple naturally occurring isotopes. This condition is reflected in the atomic masses of the elements as presented in the periodic table. For example, the most common isotope of carbon is carbon-12 (6 protons, 6 neutrons, or atomic weight of 12.0) but the atomic mass of carbon is 12.011, reflecting the weighted average of 98.9% carbon-12 and 1.1% carbon-13. This small percentage of carbon-13 may then be fractionated between specific compounds in a system (for instance, the degradation intermediate product is slightly 'lighter' in carbon-13, and the parent compound is slightly 'heavier'), but the overall abundance of the isotopes is not impacted.

The natural abundance of the stable isotopes that are of environmental interest are shown in Table C-3. It is important to notice in this table that the heavier isotope is always present in lower abundance than the lighter isotope.

Table C-3. Natural Abundances of Stable Isotopes. Source: Data from Rosman and Taylor 1998.

Element	Isotope of Interest (second most abundant stable isotope)	Ratio measured
Hydrogen	$^{1}H/^{2}H$	99.9885/0.0115
Carbon	$^{12}C/^{13}C$	98.93/1.07
Nitrogen	$^{14}N/^{15}N$	99.63/0.37
Oxygen	$^{16}O/^{18}O$	99.759/0.037
Sulfur	$^{32}S/^{34}S$	94.93/4.29
Chlorine	$^{35}Cl/^{37}Cl$	75.78/24.22

Many common environmental contaminants are derived from crude oil or other fossil fuel sources. Fossil fuels are originally sourced from living organic materials, such as higher plants, phytoplankton, and bacteria. Most of these living systems are photosynthetic systems, meaning that they derive their carbon from the CO_2 in the atmosphere by the process of photosynthesis and the hydrogen from groundwater. During the process of photosynthesis the lighter isotope is typically incorporated at a faster rate than the heavier isotope; this process is known as isotopic fractionation.

As a result of different plant types and phytoplankton having different photosynthetic cycles, the extent of fractionation will vary for different species. In living systems, the isotopic composition of a plant or algae can be used to obtain information on the actual photosynthetic cycle. However by the time all this material has accumulated in a sedimentary environment, been buried, and been converted into a fossil fuel, much of the unique isotope signature is lost due to the heterogeneous mixture of organic matter deposited. Despite this loss, crude oils derived from different source materials will have different isotopic signatures, which means individual compounds that are manufactured industrially will often have different isotopic signatures if derived from different feedstocks.

C.11 What do these isotope ratios represent and why are they generally negative?

The δ values are an expression of the difference in the isotope ratio of the sample relative to the appropriate international standard, for example a compound that has a carbon isotope value of -25 per mil and is depleted by 25 parts per thousand in the heavier isotope relative to the isotopic composition of the standard. Note that for two samples that are (as an example) -20 and -30 per mil, one can say that the -20 sample is isotopically heavier than the -30 per mil sample. This is sometimes confusing since these are negative numbers. However, the key is to remember that the numbers are reflecting the heavier isotope content of the particular sample being characterized

The majority of environmental hydrocarbon and chlorohydrocarbon samples will have negative isotope ratios. The values for isotopic fractionation at sites are generally isotopically light (negative) compared to the standards due to the selection of specific standards. The carbon standard consists of carbonate, or inorganic carbon, which tends to be isotopically heavy compared to the organic carbon compounds of interest at environmental sites.

C.12 What is the Rayleigh equation and how does it relate fractionation to degradation?

The stable isotope compositions of individual compounds can provide two basic types of information in environmental studies: source discrimination (or correlation), and the extent of biodegradation. Source discrimination or apportionment of mixed sources is dependent on sources that are isotopically distinct, due to differing production processes and degree of source degradation. The Rayleigh equation is used to relate degradation-induced decreases in concentrations directly to concomitant changes in bulk (average over the whole compound) isotope ratios.

The most commonly used form of the Rayleigh equation used in environmental studies is shown below:

$$\delta^{13}C_t = \delta^{13}C_{t=0} + \epsilon \cdot \ln F$$

where:
$\delta^{13}C$ at t=0 is the carbon isotopic ratio of the original material (known or estimated)

$\delta^{13}C$ at t is the isotopic fractionation measured at the present time (measured during the study)

ε is the enrichment factor (determined in laboratory or microcosm studies)

ln *F* is the natural logarithm of the remaining concentration of the contaminant

The value of the enrichment factor is needed to calculate the degree of degradation. The isotopic enrichment factor will vary for each compound depending on the microorganisms present and the site conditions. This factor may be measured in microcosm studies, which generate a value for the site organisms under site conditions, or potentially estimated based on a knowledge of organisms present at the site and enrichment factors measured in pure cultures of the organisms.

The extent of in situ transformation, or degradation, may therefore be inferred from measured isotope ratios in field samples, provided that an appropriate enrichment factor (bulk) is known. This bulk value, however, is usually valid for a specific compound and for specific degradation conditions. In other words, enrichment factors that are generally determined from laboratory microcosm studies must be determined for each strain of bacteria thought to be active at a particular site. Because of the time required for microcosm experiments, or the absence of pure cultures, a direct comparison of bulk values for different compounds and for different types of reactions is often not possible.

C.13 What is an Enrichment Factor?

The enrichment factor is an indication of the degree of isotopic fraction between the parent and intermediate compound during a specific degradation reaction, and it is derived from the fractionation factor α through the relationship $\varepsilon=1000\ (\alpha-1)$. The factor α reflects the ratio of the rate constants for the heavy/light isotopes. In other words, the different rates at which these species react reflects the extent of changes expected in the isotopic composition of a particular compound during its degradation.

Application of the Rayleigh equation to determine the remaining contaminant from the bulk isotopic fractionation requires the value of the enrichment factors. Different types of bacteria degrading the same compound under different conditions may have different enrichment factors. Additionally, abiotic processes can also cause isotopic shifts.

Enrichment factors are fundamentally a kinetic parameter. Fractionation in degradation reactions results from a slight difference in the energy required to break a bond between two light atoms versus the energy required to break the bond between two atoms of that same type but with one atom being heavy. This relationship is shown in Figure C-1.

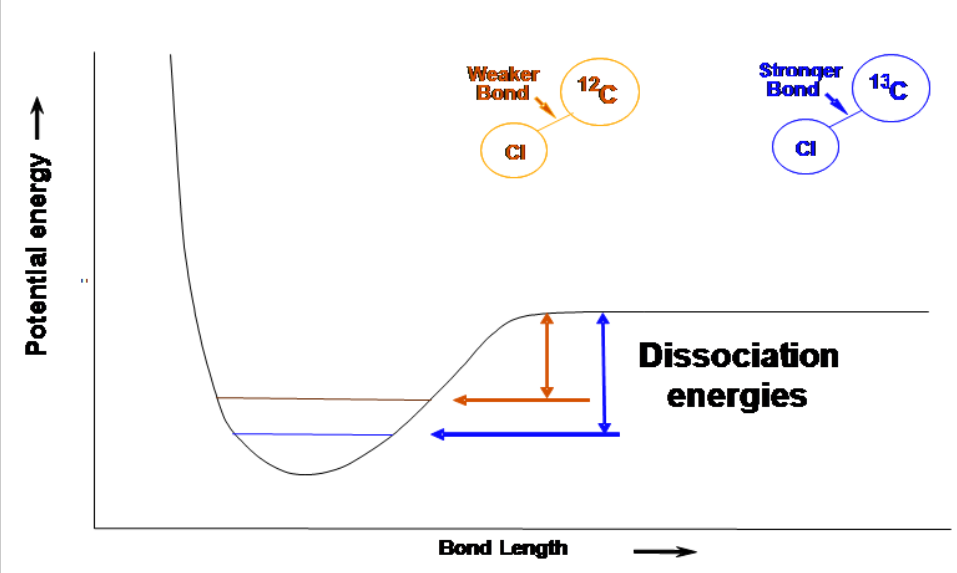

Figure C-1. Schematic indicating the difference in bond dissociation energies that is the origin of isotopic fractionation.

Source: Microseeps, Inc. Used with permission.

When the enrichment factors describe biodegradation, they are a function not just of the small difference in the bond energy portrayed in Figure C-1 but also of the microbial ecology, the strains of bacteria involved, the mass transfer limitations across the cell membrane (see Appendix D, Microbiology FAQ) for the contaminant, the enzymes involved, the availability of contaminant and the toxicity of both the reactants and products in regards to the microbes responsible for the transformation. Thus for biodegradation, enrichment factors are site specific. Further, they are often different at different points in the plume and over the life cycle of the plume.

C.14 What can be learned by the simultaneous CSIA of two types of atoms?

Studies have shown the mechanism of MTBE biodegradation can be discerned by plotting $\delta^{2}H$ vs. $\delta^{13}C$ of MTBE, as shown in Figure C-2 (Zwank et al. 2005).

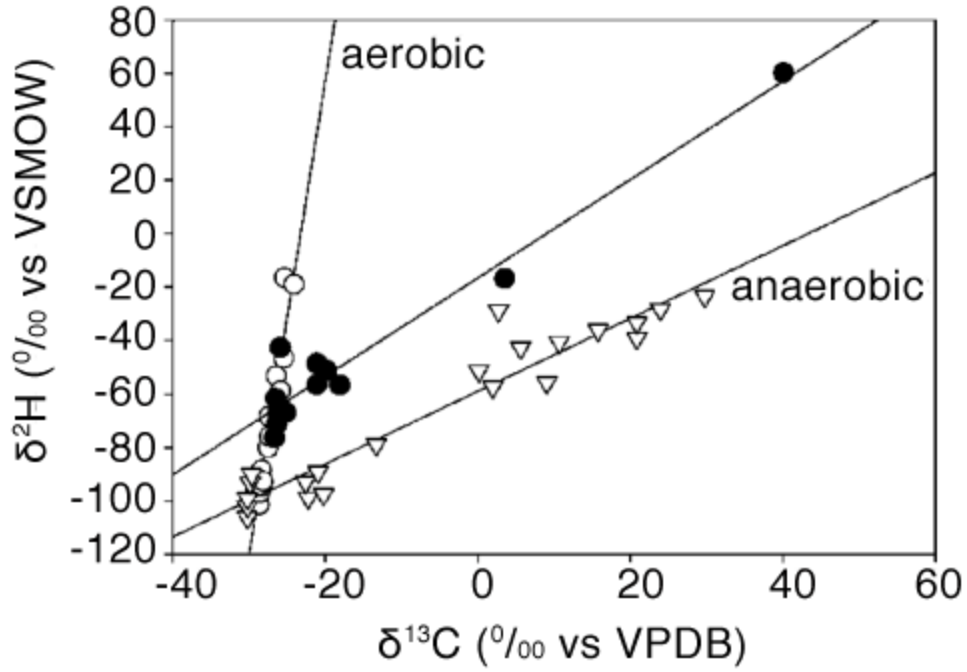

Figure C-2. Graph of δ^2H versus $\delta^{13}C$ of MTBE. Data points labeled (○) for aerobic biodegradation in a laboratory experiment (Gray et al. 2002); (●) for anaerobic biodegradation at field sites (Kuder et al 2002); (▽) field data reported in Zwank et al. (2005).

Source: Reprinted with permission from Zwank, L., M. Berg, M. Elsner, T.C. Schmidt, R.P. Schwartzenbach, and S.B. Haderlein. 2005. New evaluation scheme for two-dimensional isotope analysis to decipher biodegradation processes: Application to groundwater contamination by MTBE. Environmental Science & Technology 39:1018-1029. Copyright 2005 American Chemical Society.

The slope of the line is the ratio of the enrichment factors εH/εC. Since the data available to that point indicated that anaerobic processes were marked low εH and high εC whereas aerobic processes had higher εH and lower εC, a plot such as that presented in Figure C-2 could be used to discern the biodegradation mechanism.

However, new data has become available (Rosell et al. 2012) that does not fit this model. As of this writing, that data has only been available for several months and the scientific community is still weighing its implications. Future studies may clarify this result. Regardless, at this point two conclusions can be made:

1. If enrichment in either the $\delta^{13}C$ or the δ^2H of MTBE has been documented, it can be interpreted as evidence of the degradation of MTBE.
2. Biodegradation can proceed with neither significant $\delta^{13}C$ nor significant δ^2H enrichment. If concentrations appear to be attenuating the absence of corroborating enrichment does not rule out biodegradation. Techniques such as stable isotope probing (see Section 7.0) should be employed.

In summary, while exact interpretations are in flux, for some microbes biodegradation of MTBE leads to isotopic enrichment, while biodegradation by other microbes does not lead to enrichment. Observation of enrichment at one site is evidence of biodegradation at that site. Other microbes may be degrading MTBE at a second site and those microbes may not be isotopically enriching the MTBE, so the lack of enrichment at that second site is not conclusive evidence that there is no biodegradation at the second site.

C.15 Is CSIA useful for "abiotic" remediation?

One subset of abiotic remediation is called biogeochemical transformation. This name recognizes the importance of biology and geochemistry in a remediation that relies upon abiotic reactions and is often passive. ESTCP maintains a project on this subject (ER-201124) and held a workshop in February 2008 that issued a report (ESTCP 2008) discussing these transformations in detail. Because the carbon enrichment factors for this process are of a larger magnitude than those of microbial mediated reductive dechlorination, the use of CSIA to distinguish biogeochemical transformation from reductive dechlorination is being pursued (Liang et al. 2007). Similarly, the carbon enrichment factor for the biogeochemical transformation of 1,2-dibromoethane (ethylene dibromide or EDB) is also of a much larger magnitude than the microbial process (USEPA 2008b).

C.16 What are some applications of stable isotopes in environmental cases?

Stable isotopes are useful for source discrimination and for determining the extent of natural attenuation.

C.16.1 Source discrimination

Stable isotopes can be a powerful tool for discrimination of multiple sources of a compound. Determination of multiple isotopes (C and H or C and Cl) present in the contaminant can strengthen the evidence for source apportionment. This approach may be limited if the compound of interest from different sources are isotopically similar. For relatively small molecules (typically less that ^{10} C atoms) it is necessary to establish whether any degradation has occurred – either microbial or abiotic – since this degradation impacts the resulting isotopic composition. Thus it must be established that isotopic differences in these smaller molecules does not result from attenuation processes before concluding that they are coming from different sources.

However, in the case of larger molecules, the use of stable isotopes for the purpose of source discrimination is somewhat easier than with smaller molecules. A larger number of carbon atoms in molecules will cause a smaller, or no, observable fractionation because of an effect known as "internal dilution." This effect reduces the measurable fractionation in biodegradation of larger molecules such as pesticides. This result implies that differences in the $\delta^{13}C$ of pesticides are most likely due to differences in the undegraded pesticides, so material from source A can more readily be distinguished from material from source B, even if significant degradation of either or both sources occurred. Thus, carbon CSIA of pesticides is ideal for forensic applications. Additionally,

since chlorinated pesticides have many chlorine atoms in each pesticide molecule, CSIA of chlorine is also ideal for forensic applications of pesticides by the same reasoning. This method has been used by Aeppli et al. 2010.

