

## **Starting Soon: Environmental Molecular Diagnostics – New Tools for Better Decisions**



- ▶ ITRC Environmental Molecular Diagnostics Technical and Regulatory Guidance (EMD-2, 2013) at <http://www.itrcweb.org/emd-2/>
- ▶ Download PowerPoint file
  - Clu-in training page at <https://clu-in.org/conf/itrc/EMD/>
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No associated notes.

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Welcome – Thanks for joining  
this ITRC Training Class



## Environmental Molecular Diagnostics New Tools for Better Decisions



EMD Technical and Regulatory Guidance  
Web-Based Document

Sponsored by: Interstate Technology and Regulatory Council ([www.itrcweb.org](http://www.itrcweb.org))  
Hosted by: US EPA Clean Up Information Network ([www.cluin.org](http://www.cluin.org))

Environmental molecular diagnostics (EMDs) are a group of advanced and emerging analytical techniques used to analyze biological and chemical characteristics of environmental samples. Conventional data (e.g., 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, of the relative contributions of abiotic and biotic processes, and of the relative rates of various degradation processes. The information provided by EMDs 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. EMDs have application in each phase of environmental site management (including site characterization, remediation, monitoring, and closure activities), address a wide variety of contaminants (including PCE, PCBs, radionuclides, perchlorate, fuels), and work with various media (including groundwater, soil, sediments, soil vapor).

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. The [ITRC Environmental Molecular Diagnostics Fact Sheets](#) (EMD-1, 2011), ITRC Environmental Molecular Diagnostics Technical and Regulatory Guidance (EMD-2, 2013) and this 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. At the conclusion of the training, learners will be able to determine when and how to use the ITRC Environmental Molecular Diagnostics Technical and Regulatory Guidance (EMD-2, 2013); define when EMDs can cost-effectively augment traditional remediation data sets; and describe the utility of various types of EMDs during remediation activities.

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ITRC Training Program: [training@itrcweb.org](mailto:training@itrcweb.org); Phone: 402-201-2419

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  - All 50 states, PR, DC
- Federal partners



- ITRC Industry Affiliates Program



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▶ Available from [www.itrcweb.org](http://www.itrcweb.org)

- Technical and regulatory guidance documents
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The Interstate Technology and Regulatory Council (ITRC) is a state-led coalition of regulators, industry experts, citizen stakeholders, academia and federal partners that work to achieve regulatory acceptance of environmental technologies and innovative approaches. ITRC consists of all 50 states (and Puerto Rico and the District of Columbia) that work to break down barriers and reduce compliance costs, making it easier to use new technologies and helping states maximize resources. ITRC brings together a diverse mix of environmental experts and stakeholders from both the public and private sectors to broaden and deepen technical knowledge and advance the regulatory acceptance of environmental technologies. Together, we’re building the environmental community’s ability to expedite quality decision making while protecting human health and the environment. With our network of organizations and individuals throughout the environmental community, ITRC is a unique catalyst for dialogue between regulators and the regulated community.

For a state to be a member of ITRC their environmental agency must designate a State Point of Contact. To find out who your State POC is check out the “contacts” section at [www.itrcweb.org](http://www.itrcweb.org). Also, click on “membership” to learn how you can become a member of an ITRC Technical Team.

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## Meet the ITRC Trainers



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**Jennifer Weidhaas** is an Associate Professor of Civil and Environmental Engineering at University of Utah. Previously, she was an Assistant Professor of Civil and Environmental Engineering at West Virginia University, from 2010-2016 where she focused on determining the fate, transport and bioremediation of emerging contaminants. Prior to taking a position at WVU, Jennifer worked for six years with North Wind Inc. as a professional environmental engineer on hazardous waste remediation projects. At North Wind, Inc. she directed a molecular biology laboratory which routinely analyzed groundwater, soil and sediment samples by various EMD methods for microorganisms involved in contaminant bioremediation. Additionally she has work on numerous groundwater, soil and sediment bioremediation projects throughout the western United States. She has been a member of the Environmental Molecular Diagnostics Team since 2010. Jennifer earned a bachelor's degree in Civil Engineering in 1999 from Montana State University at Bozeman and a master's in 2002 and PhD in 2006 from the University of California at Davis in Civil and Environmental Engineering. Jennifer is a registered professional engineer in Idaho.

**Paul Hatzinger** is a senior research scientist working with SERDP in Lawrenceville, NJ. Paul's current areas of research focus on the development of *in situ* and *ex situ* bioremediation technologies for emerging contaminants and the use of stable isotope methods to distinguish contaminant sources and to document contaminant biodegradation. Paul has served as the Principal Investigator on several research grants focused on the use of stable isotopes to delineate natural from anthropogenic perchlorate in the environment, and to document the biodegradation of perchlorate and explosives in groundwater. He has authored more than 50 peer-reviewed research papers and book chapters, including several on perchlorate forensics. Paul has been a member of the ITRC Environmental Molecular Diagnostics (EMD) team since its inception. He earned a bachelor's degree in Biology and Environmental Science from St. Lawrence University in Canton, NY in 1986, and he holds both a master's degree (1991) and a doctoral degree (1996) in Environmental Toxicology from Cornell University in Ithaca, New York.

**James Fish** is an Environmental Program Specialist with the Contaminated Sites Program of the Alaska Department of Environmental Conservation (DEC), in Fairbanks, Alaska. He has worked for the Alaska DEC since 2009. As a Project Manager, he oversees the characterization and remediation of contaminated sites across Alaska's Interior. In addition to environmental cleanup, he has worked in many aspects of environmental microbiology, including water quality and treatment processes, aquaculture and fish health. His interests in microbial ecology involve biodegradation, microbial survival, and the activities of microorganisms in cold environments. He is currently the president-elect of the Alaska Chapter of the American Society of Microbiology. He joined the ITRC EMD team in 2011. He earned a bachelor's degree in Fisheries Biology in 1986 and a master's degree in Microbiology in 1991, both from the SUNY College of New York at Buffalo in Buffalo, New York.

**Dr. Aaron Peacock** has worked as an environmental scientist at Haley & Aldrich Inc. in Oak Ridge, Tennessee since 2006. Since 1995, he has worked both in academia and industry, specializing in biotechnology and environmental molecular diagnostics (EMDs). He has worked extensively in the development, evaluation, and implementation of new technologies for environmental surveillance, monitored natural attenuation (MNA), and enhanced bioremediation of metals and organic contaminants. His current focus has been on the use and development of electronic sensors and EMDs to aid in the evaluation of sites for in-situ bioremediation/MNA potential, performance of remediation programs, and forensics of non-performing sites. Aaron has been a member of the ITRC Environmental Molecular Diagnostics team since 2010. He earned a bachelor's degree in Soil Science in 1997, a master's degree in Biosystems Engineering Technology in 2000, and a doctoral degree in Environmental Science in 2007, all from The University of Tennessee in Knoxville, TN.

## Today's Training Outline



Training Topics	Tech Reg Sections	Training Progress
Introduction to Training and EMDs	1, 2, 11	↓
CSIA and Case Studies	3, App. C	
qPCR and Case Study	4, App A	
Question & Answer Break		
Biological EMDs	5-10, App. D	
Case Studies using Multiple EMDs	App. A	
Web-Based Tech Reg Navigation		
Question & Answer Break		

No associated notes.

## What If...

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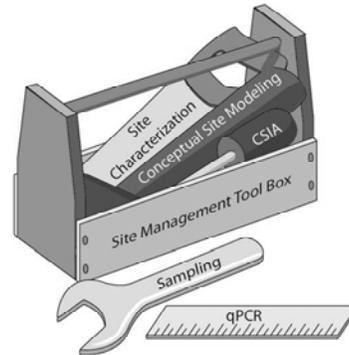


- ▶ You could tell whether the contaminants at your site are being degraded through biodegradation or being decreased through dilution?
- ▶ You could tell at what rate these contaminants are being degraded?
- ▶ You could decrease the size of your monitoring network and thereby reduce the cost of monitoring?
- ▶ You could secure that final piece of evidence needed to close a site?

No associated notes.

## What Are Environmental Molecular Diagnostics (EMDs)?

- ▶ Group of analytical techniques
  - Used to analyze biological and chemical characteristics of soil, sediment, and water samples
- ▶ Developed for medicine
  - Adapted for environmental site management
- ▶ Two major categories
  - Chemical techniques
  - Molecular biological techniques (MBTs)



No associated notes.

## Hypothetical Case Study Using EMDs



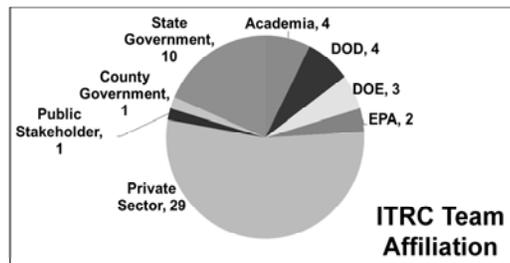
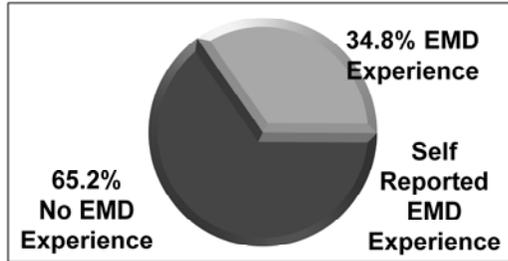
- ▶ Investigation of BTEX contaminated groundwater
- ▶ Are microorganisms actively degrading BTEX?
- ▶ EMDs utilized and information provided direct evidence of
  - Anaerobic BTEX biodegradation
    - RT-qPCR benzylsuccinate synthase (*bssA*) genes
  - Aerobic BTEX biodegradation
    - RT-qPCR for aromatic oxygenase genes
  - Biological or chemical degradation
    - CSIA for BTEX isotopic enrichment
- ▶ Allows for informed selection of remedy in a more timely manner compared to conventional monitoring

No associated notes.

# What is the Level of Regulatory Knowledge and Acceptance?



- ▶ Survey results
  - 47 regulators from 29 states
  - Most EMDs are new to regulators
  - Expressed willingness to become educated
  - Agreed that EMDs can contribute toward regulatory decision making
  - Expressed concern about site implementation



Survey results:  
 78 people completed the survey  
 60% were regulators, 21% were consultants  
 29 states were represented.

## EMD Training Objectives

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- ▶ Describe the **utility of EMDs during site management** activities
- ▶ Define when EMDs can **augment traditional data sets**
- ▶ Understand **when and how to utilize the EMD Tech Reg**

ITRC, EMD, Section 1.1

More information on ITRC is available at [www.itrcweb.org](http://www.itrcweb.org).