C.16.2 Natural attenuation

For many years, determination of natural attenuation has been determined through time-consuming laboratory microcosm studies. With the introduction of GC-IRMS and its ability to determine isotopic compositions of individual compounds, isotopic enrichment of individual compounds has become a powerful tool to determine the onset and extent of natural attenuation. Ranges of source signatures for unaltered groundwater contaminants are fairly well established and compounds that are isotopically heavier than these source signatures can be assigned as being degraded. Use of two or even three isotopes within the same compound provides an even more powerful approach.

Semi-quantitative estimates of the extent of degradation can be obtained through the use of a simplistic form of the Rayleigh equation which equates changes in isotopic composition with concentration changes. More recent efforts have been aimed at incorporating isotope data into flow transport models to provide a more realistic model of changes in isotopic and concentration data over time at specific sites.

C.17 Are there common sense rules for CSIA applications?

Isotopic fractionation provides definitive evidence of degradation of compounds. As mentioned above, isotopic fractionation is used to assess source discrimination and natural attenuation at environmental sites. Stable isotope fractionation data are only useful in the context of a well-developed conceptual site model.

Many of the source discrimination studies involve contaminated groundwater plumes. While some guidelines have been provided in a recent USEPA publication (USEPA 2008a), ideally a good coverage over the complete plume is desired, including the margins as well as the central part of the plume. In many studies, cost and budgetary restraints do not permit large numbers of samples to be collected and analyzed, but as a minimum collect at least 6-10 samples over the plume.

Once samples have been collected and concentrations determined, those samples that are suitable for CSIA can be determined. Initially carbon isotopes should be determined and evaluated in the context of the overall problem being investigated. At that time, a decision can be made as to whether additional isotopes (such as H and Cl) should be determined. In many cases, the two or three isotope approach can be more beneficial than simply using one isotope for source differentiation or evaluation of natural attenuation.

C.18 What are the interpretations of environmental CSIA applications?

Interpretation of environmental CSIA applications include source correlations; source isotopic signatures; degree of biodegradation (and impact on source isotopic signature); travel distance,

hydrogeologic factors, potential for degradation between sources and the site of interest; and natural attenuation.

C.18.1 Source correlations

Several steps are involved when using the stable isotopes for correlation purposes. First, establish that no biodegradation has occurred at the site that may have affected the stable isotope composition of the contaminant of interest. This step can normally be established through the presence of degradation products such as TBA or *cis*-1,2-DCE. Once this has been established, the isotope ratios can be evaluated in terms of source relationships. Whether samples are related to each other depends to some extent on the precision of the analytical measurements. It is possible to determine the stable carbon isotope compositions of individual groundwater contaminants with a precision of +/- 0.3 per mil. Thus, if a group of samples in a groundwater plume all fall within a range of +/-1 per mil then they may all come from the same source and they are probably related. However, if some samples have isotope ratios that differ by 1.5 per mil or more, and there is no degradation, then those samples may come from a different source.

If there are two or more sources for the plume, then it is possible that the contaminant from both sources may have the same or similar isotopic compositions. In such a situation, it may not be possible to conclusively establish the source of the contaminants in the groundwater. The isotopes will not resolve the sources in every case, but in the situations where isotopic analysis does not provide a solution, none of the other techniques commonly used in these types of problems will provide a solution either. The use of the H and or Cl isotopes may resolve this problem if the concentrations are sufficient to determine the additional isotopes.

C.18.2 Source isotopic signatures

Stable isotopes cannot be used to determine the manufacturer of a specific contaminant. Variations in feedstocks, manufacturing conditions, mixing of products from different plants, and many other variables make this an impossibility. When using CSIA, the term “source” is referring to point of release—not the manufacturer of origin.

C.18.3 Degree of biodegradation (and impact on source isotopic signature)

Biodegradation affects the isotopic signature of the compound of interest. The extent of isotopic enrichment primarily depends upon the compound, mechanism of degradation, and environmental conditions. Changes in isotopic signatures resulting from biodegradation must be recognized or else samples thought to be coming from different sources may simply differ as a result of the degradation process.

C.18.4 Travel distance, hydrogeologic factors, potential for degradation between sources and the site of interest

Other factors that may affect the source signature of a particular compound may include adsorption, evaporation, and remediation processes such as soil vapor extraction, or partitioning between various phases. However, for most of these physical processes the extent of isotopic fractionation is relatively small (~1 per mil or less) and much smaller than shifts associated with biodegradation.

C.18.5 Natural attenuation

For most of the common groundwater contaminants ranges of isotopic signatures for unaltered or nondegraded compounds have been clearly established and are summarized in Figure C-3. As the compounds degrade they become isotopically heavier or enriched as a result of preferential cleavage of ^{12}C-^{12}C bonds, leaving the residual substrate becoming enriched in the heavier ^{13}C isotope. If these values are heavier than the source signatures by at least 2 or 3 per mil, a high level of confidence exists for contaminant degradation. Certain limitations are associated with these interpretations are summarized below.

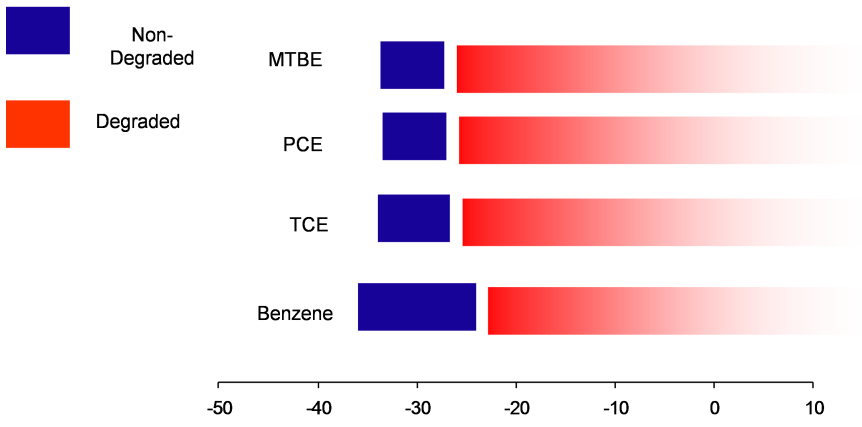

Figure C-3. Comparison between the isotopic compositions of non-degraded versus corresponding degraded components.

Source:Adapted from US Navy 2008. Data obtained from papers cited in Philp, R. P. and Jardé, E. (2006). Application of Stable and Radioisotopes in Environmental Forensics. In: Introduction to Environmental Forensics (Murphy and Morrison, Eds. 2007).

In reality, the situation may be far more complex than simply comparing the isotopic composition of the final degradation product, since the isotopic ratios and parent and degradation product are constantly changing. If compound A is simply degrading to compound B, (for example, MTBE to TBA) then the initial TBA will be isotopically light and the MTBE relatively heavy. As the reaction progresses the TBA that is formed continues to become heavier, since the precursor MTBE is also becoming heavier and at the end of the reaction (assuming no TBA has been lost), then the TBA will ultimately have the same isotopic composition as the original MTBE.

In a more complex degradation sequence, for example PCE degrading to TCE, TCE may initially degrade to *cis*-1,2-DCE. In this case, initially the TCE will be isotopically light but, as it starts to degrade, it will become heavier. At the same time, TCE will also be diluted by more TCE being produced from degradation of the PCE. This is a far more complex situation, but at the end, if all the PCE is totally converted to ethane, then the ethane will have the same isotopic composition as the original PCE. Reactive fate and transport models are currently being developed to take these multiple production and degradation processes into consideration.

C.19 What are the limitations of CSIA?

CSIA is subject to several limitations.

C.19.1 Biodegradation-natural attenuation

Reports at a number of sites indicate that no isotope signature of biodegradation was detected even when standard site evaluation criteria were suggestive of degradation. Elsewhere, isotope enrichment was observed, but poor correlation of isotope ratios and concentration attenuation was apparent. While it is possible that attenuation at those sites was primarily due to dispersion, other factors could lead to a false negative interpretation of CSIA. The following general situations regarding potential false negatives are applicable to all common groundwater contaminants.

- A monitoring well in contact with residual contaminant/analyte—The measured isotope ratio of the contaminant in the groundwater sample would reflect an average of that contaminant from the sampling radius of the monitoring well. “Fresh” isotope composition of newly dissolved contaminant may overwhelm the signature of the degraded contaminant.
- Heterogeneity of an aquifer with respect to degradation distribution—If a monitoring well intercepts groundwater with the target analyte that had been degraded to varying degrees, the net isotope ratio would reflect relative contributions from more- and less-degraded zones along the screen of the monitoring well. The degradation signature may be overwhelmed in the undegraded compound if it occurs at high concentration in some spots within the sampling radius.
- Insufficient sample coverage—At some sites, only a limited portion of the contaminant plume showed a biodegradation signature. If the portion of the plume where biodegradation is occurring is not sampled, no isotope effects will be detected. Biologically active parts of

the plume may be located in the center of the plume as well as near the source or at the leading edge.

- Certain degradation processes result in a relatively minor carbon isotope effects—For example, aerobic MTBE degradation may have ε=-2.4 or less. Carbon data may not be sufficient to detect such a process unless the extent of biodegradation is significant (> 90%) and no other restrictions occur. In other words, carbon isotope fractionation from aerobic biodegradation may not rise above the signal-to-noise ratio of the analytical method. In a hypothetical case, a degrading organism may not induce any isotope effect at all.
- Inadequate analytical methodology—The most degraded samples, in which the largest diagnostic isotope effects are expected, may be missed due to insufficient analytical sensitivity. This effect is important for potential carbon CSIA application to aerobic MTBE biodegradation. Additionally, isotope analysis requires good baseline separation in the GC step, in excess of the standard concentration analysis. Purge and trap extraction combined with good GC practice offers the best probability of success.

False positive scenarios are not likely. Non-degradative attenuation pathways under specific hydrological conditions can result in measurable isotope effects. In the studied scenarios, volatilization, air sparging, and SVE resulted in minor carbon isotope effects only. Another interference is a growing number of in situ applications using stable isotope-labeled MTBE and TBA (such as the Bio-Sep® technology). Labeled substrates migrate into groundwater and mimic biodegradation signatures when studied by CSIA (^{13}C labeled MTBE or TBA) or interfere with the instrumental performance of CSIA (any ^{2}H-labeled VOC compounds will potentially interfere with measuring $\delta^{2}H$ of MTBE). Site managers should take special care to avoid false conclusions if CSIA and stable isotope-label technique are scheduled for the site assessment.

C.19.2 Source identification

It is sometimes assumed that the isotopic compositions of certain groundwater contaminants can be used to relate these contaminants to a specific manufacturer. This assumption is based on studies published in the early 1990's in which samples of PCE from different manufacturers were analyzed and led to specific manufacturers. Subsequent research has proven that isotopic analyses cannot reliably relate contaminants to manufacturers. Feedstocks vary on a frequent basis, processes change, and products are fungible. All of these variables and many others render it virtually impossible to associate a certain isotope value with a particular manufacturer.

However, correlations do exist between the suspected release point of a contaminant and samples in the plume. Once it has been established the contaminant is not degraded, then the correlations can be attempted (if possible using two or more isotopes).

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APPENDIX D. MICROBIOLOGY FAQS

The following are lists of frequently asked questions (FAQs) related to microbiology. The FAQs provide the background information needed to better understand the content in the main body of this document. In order to provide useful examples, references are made back to content in the main body of this document.

MICROBIAL STRUCTURE AND FUNCTION

1. What are microorganisms?
2. What do bacteria look like?
3. What makes up bacteria?
4. What are lipids?
5. What are sugars?
6. What are proteins?
7. What are enzymes?
8. What are nucleic acids?
9. What are chromosomes?
10. What are genes?
11. What are ribosomes?

MICROBIAL GROWTH AND BIODEGRADATION

12. How do bacteria grow?
13. What do bacteria need to grow?
14. What are electron donors?
15. What are electron acceptors?
16. What is cellular respiration?
17. What are carbon sources?
18. Besides carbon, what other nutrients are needed for growth?
19. What is microbial metabolism, and why is it important?
20. What are some examples of aerobic respiration important to bioremediation?
21. What are some examples of anaerobic respiration important to bioremediation?
22. What types of respiration occur in a contaminated environment?
23. How does biodegradation relate to microbial metabolism?
24. What factors determine which contaminants can be biodegraded?

MOLECULAR BIOLOGY AND MICROBIOLOGY

25. What is molecular biology?
26. How is molecular biology useful in biodegradation studies?
27. What is PCR, and how does it work?

28. What is automated DNA sequencing?
29. What do molecular biology studies detect?
30. What genes are important in molecular biology studies?
31. What are 16S rRNA genes, and what can they tell me?
32. What are functional genes, and what can they tell me?
33. Why study microbial enzymes if you can study microbial genes?
34. Why study microbial lipids if you can study microbial genes?

FUTURE DIAGNOSTIC TOOLS:

35. What will molecular biology tools be able to measure in the future, and how will this help me understand biodegradation processes?
36. What are the emerging techniques for chemically characterizing contaminants, and what can they tell me?
37. What are the emerging molecular biology techniques, and what can they tell me?
38. What is metagenomics?
39. What is metatranscriptomics?
40. What is metabolomics?
41. What is proteomics?
42. What are the limitations of emerging technologies?
43. What else can I expect?

D.1 Microbial Structure and Function

1) What are microorganisms?

Microorganisms are organisms that are too small to be seen with the naked eye. Most microorganisms are smaller than 0.2 mm in length and may be no more than 1 or 2 micrometers (μm) or even smaller. Living microorganisms include bacteria and archaea, fungi, and some protozoa. Viruses are the smallest microorganisms but are not living organisms. Only some microorganisms (bacteria, archaea, and fungi) contribute significantly to processes that remove contaminants from the environment. These FAQs focus primarily on bacteria and only mention other microorganisms as relevant.

2) What do bacteria look like?

Bacteria are single-celled organisms that come in many shapes and sizes. The structure of a typical bacterium is shown in Figure D-1. The outermost layer of the bacterium is the capsule. This layer protects the bacterium from the environment. Inside the capsule is the cell wall, which maintains the shape and structural integrity of the microorganism. Inside the cell wall, a cell membrane acts as a selective barrier between the outside aqueous environment and the inside of the cell. The cell membrane surrounds the gel-like cytoplasm, where most of the biochemical reactions occur within the bacterium. The cytoplasm also contains many small organic and inorganic chemicals. Inside the

cytoplasm are two key structures: the chromosome and the ribosome. Some bacteria also have protein-based appendages (called flagella or pili), which help bacteria move in their environment.

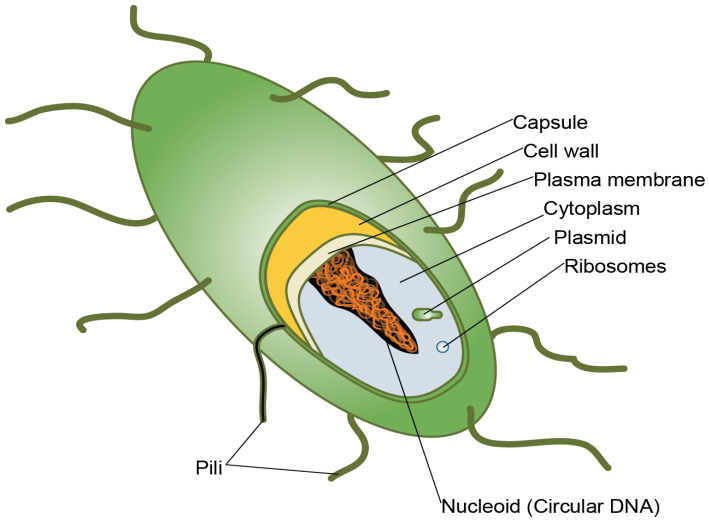

Figure D-1. The structure of a typical bacterium.