## Questions that will be answered in today's training

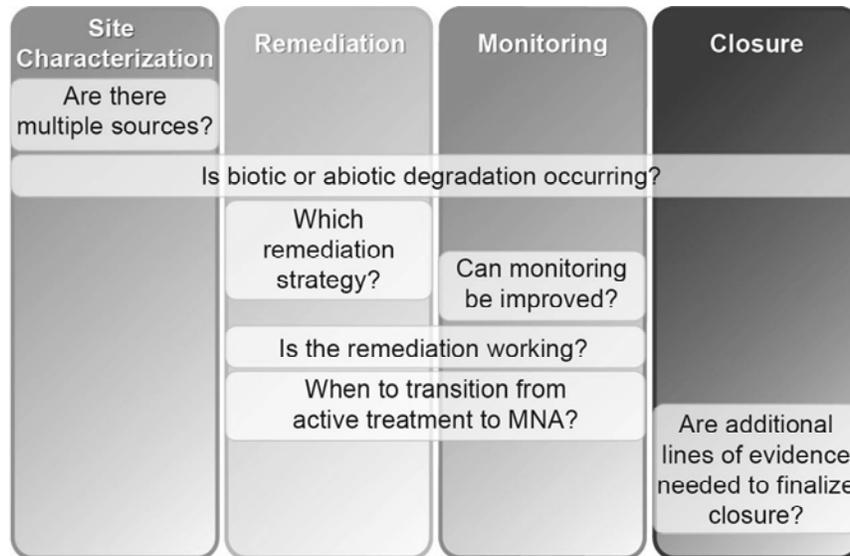
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- ▶ What are EMDs?
- ▶ How can they help me?
- ▶ What benefits do they provide compared to traditional tools?
- ▶ How do I select an EMD?
- ▶ How commonly are these tools used?
- ▶ What is the level of regulatory knowledge and acceptance?

No associated notes.

# 13 Using EMDs Throughout Site Management



ITRC, EMD, Section 2, Figure 2-1

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 and closure.

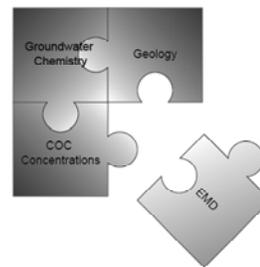
The questions presented are only examples of the types of questions that EMDs can help to answer by providing supplemental information to the site-specific characterization data and information. The project life cycle stages are depicted as linear steps for simplicity. Often at sites the stages overlap; 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.

## What Can EMDs Tell You?

### Information not provided by traditional methods

- ▶ Microbial presence / abundance
  - For example, *Dehalococcoides mccartyi* (Dhc)
- ▶ Direct evidence of contaminant (bio)degradation
- ▶ Microbial cellular activity (e.g., expression of TCE degradation gene)

EMDs can provide the “*missing piece*” to make the best decision



The biological degradation pathway for the chlorinated solvent tetrachloroethylene (by *Dehalococcoides* spp.) is as follows:

tetrachloroethylene (PCE) to trichloroethylene (TCE) to dichloroethylene(DCE) to vinyl chloride (VC) to ethene

*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 the EMD-2 document, "Dhc" refers to *Dehalococcoides mccartyi*, including all of the strains that reductively dechlorinate chlorinated ethenes to environmentally benign ethene and inorganic chloride.

ADD Loeffler 2013 citation.

## Which EMDs Support Site Management Decision Making?

- ▶ Cells make a living by
  - Eating
  - Breathing
  - Getting rid of waste
  - Reproducing
  - Adapting to environment
- ▶ Cells use enzymes to facilitate these processes
- ▶ Molecular biological techniques (MBTs) 
  - Quantitative polymerase chain reaction (qPCR)
  - Fingerprinting methods (DGGE, TRFLP, PLFA)
  - Microarrays
  - Stable isotope probing (SIP)
  - Fluorescence in situ hybridization (FISH)
  - Enzyme activity probes (EAPs)
- ▶ Chemical techniques
  - Compound specific isotopic analysis (CSIA) 

No associated notes.

## Where Are EMDs Most Applicable?

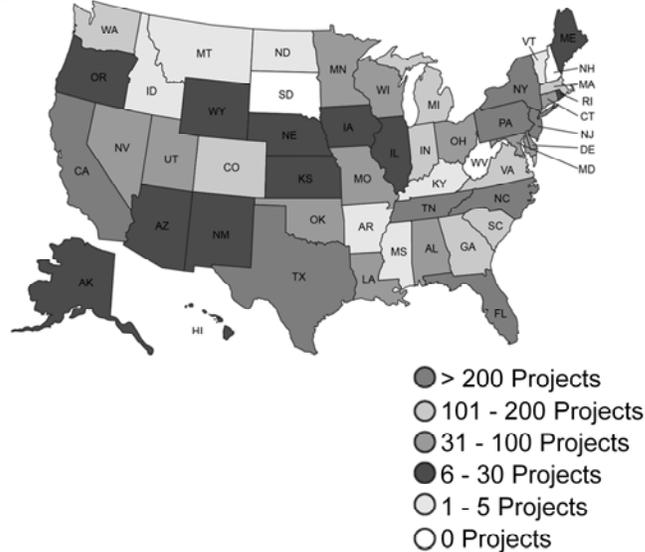


- ▶ EMDs are applicable to a variety of contaminants
  - Chlorinated solvents
  - Petroleum hydrocarbons
  - Inorganics
  - Pesticides
  - Explosives
- ▶ In a variety of media types
  - Groundwater
  - Soil/sediment
  - Soil Gas
- ▶ Under different geochemical conditions
  - Anaerobic
  - Aerobic
- ▶ For the detection and/or quantification of:
  - Different microorganisms conducting biodegradation
  - Biological or chemical degradation products and pathways
  - Microbial enzymes facilitating biodegradation reactions

No associated notes.

## EMDs Are Not New

- ▶ Data from 2009-2012
- ▶ Expand decision making tool box



Environmental molecular diagnostics (EMDs) are a group of advanced and emerging analytical techniques used to analyze biological and chemical characteristics of environmental samples. Many of these techniques were originally developed for applications in medicine, defense, and industry.

Over the last decade, however, EMDs have proven effective in environmental site management. EMDs have applications in each phase of environmental site management and provide additional lines of evidence for making better remediation decisions. EMDs provide key information not available using traditional analytical methods (e.g., groundwater analysis for volatile organic compounds).

Decision makers pursue traditional strategies because they lack sufficient data to support alternatives that could result in equal or more efficient remediation at a lower cost and perhaps in less time.

What EMDs can tell you:

- Microbial presence / abundance
- Microbial cellular activity (e.g., transcription)
- Biodegradation activity
- Direct evidence of contaminant biodegradation

The data are cumulative numbers from 2009-2012

The data are from two commercial labs, so they don't represent all of the EMD work that was completed in that time period

The states are assigned based on the information available to the labs, there is uncertainty in some of the project locations due to client confidentiality.

The map is intended to provide a relative understanding of the usage of EMDs and not provide definitive numbers of projects.

## EMDs Can Be Used in Site Decisions



- ▶ Air Force Civil Engineer Center (formerly AFCEE) guidance documents for MNA [www.afcec.af.mil](http://www.afcec.af.mil)
- ▶ National Academy publication “Alternatives for Managing the Nation’s Complex Contaminated Groundwater Sites,” [www.nap.edu/catalog.php?record\\_id=14668](http://www.nap.edu/catalog.php?record_id=14668)

Many may be familiar with technical protocols or guidance for MNA; based on three lines of evidence: contaminant concentrations, geochemistry, and microbiology.

Microbiological information has traditionally come from laboratory microcosms based on culturing microorganisms. EMD are culture-independent, can often provide in situ information, and can provide information on contaminant molecules (e.g., CSIA). Thus, additional lines of evidence are provided and strengthened with EMDs.

## EMD Cost and Availability



EMD	Cost per sample
<b>Widely commercially available</b>	
Quantitative Polymerase Chain Reaction (qPCR)	\$ 275 - 425
Compound Specific Isotope Analysis (CSIA)	\$ 100 - 2,500
Microbial Fingerprinting Methods	\$ 300 - 570
<b>Minimally commercially available</b>	
Microarrays	\$ 1,250 - 5,000
Stable isotope probing (SIP)	\$ 1,500 and up
Enzyme activity probes (EAPs)	\$ 250 - 2,500
Fluorescence in situ hybridization (FISH)	\$ 250 - 5,000

ITRC EMD-1, Introduction to EMDs Fact Sheet

Costs are per sample. Depends on how many samples and how many analyses are being done for the project. The low end of the cost range represents a very restricted analysis and may not be applicable to every site; for example for only one compound or target with very limited quality control documentation.

Some analyses for some methods (for example for some compounds or targets) are currently available through university or research laboratories.

CSIA costs per sample depend on the number and type of isotopes and the number of compounds being analyzed.

Availability and costs will be changing going forward. Information as of 2011.

## Regulatory Approval Process for EMDs



- ▶ Involve the regulator as early as possible
- ▶ Work plan should include:
  - Explain site status with traditional analytical chemistry methods
  - Identify EMDs to be used
  - Explain how EMD data complements existing data, or detail what is expected to be learned by using the EMD
  - Identify which stage of the life cycle process the EMD is to be used
  - Identify sample locations
  - Identify data quality objectives: type, quality and quantity of data to be collected.
- ▶ Identify any permitting requirements

ITRC EMD Tech Reg, Section 11

The work plan can easily be adjusted to reflect all agreements reached during the meeting and can be submitted later as a final document.

## Permitting Requirements for EMDs



- ▶ Most EMDs are laboratory techniques, permitting not required
- ▶ Permitting may be needed for SIP and EAPs performed in situ
  - Varies from state to state, may include
    - Notification
    - Work plan
    - Discharge permit
    - Underground Injection Control (UIC) Permit

ITRC EMD Tech Reg, Section 3 and Appendix C

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, one can expect at a minimum to acquire approval for one or more, or all of the following: notification, a work plan, a discharge permit or a 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.