3) What are bacteria made of?

Like all living organisms, bacteria are made of four major classes of biomolecules: lipids, polysaccharides, proteins, and nucleic acids. All of these major biomolecules consist of six key elements: carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), and sulfur (S). These elements are collectively referred to as CHONPS.

4) What are lipids?

Lipids are a large and diverse group of organic compounds that are insoluble in water but soluble in nonpolar solvents such as chloroform. In bacteria the most important lipids are called phospholipids. Phospholipids consist of glycerol backbone with two attached fatty acid chains and a phosphate group linked to an organic molecule such as choline. Phospholipids are important because they align themselves with their hydrophobic (water-hating) fatty acid tails away from the water, and their hydrophilic (water-loving) phosphate heads towards the water. This alignment, as shown in Figure D-2, results in the formation of a membrane. The cells of all living organisms have a membrane that acts as a semi-permeable barrier that isolates and protects the processes that occur within the cell from the outside environment.

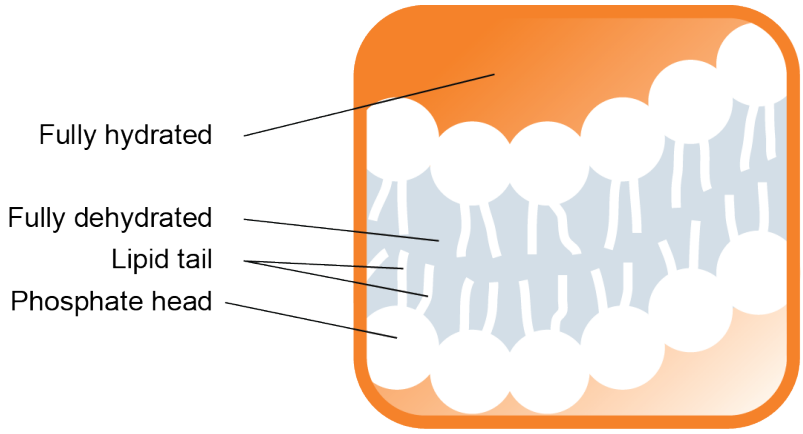

Figure D-2. Phospholipid.

The analysis of the fatty acid components of phospholipids (phospolipid fatty acids, PLFAs) can aid in understanding the microbial processes occurring at a contaminated site. For example, different microorganisms produce specific types of phospholipids. The variety and relative abundance of PLFAs can identify the types of microorganism present in a sample (see Section 5.0). The PLFA composition of bacteria can also change in response to environmental factors. These changes can determine whether bacteria in a sample are under stress. The analysis of PLFAs can also identify groups of microorganisms that are metabolically active in an environment. See Section 7.0).

5) What are sugars?

Sugars, also known as carbohydrates, are simple molecules composed of carbon, oxygen, and hydrogen. Sugars either exist as single molecules (monosaccharides) or as polymers (polysaccharides). Monosaccharides are often used by bacteria as energy sources and are components of other important biomolecules. Polysaccharides are components of the bacterial cell wall and the capsule. Capsules help bacteria attach to surfaces, form biofilms and provide protection from adverse environmental conditions. The sugars present in microorganisms typically do not provide useful information about the types or numbers of contaminant-degrading bacteria in an environment. None of the current EMDs described in this document characterize sugars derived from bacteria and other microorganisms.

6) What are proteins?

Proteins are polymers consisting of linear chains of amino acids. These chains can fold over on themselves to form complex molecular structures. Depending on their three-dimensional structure and amino acid sequence, proteins can serve a wide variety of biological functions. In simple organisms like bacteria, many proteins serve as catalysts and are responsible for the biochemical reactions that are required for a bacterium to live, grow, and reproduce. Despite the importance of proteins in bacterial processes and contaminant-degrading activities, the diversity of these biomolecules currently prevents standardized methods for their extraction, analysis, and identification. None of the current EMDs described in this document characterizes microbial proteins at the molecular (amino acid sequence) level. Figure D-3 illustrates protein structure.

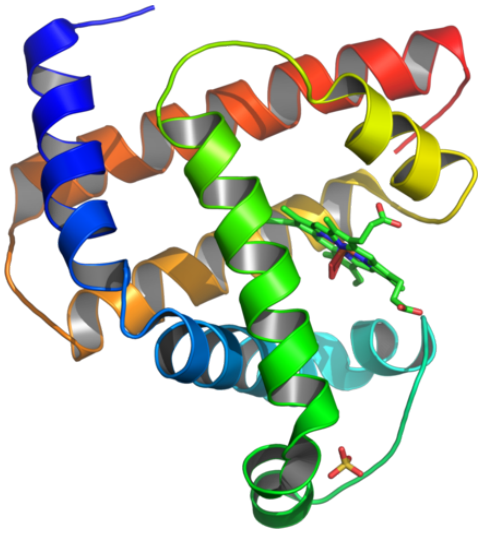

Figure D-3. Example of a protein.

Source: Myoglobin 3D structure. Aza Toth at en.wikipedia.org. 05:39, 27 February 2008. http://en.wikipedia.org/wiki/File:Myoglobin.png

7) What are enzymes?

Enzymes are specialized proteins that catalyze biochemical reactions. Catalysts increase the rate of a chemical reaction by lowering the activation energy (the energy required to initiate the reaction - see Figure D-4). As a catalyst, enzymes are not destroyed in this process and can facilitate the same reaction many times. Enzyme-catalyzed reactions convert one or more starting compounds (substrates) into one or more products. Enzymes are often specific for the type of reaction that they catalyze and typically only catalyze reactions with a limited range of substrates. One type of EMD, enzyme activity probes (EAPs) which are described in Section 8.0, detects the presence of specific enzymes in bacteria by using alternative substrates for enzymes that normally catalyze key reactions involved in contaminant biodegradation.

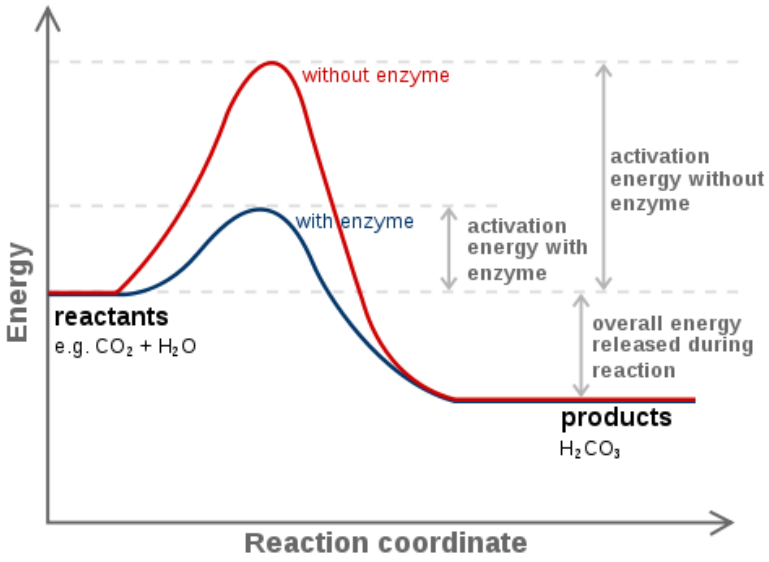

Figure D-4. Schematic of an enzyme lowering the activation energy of a reaction.

Source: Fvasconcellos at en.wikipedia.org. 28 May 2008. http://en.wikipedia.org/wiki/File:Carbonic_anhydrase_reaction_in_tissue.svg

8) What are nucleic acids?

Nucleic acids occur in all living organisms in two forms known as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). DNA is the key information-containing molecule in all living cells. The information in DNA enables a bacterium to produce all of the proteins needed for the organism to live and grow.

Nucleic acids are polymers of nucleotides. Nucleotides consist of a sugar, a phosphate group, and one of several nitrogen-containing, ring-shaped components called bases. Nucleotide acids are joined together in a linear chain by bonds formed between the sugar of one nucleotide and the phosphate group of another. This bonding produces a sugar-phosphate backbone common to all nucleic acids. The information in DNA and RNA is present in the bases attached to this backbone. In DNA these bases are either adenine (A), guanine (G), cytosine (C), or thymine (T). In RNA the bases are the same except thymine (T) is always replaced by uracil (U).

DNA and RNA have small but important differences. In RNA, the sugar in the nucleotide monomers is a called ribose. In DNA this sugar is missing an oxygen atom and is therefore called deoxyribose. RNA is also usually a single-stranded molecule, whereas DNA is usually a double-stranded molecule consisting of two anti-parallel strands bound together through bonding (base pairing) between the bases on each strand (A:T and G:C). Consequently, the sequence of bases on one strand of DNA has the complementary sequence of bases running in the opposite direction.

For example, if the sequence of bases in one strand of DNA is ATCG, the complementary sequence on the opposite strand would be TAGC. Figure D-5 illustrates this relationship.

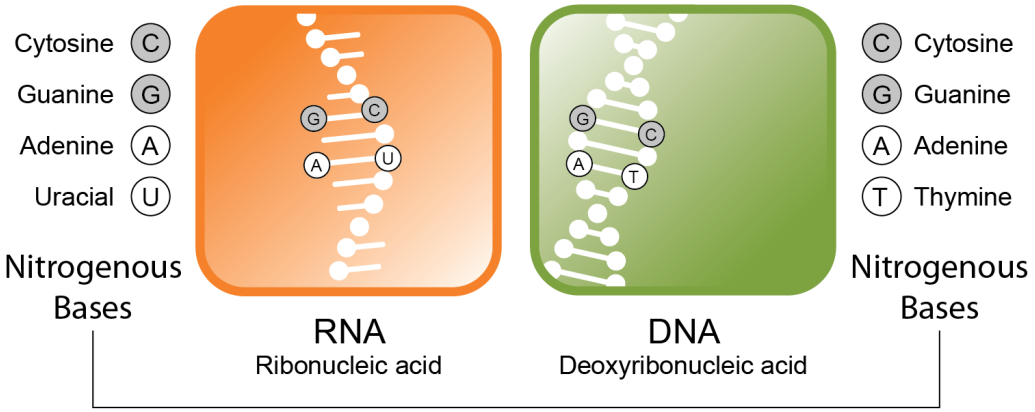

Figure D-5. Nucleic acids, DNA, and RNA.

9) What are chromosomes?

The chromosome is the organized structure of DNA found in a bacterial cell. In bacteria, often only one circular chromosome exists and may consist of as little as 600,000 bases pairs (600 kilo-base pairs [kbp]) of DNA to over 9,000,000 base pairs (9 megabase pairs [Mbp]). Also in bacteria, smaller amounts of non-essential DNA are often present in the cytoplasm, which are separate from the chromosome. These smaller pieces of DNA are called plasmids and may range in size from ~10 kbp to almost 2 Mbp.

10) What are genes?

The DNA in chromosomes and plasmids is organized into smaller functional sections called genes. Individual genes typically contain all the information required for a cell to make a single specific protein. The information in genes is encoded in the sequence of bases (A, T, C, and G) in the DNA. A typical bacterium such as *Escherichia coli* (*E. coli*) has approximately 4,500 protein-encoding genes in its approximately 4.5 Mbp chromosome, suggesting that the average gene is approximately 1kbp in size. The totality of genetic information of a bacterium (chromosome plus plasmids) is called the genome of that bacterium.

Many of the EMDs discussed in this document focus on detecting and quantifying genes. Some genes are particularly important because they encode the enzymes that catalyze the reactions involved in contaminant biodegradation. The presence of a specific bacterial gene in a sample can often indicate that microorganisms in that sample can catalyze a specific reaction. Furthermore, individual bacteria typically only have a limited number of copies of specific genes in their genomes. Measurement of the number of copies of a specific gene in an environmental sample can therefore indicate the abundance of organisms with a specific activity.

11) What are ribosomes?

Ribosomes are responsible for producing the proteins a bacterium needs. Ribosomes consist of

both protein and RNA. Bacterial ribosomes use three major forms of RNA to make protein: messenger (mRNA), ribosomal (rRNA), and transfer (tRNA). mRNA contains the information the ribosome uses to determine which protein to make. This form of RNA is generated when a gene in the chromosome is "read" (transcribed), by an enzyme called RNA polymerase. The transcription process uses the sequence of DNA bases in a gene as a template to make a copy in the form of mRNA. The sequence of bases in the mRNA is then used to by the ribosome to join the correct sequence of amino acids together as a linear chain. The process of converting the information from the mRNA into protein is called translation and is shown in Figure D-6.

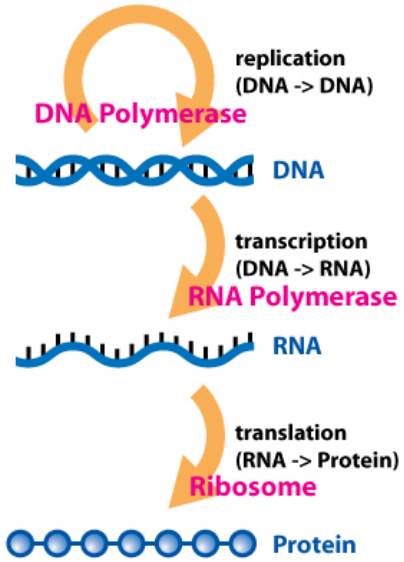

Figure D-6. Transcription and translation.

Source: Dhorspool at en.wikipedia.org. 28 November 2008. http://en.wikipedia.org/wiki/File:Central_Dogma_of_Molecular_Biochemistry_with_Enzymes.jpg

D.2 Microbial Growth and Biodegradation

12) How do bacteria grow?

Unlike most organisms, in which growth indicates an increase in size and mass, most bacteria grow by dividing one cell into two. This process is called binary fission. The two cells generated by binary fission are identical to the original cell, contain the same biomolecules, and have identical genetic information.

13) What do bacteria need to grow?

All microorganisms need five things to grow: an electron donor, an electron acceptor, a carbon source, other nutrients (such as HNOPS and trace metals), and water. Bacteria obtain all of these materials from the outside the cell.

14) What are electron donors?

Electron donors are energy sources for bacteria. Energy is extracted from electron donors by

removing electrons from the compound and transferring them to electron acceptors. Many of the bacteria relevant to understanding contaminant biodegradation in soils, sediments, and groundwater use organic compounds as electron donors. Because organic chemicals contain carbon, they can act as both the electron donor and the carbon source for bacteria. Bacteria that use organic compounds as both their electron donor and carbon source are called chemoheterotrophs. In contrast some bacteria, called lithoautotrophs, grow only using inorganic chemicals such as ammonia, iron, or hydrogen as their electron donor. Since there is no carbon in these electron donors, these organisms must use use carbon dioxide (CO_2) as their carbon source.

15) What are electron acceptors?

Once the energy has been extracted from an electron donor by a bacterium, the electrons are finally transferred to another chemical called the terminal electron acceptor. By definition, aerobic organisms use oxygen (O_2) as their terminal electron acceptor. Conversely, anaerobic microorganisms use chemicals other than oxygen. Common terminal electron acceptors that anaerobic bacteria use include, but are not limited to, nitrate (NO_3^-), iron (Fe^{3+}), manganese (Mn^{4+}), and sulfate (SO_4^{2-}). Some bacteria and archaea can also use carbon dioxide (CO_2) as an electron acceptor.

16) What is cellular respiration?

The process of removing electrons from an electron donor is called oxidation, while the process of adding electrons to an electron acceptor is called reduction. Oxidation and reduction processes are often coupled and are called redox reactions. Bacteria conserve the energy released during redox reactions to generate a biochemically useful form of energy called adenosine triphosphate (ATP). The biochemical process of oxidizing an electron donor, generating ATP, and reducing an electron acceptor is called cellular respiration. Collectively, bacteria and other microorganisms can use a wide variety of chemicals as electron acceptors. Some of the most important of these processes in the environment are listed in Table D-1.

Table D-1. Major forms of electron donors and electron acceptors used by microorganisms. Adapted from Sullivan and Baross 2007.

Respiratory process	Electron donor	Electron acceptor	Product
Aerobic respiration	Various inorganic/organic compounds	oxygen (O_2)	water (H_2O)
Denitrification	Various inorganic/organic compounds	nitrate (NO_3^-) nitrite (NO_2^-) nitric oxide (NO) nitrous oxide (N_2O)	nitrite (NO_2^-) nitric oxide (NO) nitrous oxide (N_2O) nitrogen (N_2)

Table D-1. Major forms of electron donors and electron acceptors used by microorganisms. Adapted from Sullivan and Baross 2007. (continued)

Respiratory process	Electron donor	Electron acceptor	Product
Iron reduction	H_2, organic compounds	ferric iron (Fe^{3+})	ferrous iron (Fe^{2+})
Sulfate reduction	H_2, organic compounds	sulfate (SO_4^{2-})	hydrogen sulfide (H_2S)
Acetogenesis	H_2, organic compounds	carbon dioxide (CO_2)	acetate
Methanogenesis (Archaea only)	H_2, organic compounds	carbon dioxide (CO_2)	methane (CH_4)
Fermentation	Organic compounds	No external acceptor; internal only	CO_2, H_2, acids, alcohols

17) What are carbon sources?

All living cells use carbon obtained from their immediate environment to build the carbon-containing biomolecules (proteins, sugars, lipids, and nucleic acids) that are essential for growth. Carbon is the single most abundant element in biomolecules and represents approximately 50% of the dry weight of a bacterial cell. Some bacteria only use CO_2 as a carbon source while other bacteria can use thousands of different organic chemicals as carbon sources. In many instances, these carbon sources are contaminants found in the environment.

18) Besides carbon, what other nutrients are needed for growth?

The four major biomolecules (proteins, sugars, lipids, nucleic acids) that make up all living organisms consist mainly of six major elements (CHNOPS). Carbon is the most common element found in biomolecules, while other major elements are required in lesser amounts. Nutrients like nitrogen (N), phosphorus (P), and sulfur (S) can often be obtained from minerals present in the soil and groundwater immediately surrounding the bacterium. The same is true for trace nutrients such as molybdenum (Mo), cobalt (Co), and iron (Fe).

19) What is microbial metabolism, and why is it important?

Microbial metabolism includes all of the biochemical reactions that enable microorganisms to biochemically break down (catabolize) chemicals and to concurrently use the products of these reactions to make (anabolize) new lipids, proteins, polysaccharides, and nucleic acids required for growth. Bacteria and other microorganisms, including archaea and fungi, can grow on an enormous number of organic and inorganic chemicals. These chemicals include many compounds

considered to be environmental contaminants. Understanding the microbial metabolism of contaminants helps environmental site managers to better understand the processes involved in natural and enhanced biodegradation processes and to improve the remediation of contaminated sites.

20) What are some examples of aerobic respiration important to bioremediation?

Many microorganisms can use oxygen as a terminal electron acceptor during the oxidation of organic compounds. During aerobic respiration, the organic electron donor is biochemically oxidized to carbon dioxide while oxygen (the electron acceptor) is reduced to water. Humans and other animals use this same respiratory process. Bacteria can use this respiratory process to biodegrade a wide range of contaminants such as petroleum hydrocarbons, methyl tertiary butyl ether (MTBE), and vinyl chloride (VC), among others. Some specialized bacteria can use oxygen as the terminal electron acceptor while oxidizing reduced inorganic compounds (such as Fe^{2+}, H_2S, or NO_2^-) as electron donors. For example, iron-oxidizing bacteria such as *Acidothiobacillus* gain energy from oxidizing ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) and by using oxygen as a terminal electron acceptor. This process is important in acid mine drainage because it affects the mobility of metals. Ammonia-oxidizing bacteria such as *Nitrosomonas* oxidize ammonia (NH_3) to nitrite (NO_2^-) using oxygen as a terminal electron acceptor. This process, called nitrification, is important in wastewater treatment processes to eliminate ammonia, which is toxic to fish and other organisms.

21) What are some examples of anaerobic respiration that are important to bioremediation?

A wide range of anaerobic respiratory processes are important to bioremediation. As with aerobic respiration, many forms of anaerobic respiration use organic contaminants as electron donors and oxidize these compounds to carbon dioxide. Although the biochemical steps involved are often different from those used by aerobic bacteria, petroleum hydrocarbons can often be degraded under anaerobic conditions by microorganisms that can use nitrate (NO_3^-) or sulfate (SO_4^{2-}) as terminal electron acceptors. Organisms that use nitrate as an electron acceptor generate compounds such as N_2O (nitrous oxide), NO (nitric oxide), and N_2 (nitrogen) and are called denitrifiers. Microorganisms that use and generate sulfate generated H_2S (hydrogen sulfide) are called sulfate-reducing bacteria (SRB).

Under anaerobic conditions hydrogen (H_2) is often generated during biodegradation processes. Hydrogen is also widely used as an electron donor by anaerobic bacteria. Some important microorganisms can also use H_2 as an electron donor. For example, acetate-generating (acetogenic) bacteria use H_2 as an electron donor and use energy from hydrogen oxidation to reduce CO_2 to acetate (CH_3COOH). Some microorganisms (methanogens) can also use H_2 oxidation to reduce CO_2 to methane (CH_4). This methane-generating activity is only found in archaea and not in bacteria.

Other bacteria can reduce inorganic chemicals under anaerobic conditions. For example, bacteria such as *Geobacter* use ferric iron (Fe^{3+}) as an electron acceptor and reduce it to ferrous iron (Fe^{2+}). Other bacteria can reduce metals such as manganese (Mn^{4+}), arsenic (As^{5+}), chromium (Cr^{6+}), or uranium (Ur^{5+}).

Dehalorespiration is another important type of anaerobic respiration for environmental contaminants. In this process, halogenated organic compounds are used as terminal electron acceptors.

The halogenated compounds are sequentially reduced with the result that chlorine atoms are removed and replaced by hydrogen (reductive dehalogenation). An example of this process is reductive dechlorination of tetrachloroethylene (PCE) by *Dhc*. This organism uses H_2 as an electron donor and sequentially reduces PCE to trichloroethylene (TCE), to dichloroethene (DCE; both *cis*-, and trans- isomers of DCE, although *cis*- is much more commonly produced), to vinyl chloride (VC), and finally to ethene. Other examples of dehalorespiration include the reductive dechlorination of chlorobezenes, chlorinated bromines, and chlorinated phenols.

22) What types of respiration occur in a contaminated environment?

Microorganisms preferentially use electron acceptors that allow them to generate the most ATP during respiration. If available, oxygen, which allows the maximal production of ATP, will be used first and the remaining electron acceptors will be used in a defined sequence based on their respective energy yields. This sequence is as follows: $O_2 > NO_3^- > Mn^{4+} > Fe^{3+} > SO_4^{2-} > CO_2$. In a contaminated groundwater system containing readily biodegradable electron donors, the available electron acceptors will be rapidly consumed and the environment will tend to become methanogenic. Downgradient from the source area, the concentrations of dissolved electron donors will be lower and the available electron acceptors may not have been fully consumed. Under these circumstances, zones with different electron accepting processes will develop. These can be detected based on either depletion of the electron acceptor or detection of distinctive products of the prevailing electron accepting process (such as methane accumulation in methanogenic environments,or sulfide production in sulfate-reducing environments). Changes in subsurface redox potential and geochemistry (measured as either electron acceptors or metabolic byproducts) can indicate whether or not contaminant biodegradation is occurring.

No single type of bacterium can use all of the electron acceptors shown in Figure D-7, and many microorganisms only use one or two specific electron acceptors. Consequently, different electron acceptors tend to support the growth and activity of different bacteria with different metabolic capabilities.

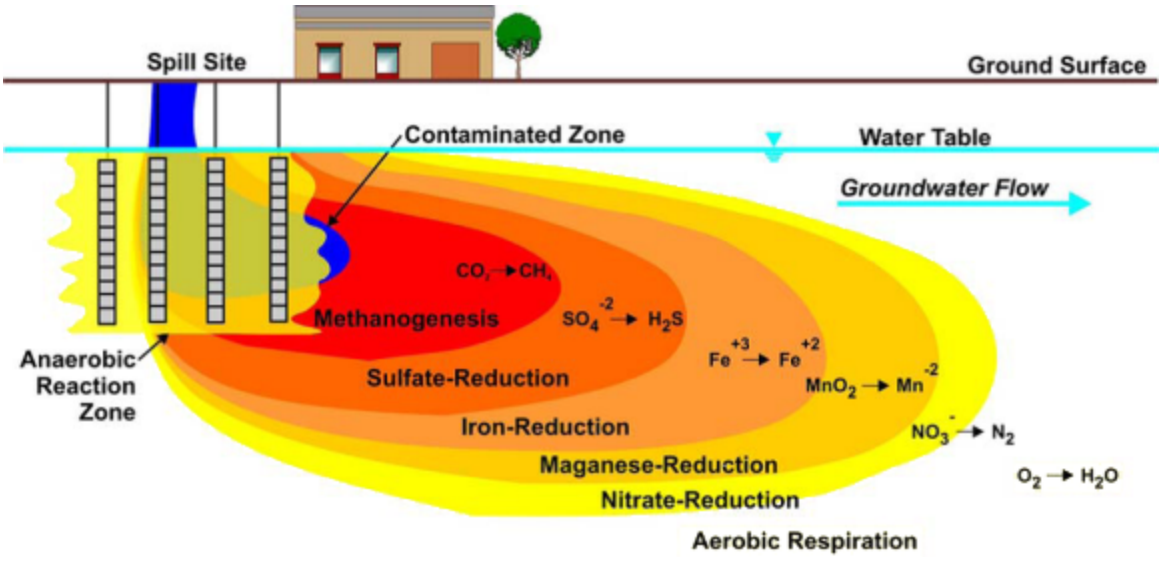

Figure D-7. Predominant terminal electron accepting processes (TEAPS) within a dissolved contaminant plume as the plume migrates through the subsurface.

Source: AFCEE 2004. Naval Facilities Engineering Service Center (NFESC), and the Environmental Security Technology Certification Program (ESTCP). 2004. Principles and Practices of Enhanced Anaerobic Bioremediation of Chlorinated Solvents. Parsons Infrastructure & Technology Group, Inc., Denver, Colorado. August.

23) How does biodegradation relate to microbial metabolism?

The term biodegradation is frequently used to describe the cellular metabolic processes that allow microorganisms to use a variety of organic compounds as carbon and energy sources for growth. In many cases, bacteria use the same organic compound (including some contaminants) as both their electron donor and carbon source. These organic compounds are broken down within the microbial cell via defined catabolic reactions. These sequential reactions are facilitated by various enzyme catalysts through a specific pathway.

In these pathways, the product of one enzyme-catalyzed reaction serves as the substrate (reactant) for the next enzyme in the pathway throughout the process. The progressively smaller carbon-containing intermediates (metabolites) generated during catabolism have two eventual fates. Some metabolites are fully oxidized to terminal products and are excreted as waste products, such as CO_2. Other metabolites are used in biosynthetic (anabolic) pathways as the starting materials for the production of new biomolecules required for growth. Anabolic processes require large amounts of energy in the form of ATP. ATP is generated by redox reactions that ultimately lead to the reduction of the electron acceptor. The connection between catabolic and anabolic pathways, electron donors, electron acceptors, and energy (ATP) production is summarized in Figure D-8.

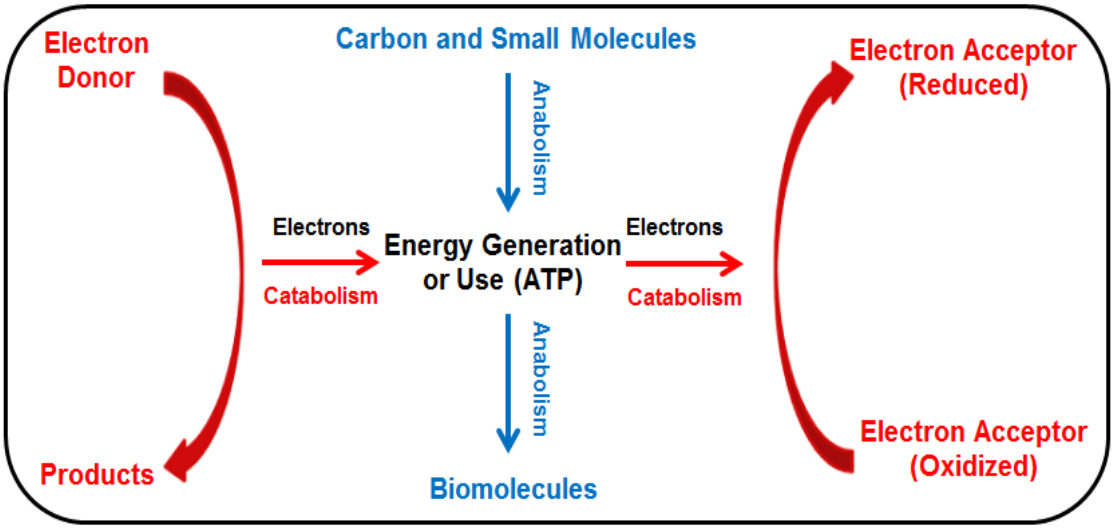

Figure D-8. Microbial metabolism and biodegradation.

Despite the structural diversity of organic compounds that microorganisms can metabolize, there are only six classes of enzymes that facilitate metabolic reactions. Certain classes of enzymes play a role in the biodegradation of certain classes of organic compounds. For example, many hydrocarbons are biodegraded by enzyme pathways that are initiated by oxygenase enzymes. These enzymes either introduce one or both atoms of oxygen (O) from molecular oxygen (O_2) into the hydrocarbon substrate. These enzymes are known as monooxygenases and dioxygenases, respectively. Table D-2 lists some examples of enzymes, the compounds they help degrade, and the gene that encodes the enzyme. Note the following in this table:

- The oxygenase enzymes are named after the compound that they help degrade. For example, benzene monooxygenase is the enzyme that initiates the pathway of benzene catabolism in organisms that can grow on benzene.
- Several hydrocarbons, such as toluene, can be oxidized by several structurally different oxygenases. Each of these enzymes introduces oxygen into a different position on the toluene molecule.
- Some hydrocarbons, such as straight chain n-alkanes, can be oxidized by more than one type of enzyme even though the products of these reactions are the same.
- Some contaminants, such as TCE, can be oxidized by enzymes that have other roles. For example, methane monooxygenase normally initiates the biodegradation of methane in aerobic methane-oxidizing bacteria (methanotrophs). Because the methane monooxygenase can react with compounds other than methane, methanotrophs can oxidize TCE even though they cannot grow on this contaminant. This lack of enzyme specificity underlies the process of cometabolism.