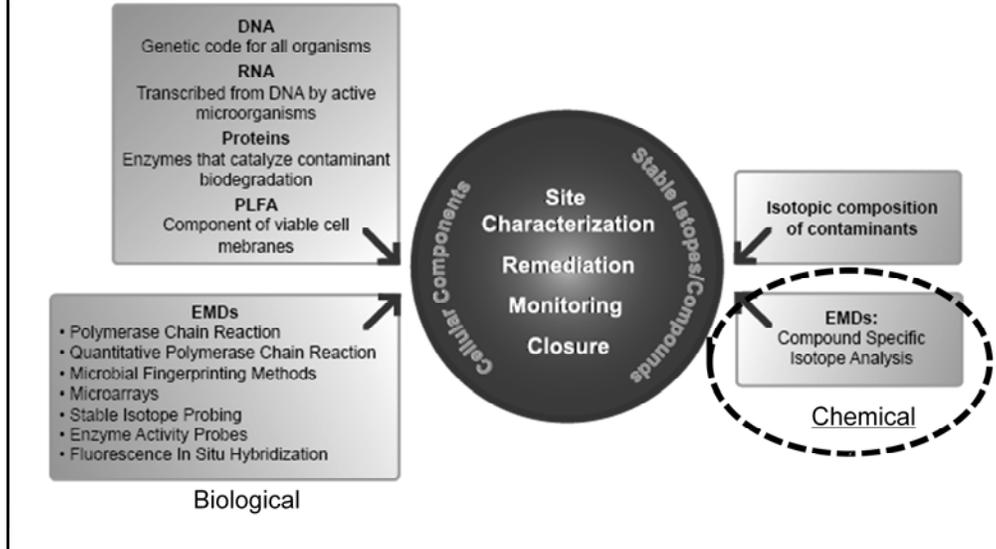
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Web-Based Tech Reg Navigation		
Question & Answer Break		

No associated notes.

## EMD Descriptions & Use: Compound Specific Isotopic Analysis



EMDs can be classified into two major categories of analytical techniques: chemical techniques, in particular compound specific isotope analysis (CSIA), and a variety of molecular biological techniques (MBTs).

What can CSIA do for you?

## What Is a Stable Isotope?



Isotopes: atoms with same number of protons, but a different number of neutrons

**Isotopes of Carbon**

$^{12}\text{C}$

6 protons  
6 neutrons  
"light" stable

$^{13}\text{C}$

6 protons  
7 neutrons  
"heavy" stable

$^{14}\text{C}$

6 protons  
8 neutrons  
radioactive

● proton    ○ neutron     $^{14}\text{C}$  not in use for CSIA

**Environmentally relevant stable isotopes**

Hydrogen	$^1\text{H}$ , $^2\text{H}$
Oxygen	$^{16}\text{O}$ , $^{17}\text{O}$ , $^{18}\text{O}$
Carbon	$^{12}\text{C}$ , $^{13}\text{C}$
Chlorine	$^{35}\text{Cl}$ , $^{37}\text{Cl}$
Nitrogen	$^{14}\text{N}$ , $^{15}\text{N}$
Sulfur	$^{32}\text{S}$ , $^{34}\text{S}$

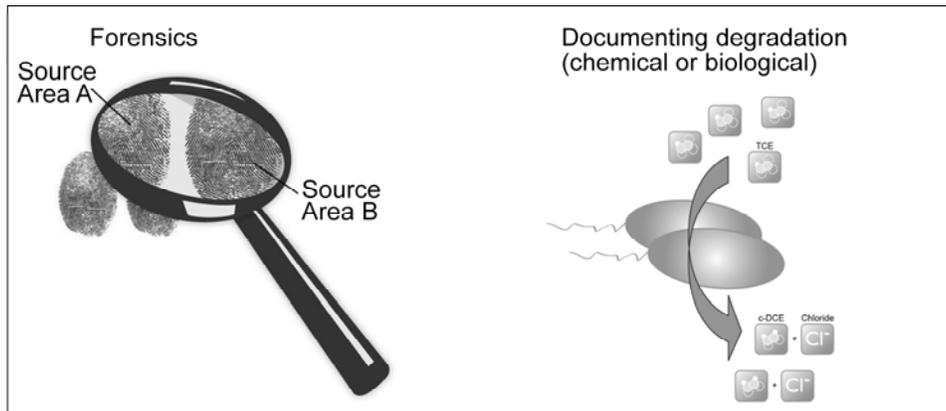
ITRC EMD Tech Reg, Section 3 and Appendix C

Source: Microseeps, Inc.

Stable isotopes have the same number of protons and electrons, but a different number of neutrons. Many environmental contaminants contain elements that have multiple stable isotopes and for which CSIA may have useful applications.

## Why Are Stable Isotopes Useful?

Compounds can have different ratios of stable isotopes (e.g.,  $^{13}\text{C}:^{12}\text{C}$ ) depending on how they were formed and whether they have been degraded since environmental release.



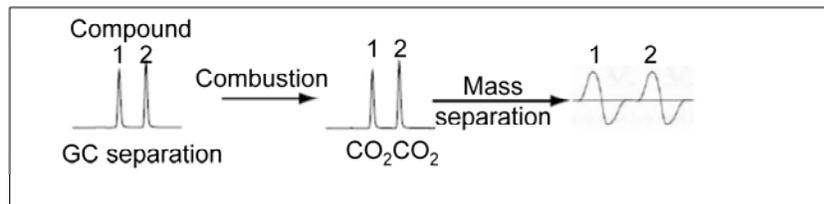
ITRC EMD Tech Reg, Section 3 and Appendix C

CSIA can be useful for forensic applications and for documenting degradation. Predictable shifts in stable isotope ratios occur during degradation of many pollutants by biological or abiotic methods.

## How Are Isotope Ratios Measured?



- ▶ Stable isotope ratios are measured using specially designed mass spectrometers: Isotope Ratio Mass Spectrometers (IRMS).
- ▶ If chemicals are analyzed individually (e.g., separated first by GC) then the process is termed **“Compound Specific Isotope Analysis (CSIA)”**



ITRC EMD Tech Reg, Section 3 and Appendix C

Source: USEPA. 2008

Source: USEPA. 2008. A Guide for Assessing Biodegradation and Source Identification of Organic Groundwater Contaminants using Compound Specific Isotope Analysis (CSIA). U.S. EPA National Risk Management Research Laboratory, ADA, OK ; EPA 600/R-08/148, 67 pg.