The enzymes described in Table D-2 are found in aerobic microorganisms and all require the presence of molecular oxygen. This requirement is an additional use for oxygen in aerobic organisms, beyond its use as a terminal electron acceptor.

Table D-2. Examples of oxygenase enzymes and some common contaminants.

Contaminant	Key enzyme	Relevant gene
benzene	benzene monoxygenase	*bmo*
toluene	toluene dioxygenase	*tod*
toluene	toluene-4-monoxygenase	*tmo*
xylenes	xylene monoxygenase	*xyl*
naphthalene	naphthalene dioxygenase	*ndo*
alkanes	alkane monoxygenase, alkane hydroxylase	*alk*
polychlorinated biphenyls	biphenyl dioxygenases	*bph*
vinyl chloride	alkene monooxygenase	*etn*
trichloroethylene	methane monooxygenase	*mmo*

In most cases, the enzymes listed in Table D-2 are also the first enzymes in pathways that allow bacteria to catabolize various contaminants. The full catabolic pathways for the contaminants listed in Table D-2 involve many other important enzymes. However, the initial reaction in a pathway is often the rate-limiting step and must facilitate reaction of otherwise unreactive compounds. For example, hydrocarbons are often unreactive compounds. However, they become relatively simple compounds to biodegrade once an oxygen atom has been introduced into a C-H bond to create an alcohol (for instance, in Figure D-9, a catechol compound is formed during aerobic respiration).

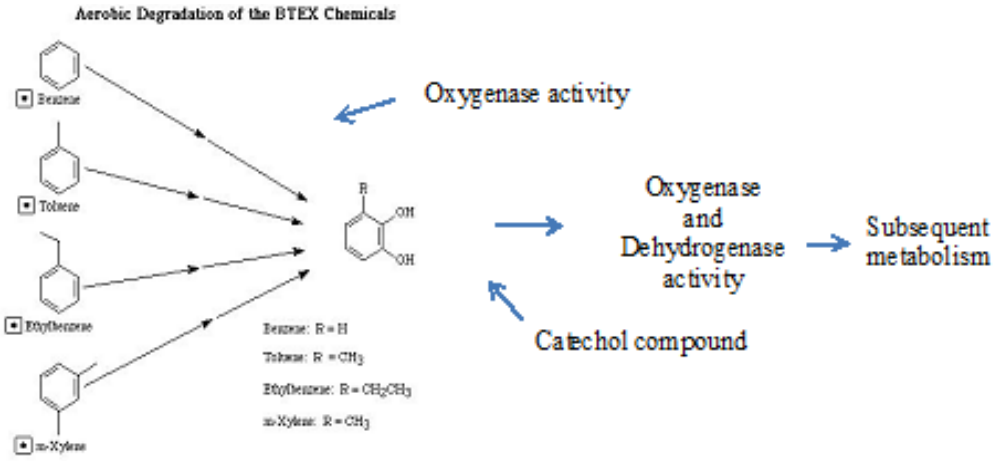

Figure D-9. Aerobic bacterial biodegradation of aromatic BTEX compounds.

Source: Adapted from the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD). Gao J, Ellis LBM, and Wackett LP (2010). "The University of Minnesota Biocatalysis/Biodegradation Database: Improving public access" Nucleic Acids Research 38: D488-D491. BTEX Metabolism Metapathway Map page author, Stephen Stephens. http://umbbd.ethz.ch/BTEX/BTEX_map.html

Figure D-10 illustrates the steps involved in the biodegradation of toluene and the enzymes associated with each individual step. Mono- and Di-oxigenases are involved in the initial phase of toluene biodegradation leading to the opening of the aromatic ring.

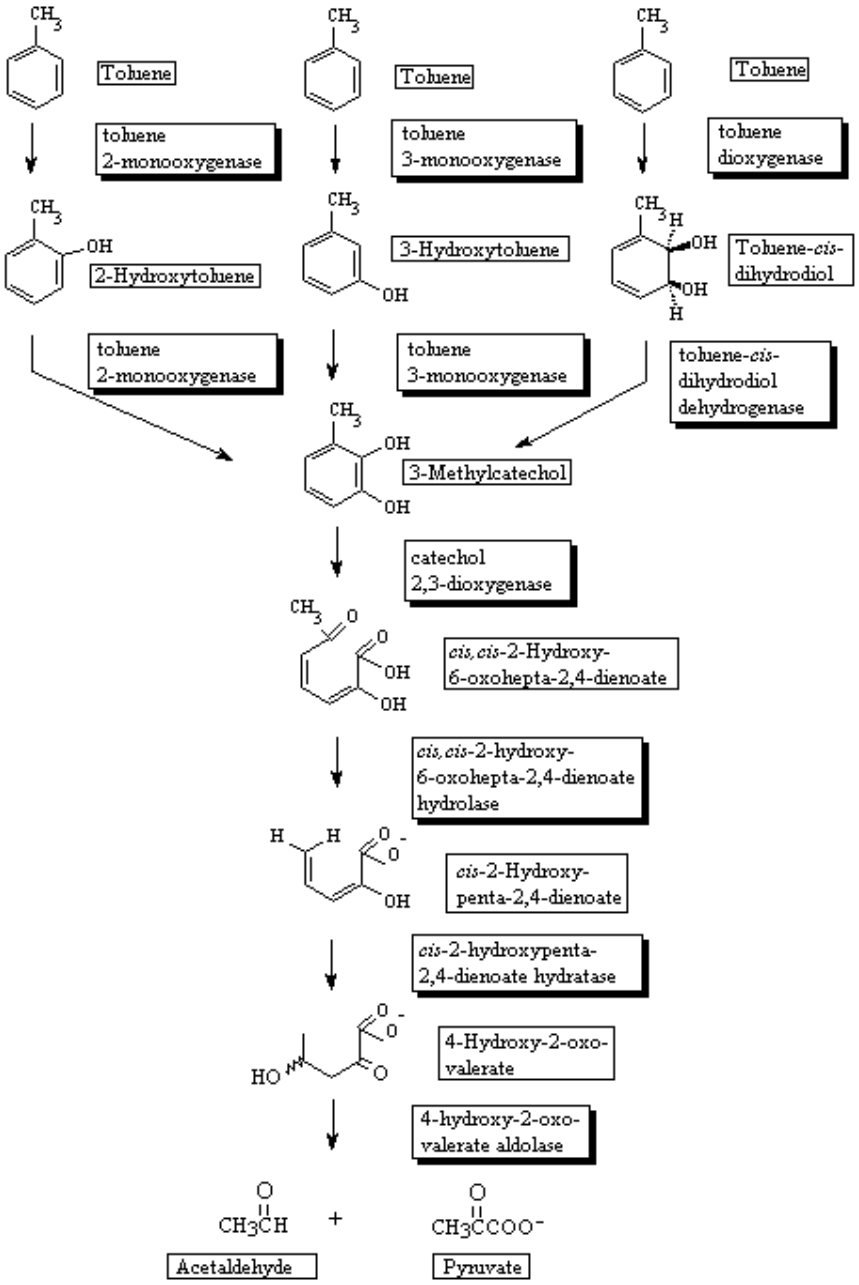

Figure D-10. Toluene oxidation.

Source: University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD). Gao J, Ellis LBM, and Wackett LP (2010). "The University of Minnesota Biocatalysis/Biodegradation Database: Improving public access" Nucleic Acids Research 38: D488-D491. Toluene Graphical Pathway Map (2) page author Dong Jun Oh. http://umbbd.ethz.ch/tol/tol_image_map2.html.

Figures D-11 and D-12 illustrate many of the key features of a typical biodegradation (catabolic) pathway. For example, the pathway is initiated by the activity of an oxygenase enzyme. The

metabolites generated by the pathway are transformed into simpler compounds and the pathway ends with small simple metabolites that can easily be converted into carbon dioxide or used to start the synthesis of new biomolecules through anabolic pathways.

Biodegradation pathways in anaerobic microorganisms have similar characteristics to those described for aerobic pathways, except that molecular oxygen is not involved. The enzymes described in Table D-3 are found in anaerobic microorganisms and do not require the presence of molecular oxygen. For example, anaerobic degradation of aromatic compounds, such as toluene, is initiated by benzylsuccinate synthase in a similar manner that aerobic degradation is initiated by a toluene oxygenase (Figure D-11).

Table D-3. Examples of key enzymes involved in the anaerobic degradation of contaminants

Contaminant	Key enzyme	Relevant gene
toluene	benzylsuccinate synthase	*bssA*
perchloroethene	PCE reductase	*pceA*
trichloroethene	TCE reductase	*tceA*
vinyl chloride	VC-reductase	*vcrA and bvcA*

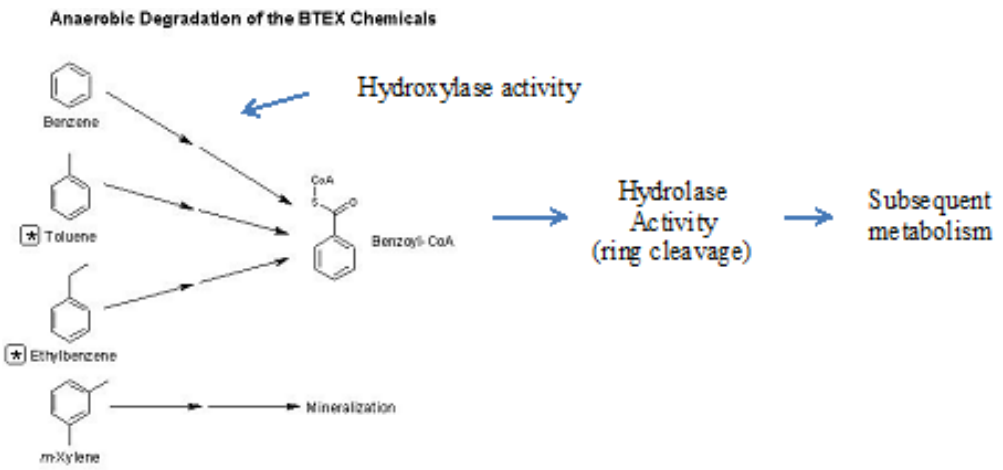

Figure D-11. Anaerobic bacterial biodegradation of aromatic BTEX compounds.

Source: Adapted from the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD). Gao J, Ellis LBM, and Wackett LP (2010). "The University of Minnesota Biocatalysis/Biodegradation Database: Improving public access" Nucleic Acids Research 38: D488-D491. BTEX Metabolism Metapathway Map page author, Stephen Stephens. http://umbbd.ethz.ch/BTEX/BTEX_map.html.

Unlike aerobic biodegradation, anaerobic biodegradation can also involve processes that use contaminants as electron acceptors. An example of this type of process is the pathway of PCE degrad-

ation to ethene by *Dhc* (Figure D-12). This organism uses a different reductase enzyme in each of the steps in this pathway.

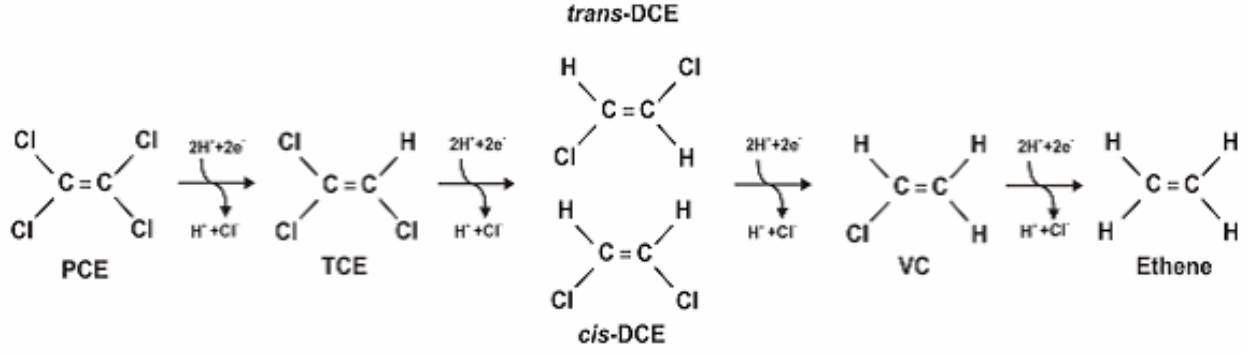

Figure D-12. Sequential reductive dechlorination of PCE to ethene.

Source: AFCEE 2004. Naval Facilities Engineering Service Center (NFESC), and the Environmental Security Technology Certification Program (ESTCP). 2004. Principles and Practices of Enhanced Anaerobic Bioremediation of Chlorinated Solvents. Parsons Infrastructure & Technology Group, Inc., Denver, Colorado. August.

24) What factors determine which contaminants can be biodegraded?

The ability of bacteria to biodegrade a specific contaminant is dictated by many factors including:

- environmental conditions, such as sufficient electron donor or acceptor, availability of water and other nutrients, temperature, and pH.
- characteristics of the contaminant, such as molecular structure, bioavailability, and toxicity
- genetic capability (do microorganism possesses the genes that encode the necessary enzymes required to degrade the contaminant?)
- nature of the contamination, such as concentration, and presence of co-contaminants

For example, even though bacteria may be present with the correct genes and metabolic capabilities required to degrade a specific contaminant, the contaminant may not be biodegraded due to the presence of a co-contaminant that inhibits biodegradation.

Note that bacteria typically live as part of microbial communities that are typically characterized by a high degree of species interdependence. Metabolically similar microorganisms can be classified into groups called guilds (such as methanogens and SRB). The relationships between these guilds are important because the complete degradation of contaminants in the environment often involves the interactions of multiple guilds within a community and depends on syntrophic relationships. For example, the anaerobic biodegradation of BTEX compounds may involve microorganisms that initially degrade the compounds to intermediates, which then serve as substrates for additional groups of microorganisms. Effective bioremediation approaches therefore need to account for not only the contaminant type, quantity, and bioavailability, but also the indigenous microbial communities at a site.

D.3 Molecular Biology and Microbiology

25) What is molecular biology?

Molecular biology is the study of the essential molecules produced by living organisms such as those used in reproduction, energy generation, and cell structures. The main biomolecules relevant to biodegradation studies are nucleic acids, proteins, and lipids. Although obviously important to microorganisms themselves, polysaccharides are not particularly useful molecules to analyze for understanding biodegradation and the microorganisms involved in these processes.

Molecular biology overlaps with genetics (the study of the genes) and biochemistry (the study of the biomolecule structure, pathways, and metabolites). Molecular biology is a relatively young science (originating in the 1930s and 1940s), and did not become a distinct discipline until the 1960s, when scientists discovered the structure of DNA and how DNA sequences direct protein synthesis. For environmental scientists, the main uses of molecular biology are to identify or quantify contaminant-degrading microorganisms, determine the genetic capability of microorganisms, and describe microbial diversity in the environment.

26) How is molecular biology useful in biodegradation studies?

Until the introduction of molecular biology techniques, studies of microorganisms involved in biodegradation were often limited to determinations of the total numbers of microorganisms that could be grown or cultured under standard laboratory conditions. The shortcoming of this approach is that, despite many years of study, microbiologists can only grow a tiny fraction of the microorganisms present in the environment in the laboratory. Consequently, culture-dependent techniques such as heterotrophic plate counts drastically underestimate both the numbers of and types of microorganisms present in environmental samples. Most modern molecular biology techniques described in this document analyze biomolecules that are generated by microorganisms in the environment, and then use these indirect measurements to determine the abundance and activities of these microorganisms. These techniques typically do not require laboratory growth of microorganisms, therefore avoiding selective and inefficient culture-dependent processes.

Most molecular biology approaches used to characterize biodegradation processes analyze nucleic acids (DNA and RNA). One reason for analyzing nucleic acids is that nucleic acids are structurally homogeneous, (unlike proteins, which have different sizes and different chemical and physical properties). Although the individual nucleotide sequences in DNA molecules vary almost infinitely, these differences have limited effects on the techniques needed to extract, purify, and characterize the biomolecule from various sources. Human DNA behaves the same as plant DNA, bacterial DNA, or fungal DNA. Another reason to analyze nucleic acids is that, unlike the other biomolecules that are investigated in biodegradation studies (proteins and lipids), there are several powerful techniques that can be used to study DNA. Two of the most prominent techniques are the polymerase chain reaction (Section 4.0) and automated DNA sequencing. These two key technical advances are described in the following two sections.

27) What is PCR?

The polymerase chain reaction (PCR) is a routine molecular procedure that harnesses and directs

the activity of DNA polymerase, a natural DNA-synthesizing enzyme. This enzyme "reads" the sequences of bases in a template DNA strand and can produce billions of identical copies of this sequence and its complementary DNA strand. The amplification is achieved through cycling a reaction mixture that contains template DNA and DNA polymerase through a carefully prescribed sequence of temperature changes and incubation conditions.

The PCR procedure has transformed the biological sciences and has many applications in environmental studies. PCR is particularly useful for generating large amounts of identical DNA, even if only small amounts of template DNA (or RNA) can be recovered from a sample. A remarkable feature of PCR is that it can specifically amplify one gene or DNA sequence—even when that target gene or sequence is present at extremely low concentrations in a DNA sample that contains many billions of other nontarget genes or DNA sequences.

DNA and RNA amplification specificity is made possible through the use of primers. Primers are short pieces of DNA (~20 nucleotides) that are complementary to the beginning and the end of the section of DNA to be amplified by PCR. A typical PCR amplification reaction mixture contains billions of copies of these primers. During the amplification procedure, the primers bind to (anneal) to their complementary sequences and serve as initiation points for DNA polymerase to start synthesizing new DNA. Without primers PCR will not work; with poorly designed primers, a PCR amplification can generate large amounts of nontarget DNA. However with well-designed primers, PCR can selectively amplify genes of DNA sequences that are specific for individual species of microorganisms.

A typical PCR amplification requires the presence of the following materials:

- Template DNA: This DNA contains the nucleotide sequence of interest (target DNA). The template DNA can be any form of DNA extracted from a sample.
- A heat-stable DNA polymerase: DNA polymerase is an enzyme that identifies existing nucleotide sequences in single-stranded DNA and concurrently synthesizes a complementary strand. A heat-stable form of the enzyme is used because PCR employs repeated high temperature cycles as part of the amplification process.
- Deoxyribonucleotides: DNA is a polymer of four deoxyribonucleotides building blocks (A, T, C, and G). These compounds are added to a PCR mixture as deoxyribonucleotide triphosphates (dNTPs) and are used by DNA polymerase to synthesize new DNA.
- Primers: Primers are short, single-strand DNA molecules that bind specifically to the target DNA sequence in template DNA. The primers are used to direct the DNA polymerase to the section of DNA that is to be amplified.

DNA amplification using PCR is conducted on a small scale (<50 µl) in sealed microtubes. These microtubes are incubated in an automated thermocycler that can very quickly and accurately change the reaction temperature of the PCR mixture within the microtubes. A typical PCR program consists of the following steps:

1. Denaturation: The reaction mixture is heated to ~95°C to melt any double-stranded DNA into a single-stranded form.
2. Annealing: The reaction mixture is cooled to 50-65°C to allow the primers to anneal (bind) to the single-stranded DNA template. The annealing temperature is critical in determining the specificity of the DNA amplification and may vary depending on the specific primers used.
3. Extension/elongation: The reaction temperature is raised to about 75°C. During this step the DNA polymerase binds to a DNA-attached primer and synthesizes a new DNA strand that is complementary to the target DNA sequence in the template DNA.

After each cycle, the number of copies of the target sequence is doubled and continues to increase exponentially throughout the reaction time course (25-40 cycles). Figure D-13 describes this process.

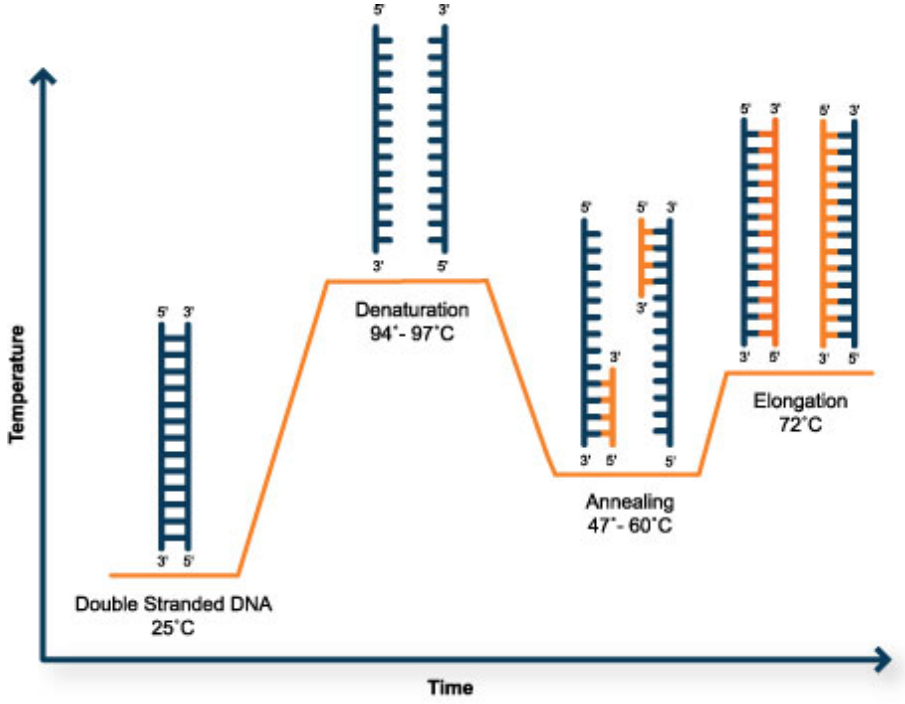

Figure D-13. PCR Process steps.

Source: USEPA 2004.

The amplification of DNA using PCR has many applications in environmental diagnostics. In some cases, PCR can simply be used to detect the presence or absence of a particular target sequence or gene or it is used simply to generate sufficient DNA to conduct other types of molecular analyses. In other cases, the basic PCR process has been modified so that the numbers of target sequences in a template DNA sample can be accurately and quickly determined, called qPCR. More information related to qPCR and its applications can be found in Section 4.0.

28) What is automated DNA sequencing?

The second key technology that underlies molecular biology is the determination of the linear order

of nucleotides (bases) in DNA molecules (DNA sequencing). This sequence of nitrogen-containing bases (A, T, C, and G) determines which amino acids are incorporated into proteins. This result in turn defines the type of reaction that an enzyme can catalyze. Consequently, the nucleotide sequence of genes can reveal what type of contaminant-degrading activities might occur for a specific organism or microbial community. DNA sequencing technologies are rapidly changing. Recently, several high-throughput systems have been developed including pyrosequencing (such as 454 sequencing) and ion semiconductor sequencing (for example, Ion Torrent™ technology). Pyrosequencing detects light generated from enzymes that use pyrophosphate released when a base is added to a growing DNA molecule while ion semiconductor sequencing detects protons (H+) released during the same process. Further advances in DNA sequencing are emerging and will continue to dramatically decrease the cost and concurrently increase the use of large scale DNA sequence analysis for characterizing microbial communities and determining the likely activities of member organisms.

29) What do molecular biology studies detect?

Like all other living cells, the flow of "information" in a microorganism goes from genes (DNA) to mRNA to proteins. As technologies become available for the rapid amplification and sequencing of DNA, many molecular studies in the environmental arena focus on detecting specific DNA molecules. The detection of particular genes or DNA sequences is then used to predict or interpret the results of other more conventional analyses.

One limitation of PCR is that the primers required in this procedure simply define the start and end of the stretch of DNA to be amplified and do not provide any information about the sequence of nucleotides between these two points. Some common checks on PCR amplification products (amplicons) are to determine whether the product is the correct predicted size (number of base pairs) and whether it has the correct physical properties (for instance, melting temperature). However, the most thorough analyses typically sequence the PCR amplicons to determine their nucleotide sequence.

Molecular biology studies also detect specific genes through the process of hybridization. Hybridization describes the non-covalent bonding that occurs between two strands of nucleic acids. The strength of this bonding is dictated by the degree to which the two strands are complementary. If two strands are highly complementary (for example, every T in one strand has a matching A in the other strand) the degree of hybridization will be strong. If the two strands are dissimilar, the degree of hybridization will be limited. This intrinsic ability of nucleic acids to form stable hybrids enables the primers used in PCR to amplify specific genes. Hybridization is also exploited in several other EMDs, including fluorescence in situ hybridization (FISH) and microarrays.

30) What genes are important in molecular biology studies?

Two different types of genes are of interest in molecular studies of biodegradation processes: 16S ribosomomal RNA (rRNA) genes and functional genes (the genes that encode for enzymes involved in specific biodegradation processes).

31) What are 16S rRNA genes, and what can they tell me?

The analysis of 16S rRNA has emerged as an important focus in biodegradation studies, as well as

in more general studies of microbial ecology. Ribosomes in bacteria are made up of two major components: the small (30S) and the large (50S) subunit. The small ribosome subunit contains several proteins as well as a single RNA molecule which is known as the 16S rRNA. The equivalent molecule in eukaryotic microorganisms such as fungi is called 18S rRNA. The 16S rRNA is a useful genetic target in bioremediation studies because the molecule is relatively easy to extract and purify from an environmental sample. This molecule also contains enough nucleotide sequences for microorganisms to be differentiated from each other. With the advent of PCR, microbiologists now focus on the gene that encodes this RNA, but the principle underlying the analysis remains the same.

The 16S rRNA serves as a molecular clock and undergoes changes in nucleotide sequence at a rate comparable to the rate at which bacteria evolve and differentiate with new capabilities. Consequently, analyses of changes in 16S rRNA nucleotide sequences can quantify how related one bacterium is to another. Bacteria typically only have one copy of this gene in their genome, and it is only transmitted when one cell divides into two—which simplifies this analysis.

In studies of biodegradation processes, analysis of 16S rRNA genes has many uses. In some cases, the number of copies of a specific 16S rRNA gene can be measured using qPCR. Conversely, an analysis of all of the 16S rRNA sequences present in a sample can be used to define which types of microorganisms are present and how the composition of a microbial community changes in response to a treatment or contaminant. The presence and number of organisms with a particular 16S rRNA sequence can also be determined by techniques such as FISH.

The central argument often made in analyses of 16S rRNA sequences is that a high degree of similarity between two 16S rRNA sequences (>97%) implies that the two microorganisms are closely related at the species level. It is often further assumed that a high degree of sequence similarity implies the two species have similar, if not identical, metabolic capabilities. However, there are a growing number of examples of organisms that have identical 16S rRNA genes sequences but have distinctly different metabolic capabilities. This realization has led to a progressive increase in interest in detecting and quantifying functional genes in environmental samples.

32) What are functional genes, and what can they tell me?

Functional genes encode enzymes involved in specific biochemical processes. Analysis of functional genes can therefore describe what biodegradation processes an individual microorganism or microbial community might be capable of, without providing any real evidence of which bacteria the gene came from.

33) Why study microbial enzymes if you can study microbial genes?

Enzymes are the actual biomolecules that catalyze biodegradation reactions. Even though a gene is detected or quantified in an environmental sample, the corresponding enzyme has not necessarily been produced by the microorganisms within the sample nor is this enzyme necessarily fully functional.

Some EMDs, such as enzyme activity probes (EAPs), detect specific enzyme activities in environmental samples. Enzyme analyses measure the potential for a given reaction (such as a key step in the degradation of a contaminant) in a given environment at a given time, so the results are useful to measure whether a reaction will occur and to evaluate the impacts of different management options on the potential for that reaction. EAPs can also be applied in the field to determine in situ rates of some biodegradation processes.

34) Why study microbial lipids if you can study microbial genes?

Phospholipid fatty acid (PLFA) analysis is useful for estimating the amount of microbial biomass in a sample that is metabolically active. Unlike DNA, microbial phospholipids rapidly decompose after microorganisms die. Therefore, PLFA analysis is an accurate quantification of live microorganisms in a sample. PLFA analyses can also be used to identify broad groups of metabolically active microorganisms as a fingerprinting technique for characterizing microbial community dynamics. Finally, some microbes modify specific PLFAs when stressed, so lipid analysis can provide some information on the health of the microbial community. The most recent development in PLFA analysis has been to combine this approach with stable isotope probing (SIP) to verify degradation of a contaminant. In this method, a contaminant labeled with a stable isotope (such as ^{13}C) is added to a culture or environmental sample. After an exposure period, the lipids are recovered and analyzed. If the label is found in the lipids, then the compound was degraded and organisms incorporated it into their membrane's biomolecules.

D.4 Future Diagnostic Tools

35) What are the emerging techniques for chemically characterizing contaminants, and what can they tell me?

CSIA is already a versatile, widely used EMD that can detect both biological and chemical transformations of contaminants. CSIA currently characterizes the isotopic composition of a whole contaminant rather than characterizing the isotopic composition of atoms in particular positions within a contaminant. Deuterium nuclear magnetic resonance (^{2}H-NMR) has the potential to contribute to source determination of MTBE and other hydrogen-containing compounds, such as chlorinated solvents that are only partially chlorinated. For example, ^{2}H-NMR could tell if the VC present in a groundwater sample is the biodegradation product of TCE or PCE.

Another growing field for isotopic analyses is its application to metals such as chromium, copper, lead, and uranium. This analysis uses a technique that measures the total isotopic ratio for all species that contain the element of interest called multi-collector inductively-coupled-plasma mass-spectroscopy (MC-ICP-MS). Much like CSIA, this technique could be used to analyze fate and transport of metals, as well as in forensic applications.

Aqueous mineral intrinsic biogeochemistry analysis (AMIBA) is a suite of analyses that provides a molecular-level examination of the geochemistry fundamental to biogeochemical transformation. More information is available about the AMIBA analyses and about collecting these samples in Technical Protocol for Enhanced Bioremediation Using Permeable Mulch Biowalls and Bioreactors (AFCEE, 2008).