Bulk analysis is addressed in the Tech Reg in section 3.3.2.2 and in Appendix C.5.

## How Are Isotope Ratios Reported?



- ▶ Isotopic ratios of light elements are generally reported relative to a known standard as “delta” ( $\delta$ ) values and measured in parts-per-thousand (denoted “‰” = per mil)

**Equation 1.**  $\delta$  (in ‰) =  $(R_x/R_s - 1) * 1000$

**R** = ratio heavy/light isotope (e.g.,  $^{13}\text{C}/^{12}\text{C}$ )

**R<sub>x</sub>** = sample (e.g.,  $^{13}\text{C}/^{12}\text{C}$  in environmental sample)

**R<sub>s</sub>** = standard (e.g.,  $^{13}\text{C}/^{12}\text{C}$  in carbon standard)

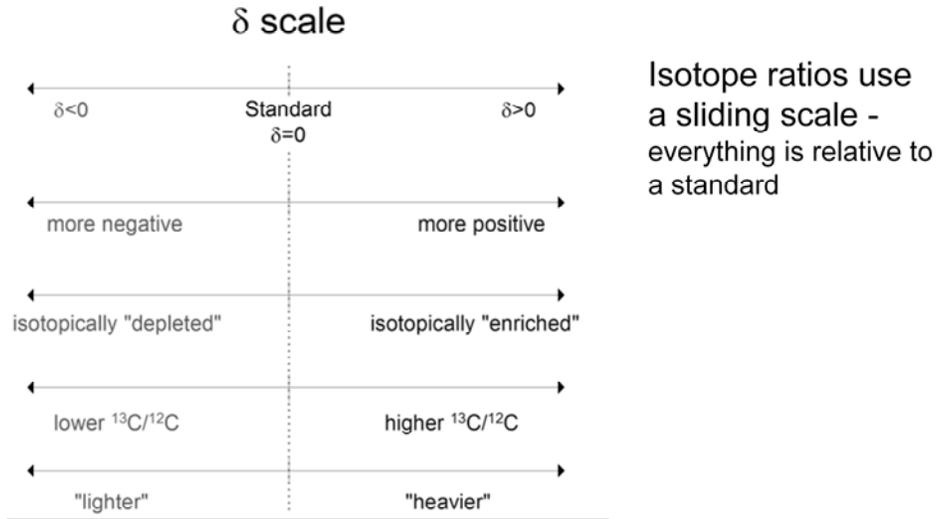
Example:  $\delta^{13}\text{C} = + 30 \text{ ‰}$

30 parts-per-thousand (3 %) higher ratio of  $^{13}\text{C}/^{12}\text{C}$  in sample relative to a known isotopic standard

ITRC EMD Tech Reg, Section 3 and Appendix C

No associated notes.

## Stable Isotope Ratios as Delta Notation ( $\delta$ )



Isotope ratios use a sliding scale - everything is relative to a standard

ITRC EMD Tech Reg, Section 3 and Appendix C

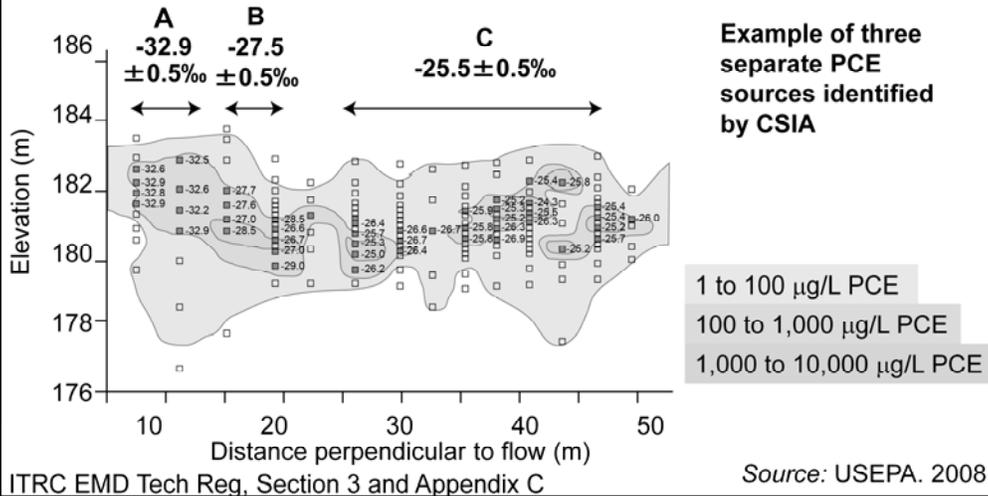
Source: Microseeps, Inc., 2010

All elements have reference standards for stable isotope ratio analysis. Generally the stable isotope ratio ( $\delta$  value) of these standards is set at 0. For example the reference standard for Carbon is Vienna Pee Dee Belemnite (VPDB).

The  $\delta$  value in a sample is compared to that in the standard for that element with values often being described as "heavier" or "lighter" than the relevant standard. Stable isotope ratios can be positive or negative. Table C-2 in Appendix C of the Tech. Reg. document includes reference standard information for common elements in environmental applications.