36) What are emerging molecular biology techniques, and what can they tell me?

Metagenomics, metatranscriptomics, metabolomics, proteomics, and high-throughput sequencing tools are among the emerging techniques that will impact the understanding of biodegradation processes in the environment. These techniques analyze the structure and functioning of entire microbial community rather than individual microorganisms. Many of these techniques are being developed as the result in advances in high-throughput methodologies which enable multiple samples to be rapidly analyzed.

37) What is metagenomics?

Metagenomics is the analysis of the genome (complete DNA sequence) of multiple organisms. In the environmental field, metagenomics analyzes the genome of all of the organisms within a specific microbial community. This analysis is useful because biodegradation of contaminants often involves the activities of multiple microbial types operating as a community. The movement towards whole community sequencing recognizes that virtually no microorganism exists in nature as a pure culture and that interactions between organisms as part of sometimes complex communities is the norm rather the exception. Metagenomic analysis of contaminated environments may prove that particular contaminants lead to the establishment of microbial communities with consistent functions and representative species. However, association of a particular species or gene with environmental processes will remain tenuous without further compelling information that links these sequences to functions in heterogeneous environments. Advanced DNA sequencing methodologies such as pyrosequencing are facilitating DNA sequencing of microbial communities.

38) What is metatranscriptomics?

Metatranscriptomics is the application of high-throughput DNA sequencing approaches to determine the transcriptional activities of entire microbial communities. Understanding the transcriptional responses of entire microbial communities to environmental conditions and perturbations can potentially provide important insights into the factors that control the activities of individual strains with required degradation capabilities.

39) What is metabolomics?

Metabolomics is the analysis of the entire suite of small metabolites that are generated by microorganisms during their normal functioning. In many instances, current analyses of metabolites focus on individual compounds or groups of related compounds. The aim of metabolomics studies is to obtain a comprehensive understanding of all major metabolites within a sample at any given time. Metabolomics studies have been successfully developed to understand the function of cells in pure culture, where concentrations of metabolites can be high. Because these metabolites cannot be amplified in the same way that nucleic acids can, the development of comprehensive metabolomics approaches must capitalize on increased sensitivities and resolution of analytical approaches, such as mass spectrometry. Applications of metabolomics to contaminant biodegradation studies can potentially provide valuable information related to the metabolic status of entire microbial communities involved in, and required for, contaminant biodegradation.

40) What is proteomics?

Proteomics determine the protein expression patterns of microorganisms. This type of analysis

comes closest to directly analyzing the functional capabilities of a microorganism because it detects and quantifies all of the proteins and enzymes currently present within the microorganism. Since protein-based enzymes catalyze the vast majority of the biochemical reactions within a cell, the proteomic profile of a bacterium effectively indicates what biochemical processes are being completed at any given time or when growing under a specific set of conditions. However, individual microorganisms can have several thousand different proteins present at any given time and the relative concentrations of these proteins may vary by many orders of magnitude. Like metagenomics, environmental proteomics extends the analysis from a single type of organism to an entire microbial community in an environmental sample. Similarly, advances in proteomic analyses will require refinements and extensions of the capabilities of mass spectrometers along with corresponding databases that will allow protein fragment data to be identified and assigned to specific proteins and enzymes. Because mass spectrometry can resolve the isotopic composition of protein fragments, environmental proteomics may be combined with stable isotope probing approaches that will enable functioning organisms and their enzymes to be identified.

41) What are the limitations of these emerging technologies?

Established and emerging EMDs can generate enormous amounts of data. Limitations to using some of these emerging techniques include handling the enormous amounts of data generated, extracting useful information from these data, and communicating these findings in effective and meaningful ways. Many of these approaches will require the development of specialized bioinformatic tools and effective curated databases. Not all emerging technologies will gain traction within the commercial market since for some, the cost of development is larger than the potential benefits. On the other hand, some methods have already been commercialized (such as pyrosequencing) and have been incorporated into certain applications.

42) What else can I expect?

The EMDs described in this document have eliminated the need to physically isolate and culture microorganisms to understand their distribution and activities in contaminated environments. This contribution has been powered in large part by laboratories' ability to extract and amplify nucleic acids (DNA and RNA) using techniques such as PCR and to use related amplification techniques such as qPCR to quantify individual genes and organisms in environmental samples. A second group of emerging technologies is moving in the opposite direction, toward the “meta” techniques that aim to analyze the genomes and metabolic capabilities of individual microbial cells.

While efforts continue to improve methods that culture and isolate microorganisms, the development of single-cell analytical approaches is an alternative approach that precludes the need to isolate organisms through culture-based approaches. For example, individual cells can be identified and isolated using flow cytometers, which are microfluidics platforms than can be used to study the activities of individual bacterial cells. The genomes of individual cells can also be sequenced after amplification of DNA using less-biased and non-thermal amplification technologies such as multiple displacement amplification.

D.5 References

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APPENDIX E. EMD FACT SHEETS

 Introduction to EMDs

 EMD Sampling Methods Fact Sheet

 EMD-1 (all Fact Sheets compiled in a single document)

Compound specific isotope analysis (CSIA)

 CSIA Fact Sheet

Polymerase chain reaction (PCR)

 PCR Fact Sheet

Quantitative polymerase chain reaction (qPCR)

 qPCR Fact Sheet

Reverse transcriptase qPCR (RT-qPCR)

 qPCR Fact Sheet

Phospholipid fatty acid (PLFA) analysis

 Microbial Fingerprinting Fact Sheet

Denaturing gradient gel electrophoresis (DGGE)

 Microbial Fingerprinting Fact Sheet

Terminal restriction fragment length polymorphism (T-RFLP)

 Microbial Fingerprinting Fact Sheet

Microarrays

 Microarrays Fact Sheet

Stable isotope probing (SIP)


SIP Fact Sheet

Enzyme activity probes (EAPs)


EAP Fact Sheet

Fluorescence in situ hybridization (FISH)


FISH Fact Sheet

APPENDIX F. ADDITIONAL RESOURCES

F.1 CSIA Methods

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F.2 PCR and qPCR Methods

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F.3 Fingerprinting Methods

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F.5 SIP Methods

Table 1 in the SIP Fact Sheet includes examples of recent applications of SIP to important contaminants.

F.6 EAP Methods

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SILVA rRNA database project: http://www.arb-silva.de/fish-probes/

- Hosted and maintained by the Max Planck Institute for Marine Microbiology in Bremen, Germany
- Background and principles of FISH analysis
- Current collection of FISH protocols
- 16S rRNA sequence database

probeBase: www.microbial-ecology.net/probebase/

- Hosted and maintained by the Department of Microbial Ecology at the University of Vienna in Vienna, Austria
- Contains a searchable list of >2600 rRNA-targeted oligonucleotide probes

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APPENDIX G. EMD TEAM CONTACTS

Robert Mueller, Team Leader
New Jersey Department of Environmental Protection
(609) 984-3910
bob.mueller@dep.state.nj.us

Lesley Hay Wilson, ITRC Program Advisor
Sage Risk Solutions LLC
(512) 327-0902
Lhay_wilson@sagerisk.com

Richard Aho
Michigan CSWMA
(906) 249-4125
rica@miuplink.com

Brett Baldwin
Microbial Insights, Inc.
(865) 573-8188
bbaldwin@microbe.com

Ramesh Belani
Pennsylvania Dept. of Environmental Protection
(484) 250-5756
rbelani@pa.gov

Caitlin Bell
ARCADIS
(510) 596-9552
caitlin.bell@arcadis-us.com

Adria Bodour
AFCEE/TDV
(210) 395-8426
adria.bodour.1@us.af.mil

Christine P. Brown
California EPA Dept. of Toxic Substances Control
(310) 704-8310
cbrown@dtsc.ca.gov

Cleet Carlton
California Regional Water Quality Control Board
(510) 622-2374
ccarlton@waterboards.ca.gov

Ramona Darlington
Battelle Memorial Institute
(614) 424-4199
Darlingtonr@battelle.org

Sophia Drugan
Kleinfelder, Inc.
(510) 628-9000
sdrugan@kleinfelder.com

David Duncklee
Duncklee and Dunham
(919) 858-9898
dave@dunckleedunham.com

Stephanie Fiorenza
BP
(281) 366-7484
Stephanie.Fiorenza@bp.com

James Fish
Alaska Dept. of Environmental Conservation
(907) 451-2117
james.fish@alaska.gov

Christopher Glenn
Treadwell Rollo
(510) 384-2626
cnglenn@treadwellrollo.com

Jessica Goin
Anchor QEA
(503) 972-5019
jgoin@anchorqea.com

Paul Hatzinger
Shaw Environmental & Infrastructure, Inc.
(609) 895-5356
paul.hatzinger@shawgrp.com

Cheryl A. Hawkins
USEPA OSWER/OSRTI/TIFSD/
Environmental Response Team
(732) 687-0487
hawkins.cheryla@epa.gov

Michael Hyman
North Carolina State University
(919) 515-7814
michael_hyman@ncsu.edu

Undine Johnson
Georgia Environmental Protection Division
(404) 362-2594
undine.johnson@dnr.state.ga.us

Tasha Kamegai-Karadi
Geosyntec Consultants
(510) 285-2765
tkamegai@geosyntec.com

Stephen Koenigsberg
Brown and Caldwell
(714) 689-4864
skoenigsberg@BrwnCald.com

David Lattier
EPA
(513) 569-7976
lattier.david@epa.gov

Carmen Lebrón
Naval Facilities Engineering Service Center
(805) 982-1616
carmen.lebron@navy.mil

M. Hope Lee
Pacific Northwest National Laboratory
(509) 375-4593
hope.lee@pnnl.gov

Frank Löffler
University of Tennessee
Oak Ridge National Laboratory
(865) 974-4933
frank.loeffler@utk.edu

Jun Lu
AECOM
(562) 213-4120
jun.lu@aecom.com

Tamzen Macbeth
CDM Smith
(208) 569-5147
macbethtw@cdmsmith.com

Patrick McLoughlin
Microseeps, Inc.
(412) 826-5245
pmcloughlin@microseeps.com

Sara Michael
California EPA Dept. of Toxic Substances Control
(714) 816-1983
smichael@dtsc.ca.gov

Ann Miracle
Pacific Northwest National Laboratory
(509) 372-4327
ann.miracle@pnl.gov

Rebecca Mora
AECOM Environment
(714) 689-7254
Rebecca.Mora@aecom.com

Dora Ogles
Microbial Insights, Inc.
(865) 573-8188
dogles@microbe.com

Aaron Peacock
Haley & Aldrich, Inc.
(865) 356-0498
apeacock@haleyaldrich.com

Ioana Petrisor
Cardno ENTRIX
(949) 273-6311
ioana.petrisor@cardno.com

Erik Petrovskis
Geosyntec Consultants
(734) 332-8004
epetrovskis@geosyntec.com

Paul Philp
University of Oklahoma
(405) 325-4469
pphilp@ou.edu

Eric Raes
Engineering and Land Planning Associates, Inc.
(908) 238-0544
eric@elp-inc.com

Chad Roper
AECOM Environment
(805) 764-4027
chad.roper@aecom.com

Devon Rowe
ENVIRON
(503) 353-1734
drowe@environcorp.com

Harvinder Singh
AECOM
(714) 689-7272
harvinder.singh@aecom.com

Claudio Sorrentino
California EPA Dept. of Toxic Substances Control
(916) 255-6656
Claudio.Sorrentino@DTSC.ca.gov

Peter Strauss
PM Strauss & Associates
(415) 647-4404
petestrauss1@comcast.net

Hans Stroo
Stroo Consulting, LLC
(541) 482-1404
hans@strooconsulting.com

Kerry Sublette
University of Tulsa
(918) 631-3085
kerry-sublette@utulsa.edu

David Tsao
BP
(630) 420-5147
david.tsao@bp.com

Yi Wang
ZymaX Forensic Isotope
(760) 781-3338
Yi.Wang@zymaxusa.com

Jennifer Weidhaas
West Virginia University
(304) 293-9952
jennifer.weidhaas@mail.wvu.edu

Kimberly A. Wilson
South Carolina DHEC
(803) 896-4087
wilsonka@dhec.sc.gov

Ryan Wymore
CDM Smith
(720) 264-1110
wymorea@cdmsmith.com

APPENDIX H. ACRONYMS

1,1,1-TCA	1,1,1-trichloroethane
ANOVA	Analysis of variance
AO	Acridine orange
ATCG	Adenine, Thymine, Cytosine, Guanine
***atzB* gene**	Hydroxyatrazine ethylaminohydrolase
bp, kbp, Mbp	Base pairs, kilobase pairs, megabase pairs
***bssA* gene**	benzylsuccinate synthase
BTEX	Benzene, Toluene, Ethelbenzene, Xylene
***bvcA* gene**	Reductive dehalogenase implicated in the vinyl chloride to ethene reductive dechlorination
C	Carbon
CARD	Catalysed reported deposition
CARD-FISH	Catalyzed reported deposition fluorescence in situ hybridization
cDNA	Complementary DNA
CERCLA	Comprehensive Environmental Response, Compensation and Liability Act
CFR	Code of Federal Regulations
***cis*-1,2-DCE or *cis*-DCE**	*cis*-1,2-dichloroethylene
Cl	Chlorine
***cld* gene**	Chlorite dismutase gene
CO	Carbon monoxide
CO_2	Carbon dioxide
CSIA	Compound Specific Isotope Analysis
CSM	Conceptual site model
Cu	Copper
DAPI	4.6-diamindino-phenylindole
DC	Dissimilarity Coefficient
DCA	Dichloroethane
DGGE	Denaturing gradient gel electrophoresis
Dhc	*Dehalococcoides mccarti*
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic acid

DNAPL	Dense non-aqueous phase liquid
EAPs	Enzyme activity probes
EDB	1,2-dibromoethane
EE/CA	Engineering Evaluation/Cost Analysis
EMD	Environmental molecular diagnostics
ENA	Enhanced natural attenuation
EPA	Environmental Protection Agency
ESTCP	Environmental Security Technology Certification Program
FAMEs	Fatty acid methyl esters
FAQ	Frequently asked questions
Fe	Iron
FISH	Fluorescence in situ hybridization
FMGP	Former manufactured gas plant
FS	Feasibility study
g	Gram
GAC	Granular activated carbon
GC	Gas Chromatograph
GC/ECD	Gas chromatograph/electron capture detector
GC/MS	Gas chromatograph/mass spectrometer
H	Hydrogen
HNOPS	Hydrogen, Nitrogen, Oxygen, Potassium, Sulfur
ICP-MS	Inductively Coupled Plasma–Mass Spectrometry
IRMS	Isotope Ratio Mass Spectrometer
ISCO	In Situ Chemical Oxidation
ITRC	Interstate Technology and Regulatory Council
L	Liter
L108	Aquincola tertiaricarbonis L108
LNAPL	Light non-aqueous phase liquid
LTM	Long Term Management
LTMO	Long-Term Monitoring Optimization
LUST	Leaking underground storage tank
MAR-FISH	Microautoradiography Fluorescence in situ hybridization
MCL	Maximum Contaminant Level
MDA	Multiple displacement amplification

mg	Milligrams
MIP	Membrane Interface Probe
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
mL	Milliliter
MNA	Monitored Natural Attenuation
Mo	Molybdenum
MPN	Most probable number
mRNA	Messenger RNA
MS	Mass Spectrometry
MTBE	Methyl tertiary-butyl ether
MW	Monitoring Well
N	Nitrogen
NAH	Naphthalene dioxygenase
NanoSIMS-FISH	Nanoscale secondary-ion mass spectrometry - Fluorescence in situ hybridization
NAPL	Nonaqueous phase liquid
NAS	Naval Air Station
NAVFAC	Naval Facilities Engineering Command
NELAC	National Environmental Laboratory Accreditation Conference
NPL	National Priority List
NTC	No Template Controls
O	Oxygen
ORP	Oxidation-reduction potential
PAH	Polycyclic aromatic hydrocarbon
Pb	Lead
PCBs	Polychlorinated biphenyls
PCE	Perchloroethylene
***pceA* gene**	tetrachloroethene reductive dehalogenase
PCR	Polymerase chain reaction
PHE	Phenol hydroxylase
PLFA	Phospholipid fatty acid
PM1	*Methylibium petroleiphilum*
PQL	Practical Quantitation Limit
QA/QC	Quality assurance/quality control

qPCR	Quantitative polymerase chain reaction
RCRA	Resource Conservation and Recovery Act
RDase	Reductive dehalogenase
RDX	Hexahydro-1,3,5-trinitro-1,3,5-triazine
RFU	Relative fluorescence units
RI	Remedial investigation
RMDs	Retrievable media devices
RMO	Ring-hydroxylating toluene monooxygenase
RNA	Ribonucleic acid
ROD	Record of Decision
rRNA	Ribosomal ribonucleic acid (ribosomal RNA)
RT-qPCR	Reverse transcriptase qPCR
S	Sulfur
SERDP	Strategic Environmental Research and Development Program
SETAC	Society of Environmental Toxicology and Chemistry
SIM	Single Ion Monitoring
SIMS	Secondary-ion mass spectrometry
SIP	Stable isotope probing
sMMO	Soluble methane monoooxygenase
SNR	Signal to noise ratio
SOP	Standard Operating Procedure
SRB	Sulfate-reducing bacteria
T	Time
TAN	Test Area North site at the Idaho National Laboratory
TBA	Tert-Butyl Alcohol
TCA	Trichloroethane
***tceA* gene**	trichloroethene reductive dehalogenase
TCE	Trichloroethene
TCFE	Trichlorofluoroethylene
TEL	Tetra-ethyl Lead
TMO	Toluene monooxygenase
TNT	2,4,6-trinitrotoluene
TOC	Total Organic Carbon
TOD	Toluene 2,3-dixoygenase

TPH	Total petroleum hydrocarbons
T-RFLP	Terminal restriction fragment length polymorphism
U(IV)	Uranium, +4 oxidation state
U(VI)	Uranium, +6 oxidation state
ug	Micrograms
UST	Underground storage tank
VC	Vinyl Chloride
vcrA	Vinyl chloride reductase (varietal A), a reductive dehalogenase gene
VFAs	Volatile Fatty Acids
VOC	Volatile organic compound
Zn	Zinc

APPENDIX I. GLOSSARY

1

16S rRNA
A subunit of the ribosome composed of ribonucleic acid (RNA). The RNA sequence is used to classify and identify microorganisms (e.g. genus and species).

A

active site
Part of an enzyme where catalysis of the substrate occurs.

activity
Refers to when a microorganism performs a specific function (e.g., sulfate reduction, metabolism of benzene)

Archaea
Microorganisms that are genetically distinct from bacteria. Methanogens are an example of archaea (www.biology-online.org, accessed online, 2013).

aseptic
Free from pathogenic microorganisms.

B

bioaugmentation
The introduction of cultured microorganisms into the subsurface environment for the purpose of enhancing bioremediation of organic contaminants (USEPA 2011)

biodegradation
A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (USEPA 2011).

biomarker
A distinctive (unique) characteristic of a biomolecule that can be measured and used as an indicator of a target microorganism or biological process. For example, a specific DNA sequence (used as a probe on a microarray) could be a biomarker for a particular microorganism (e.g., Desulfotomaculum).

biomolecules
Classes of compounds produced by or inherent to living cells including phospholipids, nucleic acids (e.g., DNA, RNA), and proteins.

bioremediation
The treatment of environmental contamination through the use of techniques that rely on biodegradation. Bioremediation has two essential components: biostimulation and bioaugmentation.

biostimulation
A remedial technique which provides the electron donor, electron acceptor, and/or nutrients to an existing subsurface microbial community to promote degradation.

C

chemoheterotrophs
Bacteria that use organic compounds as both their electron donor and carbon source.

chlorite dismutase (cld)
An enzyme that catalyzes the disproportionation (simultaneous reduction and oxidation) of chlorite (ClO_2^-) to chloride (Cl^-) and oxygen (O_2). Both perchlorate reductase and chlorite reductase are present in perchlorate respiring bacteria capable of using perchorlate or chlorate as electron acceptors during respiration.

cis-DCE stall
In biodegradation through reductive dechlorination, the parent chlorinated ethene is sequentially dechlorinated via the following process: PCE to TCE to cis-DCE to vinyl chloride to ethene. For a variety of reasons, the slowest step in the process is often the dechlorination of the cis-DCE. This phenomenon is known as “cis-DCE stall".

compound specific isotope analysis (CSIA)
Analyzes the relative abundance of various stable isotopes (e.g., ^{13}C:^{12}C, ^{2}H:^{1}H). Degradation processes can cause shifts in the relative abundance of stable isotopes of the contaminant; changes in isotopic ratios can be measured.

contaminant-degrading population
The group of organisms that are capable of degrading a particular contaminant.

D

Dehalococcoides
Dehalococcoides is a genus of organohalide-respiring bacteria (for example, bacteria that use chlorinated solvents as metabolic electron acceptors) within the phylum Chloroflexi, in the domain Bacteria, and currently represented by a single species, Dehalococcoides mccartyi (Dhc). This species is the only one known with strains that dechlorinate dichloroethenes (DCEs) and vinyl chloride (VC) to ethene and inorganic chloride.

dehalogenase
An enzyme that catalyzes the removal of a halogen atom from an organic compound.

denaturing gradient gel electrophoresis (DGGE)
Type of gel electrophoresis used to separate mixtures of PCR products based on the melting point which is reflective of the DNA sequence. DGGE is used to generate a genetic fingerprint of the microbial community and potentially identify dominant microorganisms.

DNA probe
short DNA strand (see microarray probes, Microarray Fact Sheet; FISH probes, FISH Fact Sheet; qPCR probes, qPCR Fact Sheet).

DNA—Deoxyribonucleic acid
A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication, and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (USEPA 2004a).

DNases
Enzymes that specifically degrade DNA.

dNTPs Deoxyribonucleotide triphosphates
dNTPs are incorporated into DNA during elongation (USEPA 2004a).

E

electron acceptor
A chemical compound that accepts electrons transferred to it from another compound (based on USEPA 2011).

electron donor
A chemical compound that donates electrons to another compound (based on USEPA 2011).

environmental forensics
The process of distinguishing contaminants from different sources.

enzyme activity probes (EAPs)
Transformation of surrogate compounds (probes) resembling contaminants produces a fluorescent (or other distinct) signal in cells which is then detected using a microscope.

enzymes
Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on USEPA 2004a).

epifluorescent microscope
A type of microscope that uses a high energy light source (e.g., ultraviolet light) and specialized filters to visualize fluorescently stained specimens. Epifluorescent microscopy procedures can be used to determine both the total number of cells and total number of viable or active cells in a sample.

F

FISH probes
Short sequences of single stranded DNA carrying a fluorescent label. When the probe binds to the target DNA/RNA sequence of the microorganism(s) of interest in an environmental sample, the target cell will fluoresce and can be visualized and counted using a specialized microscope or a flow cytometer.

flow cytometry
A method whereby cells or particles move in a liquid stream past a laser or light beam and a sensor detects the relative light scattering and fluorescence of the particles.

fluorescence in situ hybridization (FISH)
Detects the presence of targeted genetic material in an environmental sample and estimates the number of specific microorganisms or groups of microorganisms.

functional gene
A segment of DNA that encodes an enzyme or other protein that performs a known biochemical reaction. For example, the functional gene tceA encodes the reductive dehalogenase enzyme that initiates reductive dechlorination of TCE. Other genes can code for RNA entities which can regulate the activity of other DNA target sequences.

G

gene
A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

genus
A category of organism classification (taxonomy). A particular genus is a group of related species. For example, Pseudomonas is a genus of bacteria.

H

heterotrophic plate count
A test used to estimate the total number of bacteria capable of growing on organic compounds in an environmental sample.

I

isotope
Two atoms with the same number of protons but a different number of neutrons.

isotopic fractionation
Some processes (for example, those which involve breaking chemical bonds) have slightly different rates for different isotopes, leading to a more rapid consumption of one isotope over the other. This characteristic is manifested in a change in the isotopic ratio of the residual compound.

isotopic ratio
The concentration of the heavy isotope divided by the concentration of the light isotope.

isotopically labeled contaminants
A contaminant that has been specially synthesized to deliberately contain specific isotopes at elevated levels above those found in either natural or commercial bulk forms of the same chemical.

L

labeled cell
A microorganism in which a gene probe has bound to a matching sequencing within the microorganism and released a fluorescent dye, resulting in a cell that is emitting fluorescent light.

lipids
A diverse range of organic compounds that are defined as being insoluble in water but soluble in non-aqueous solvents. Lipids include oils, waxes, and sterols.

lithoautotrophs
Bacteria that grow only using inorganic chemicals such as ammonia, iron, or hydrogen as their electron donor.

M

metabolic product
Products generated by a microorganism whose structure and function are defined by DNA sequences also called genes. Example metabolic products include RNA and proteins or enzymes.

microarray
Detects and estimates the relative abundances of hundreds to thousands of genes simultaneously.

microarray probes
Short, defined segments of DNA that are designed to bind with the target gene if found in the environmental sample. The probes are attached to the solid surface of the microarray.

microbial community
The microorganisms present in a particular sample.

microbial community composition
Description of the types or identities of microorganisms present in a sample.

microbial diversity
Microbial diversity can have many definitions but in this context generally refers to the number of different microbial species and their relative abundance in an environmental sample (Nannipieri et al. 2003).

microbial fingerprinting methods
A category of related techniques that differentiate microorganisms or groups of microorganisms based on unique characteristics of a universal component or section of a biomolecule.

microcosm
A sample that is regarded as a small but representative portion of something larger. In environmental studies microcosm are typically small samples of soil, sediment, or water incubated in enclosed containers under laboratory conditions.

N

nitrite reductase genes
Functional genes encoding the enzymes that catalyzes nitrite reduction. Nitrite reductase genes are commonly used as the target gene to detect microorganisms capable of denitrification.

nucleic acid
A complex biomolecule consisting of a long "backbone" of organophosphate sugars with four different types of nucleotide bases attached.

O

oxygenase
An enzyme that catalyzes the incorporation of molecular oxygen into a compound (based on Madigan et al. 2010).

P

phospholipid
A type of biomolecule that is a primary structural component of the membranes of almost all cells.

phospholipid fatty acid (PLFA) analysis
A laboratory analytical techniques that differentiate microorganisms or groups of microorganisms based on quantifying PLFA groups.

phylogeny (phylogenetic analysis)
Classification of microorganisms into groups (e.g. genus and species) based in part upon the rRNA sequences.

planktonic existence
Free floating microorganisms that are not associated with particles, sediments or biofilms.

PLFA
Phospholipid fatty acids derived from the two hydrocarbon tails of phospholipids.

polymerase chain reaction
Makes copies of a specific DNA sequence within a target gene of microorganisms that can be further analyzed.

primers
Short strands of DNA that are complementary to the beginning and end of the target gene and thus determine which DNA fragment is amplified during PCR or qPCR.

protein
Large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds (US Navy 2009).

Proteobacteria
A broad phylum of gram negative bacteria that is categorized into six groups, involving many genera, based on 16s rRNA differences

Pyrosequencing
A common high throughput DNA sequencing approach that uses light-emitting enzyme couple systems to detect pyrophosphate released when one nucleotide is attached to another. This is a well-established DNA sequencing approach that regulators, consultants and others in environmental site management are likely to encounter.

Q

qPCR probes
Short, defined segments of DNA or RNA, that may or may not be labeled, that are designed to bind with the target gene if found in the environmental sample.

qPCR target (target gene)
The specific gene quantified by a particular qPCR analysis. For example, vinyl chloride reductase genes are the target genes in qPCR analyses performed to assess reductive dechlorination of vinyl chloride to ethene. Similarly, a qPCR analysis targeting the toluene dioxygenase gene is used to evaluate aerobic biodegradation of toluene and benzene.

quantitative polymerase chain reaction (qPCR)
A laboratory analytical technique for quantification of a target gene based on DNA.

R

redox conditions
Description of the oxidation/reduction potential of the subsurface (e.g. aerobic, anaerobic, sulfate reducing, or methanogenic conditions)

restriction enzymes
Restriction enzymes (also called restriction endonucleases) are bacterial enzymes that recognize and cut specific DNA sequences (typically 4 to 6 base pairs long). Each restriction enzyme has a unique recognition and cleavage site sequence.

reverse transcriptase qPCR (RT-qPCR)
A laboratory analytical technique for quantification of a target gene based on RNA.

ribosome
A multi-component biological molecule which is part of the protein-synthesizing machinery of the cell.

RNA - ribonucleic acid
Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

S

species
The lowest taxonomic rank, and the most basic unit or category of biological classification. (www.biology-online.org)

stable isotope probing (SIP)
A synthesized form of the contaminant containing a stable isotope (e.g., ^{13}C label) is added. If biodegradation is occurring the isotope will be detected in biomolecules (e.g., phospholipids, DNA).

stable isotopes
Forms of an element that do not undergo radioactive decay at a measureable rate.

substrate
Any substance that is acted upon by an enzyme.

syntrophic population
Microorganisms that are associated or mutually dependent upon one another.

T

terminal electron acceptors
Compounds used by microorganisms to support respiration. In aerobic organisms the terminal electron acceptor is oxygen (O_2). Anaerobic organisms use compounds other than O_2. These include common naturally–occurring compounds such as nitrate (NO_3^-) or sulfate (SO_4^{2-}) or anthropogenic contaminants such as chlorinated ethenes (e.g. perchloroethylene). Atoms from electron acceptors are typically not incorporated into biomolecules made by organisms that reduce these compounds during respiration.

terminal restriction fragment length polymorphism (T-RFLP)
A nucleic acid (DNA or RNA)-based technique used to generate a genetic fingerprint of the microbial community and potentially identify dominant microorganisms.

transcription
The first step in activation of a biochemical pathway where a complementary RNA copy is synthesized from a DNA sequence.

translation
The second step of gene expression where messenger RNA (mRNA) produced by transcription is decoded by the cell to produce an active protein.

V

viable biomass
In this context, viable biomass refers to living microorganisms (capable of metabolism and/or reproduction).

W

whole cell

The entirety of a microbial cell, without extraction of DNA, RNA, or similar. A whole-cell preparation does not modify the cell but evaluates it as unit.