## CSIA Forensic Applications

- ▶ If chemicals are made by different processes or with different reagents, stable isotope ratios can vary significantly
- ▶ CSIA can be a means to distinguish sources



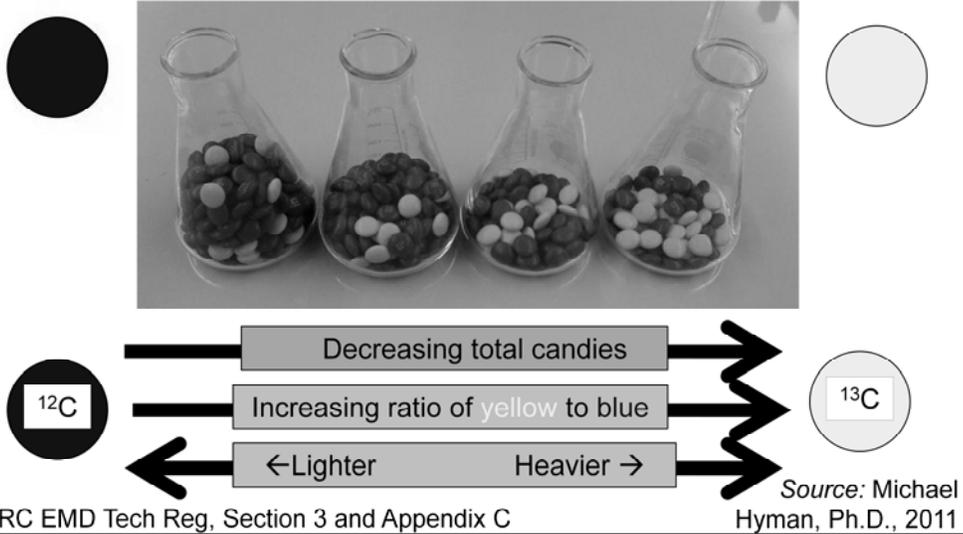
Source: USEPA. 2008. A Guide for Assessing Biodegradation and Source Identification of Organic Groundwater Contaminants using Compound Specific Isotope Analysis (CSIA). U.S. EPA National Risk Management Research Laboratory, ADA, OK ; EPA 600/R-08/148, 67 pg.

Further information available on precision of isotopic analyses in the EPA 2008 document.

On the graph above, the plume is flowing out of the page toward the reader. Delta values of PCE in nested wells are provided in the figure along with concentrations (see colors). The delta values ( $\delta$ ) for C in PCE collected from these wells suggest that there are three distinct sources (Zone A, B, C).

## CSIA: What Happens During Degradation?

- ▶ Stable isotope ratios in the parent molecule often increase during biological or chemical degradation “fractionation”

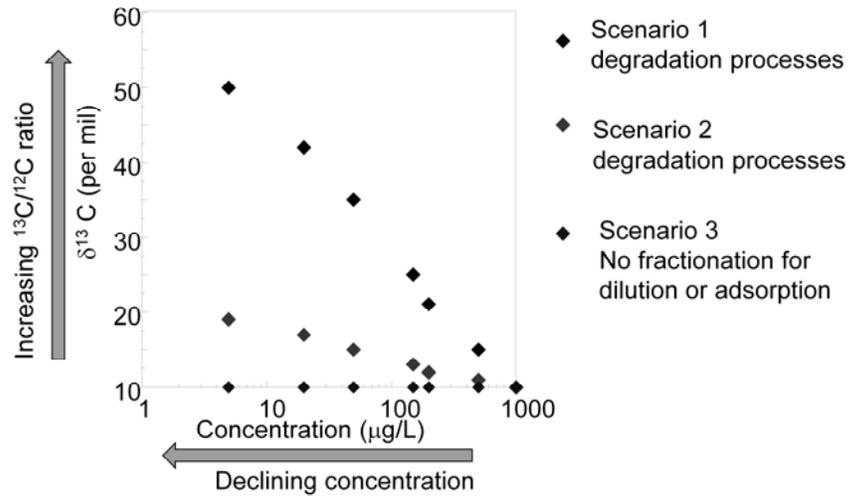


Bonds between heavier isotopes are stronger than those between lighter isotopes – broken less readily

As a result, isotope ratios often increase in parent molecules when biodegradation or chemical degradation occurs – “fractionation”

## CSIA: What Happens During Degradation?

- Example of isotopic enrichment during contaminant degradation



ITRC EMD Tech Reg, Section 3 and Appendix C

Source: Paul Hatzinger, Ph.D.

The extent of isotope fractionation during degradation is often measured by plotting chemical concentration vs. the  $\delta$  value for the isotope. This approach can be used to show that degradation is occurring (increasing  $\delta$  value with declining chemical concentration) and get an indication of mechanism (e.g., by evaluating slope of the lines in the figure above – more vs. less fractionation), particularly if you can measure isotopes of multiple elements in a molecule (e.g., Cl and C in TCE).

## CSIA Benefits and Limitations



### ► Benefits

- Provides direct evidence of biological degradation or abiotic attenuation of contaminants
- Useful for generating attenuation rates and mechanisms
- Provides information to identify multiple sources
- Commercially available

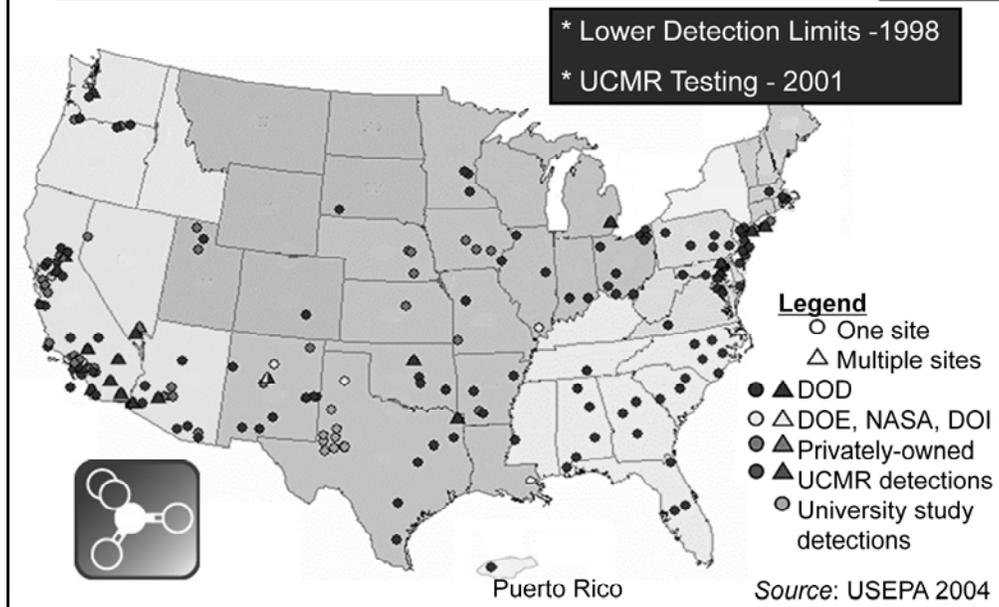
### ► Limitations

- Need laboratory *fractionation factors* for key contaminants and degrading organisms to calculate in situ attenuation rates
- Multiple samples are required to generate attenuation rates
- More commercial labs would be beneficial

ITRC EMD Tech Reg, Section 3 and Appendix C

No associated notes.

## Case Study A1: Distinguishing Perchlorate Sources with CSIA



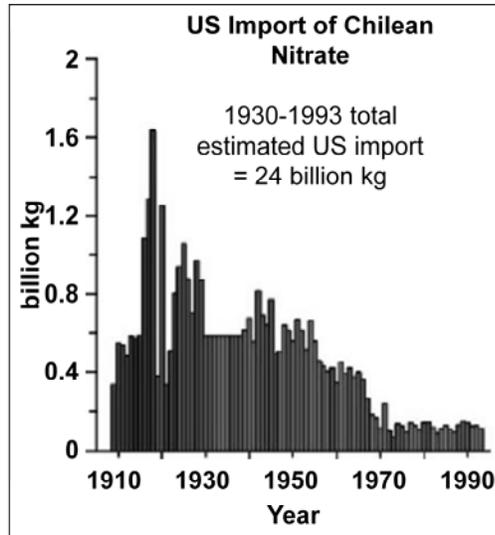
Source USEPA 2004. "National Perchlorate Detections as of September 23, 2004." Federal Facilities Restoration and Reuse Office. See also ITRC Perchlorate: Overview of Issues, Status, and Remedial Options (PERC-1, Sep-05).

Perchlorate is a solid oxidant that is widely used in rocket motors (e.g., space shuttle rocket boosters contain 380 tons each) as well as various other military items. Historically, perchlorate was considered to be a contamination issue unique to the military and aerospace industries with a limited number of potential sites.

With improved detection limits and required testing under the Unregulated Contaminant Monitoring Rule in 2001, it became apparent in the early 2000's that perchlorate in groundwater was much more prevalent than expected based on the previous paradigm? In many instances, there was no apparent correlation between perchlorate detected in groundwater and any military, aerospace, or manufacturing facility.

## Other Perchlorate Sources?

- ▶ Natural perchlorate
  - Chilean Caliche – Atacama Desert
    - Natural fertilizer
  - Mineral deposits and soils
    - Southwestern US
  
- ▶ Other synthetic sources
  - Fireworks
  - Road flares
  - Perchloric acid and salts
  - Chlorate (herbicide)
  - Chlorine bleach



Source: Figure 2. Reprinted with permission from Dasgupta, PK, JV Dyke, AB Kirk, and WA Jackson. 2006. Perchlorate in the United States. Analysis of Relative source contributions to the Food Chain. Environ. Sci. Technol. 40;6608-6614. Copyright 2006 American Chemical Society.

Source: Figure 2, Reprinted with permission from Dasgupta, PK, JV Dyke, AB Kirk, and WA Jackson. 2006. Perchlorate in the United States. Analysis of Relative source contributions to the Food Chain. Environ. Sci. Technol. 40;6608-6614. Copyright 2006 American Chemical Society.

Data references are included in Dasgupta et al 2006 – reference numbers 36, 38 and 39.

Several sources other than military/aerospace use of propellants are potentially important, including commercial synthetic sources and natural perchlorate derived from Chilean nitrate fertilizer or atmospheric production and accumulation. Perchlorate associated with Chilean nitrate fertilizer averaged 750 metric tons per year in imported materials from 1930 -1993 (Dasgupta et al). Natural perchlorate that has formed in the atmosphere and accumulated in dry areas of the Southwest is also a potential source – not going to be a focus of this case study.

## CSIA for Perchlorate

- ▶ Methods developed for analysis of both O and Cl isotopes in perchlorate ( $\text{ClO}_4^-$ )
- ▶ Analyzed by Isotope Ratio Mass Spectrometer (IRMS) with a precision of about  $\pm 0.1$  to  $0.5 \text{ ‰}$
- ▶ Special columns developed to collect perchlorate from groundwater

Elements in a compound can have widely different isotopic ratios based on mode of Formation. Stable isotope ratios can provide a unique “fingerprint”

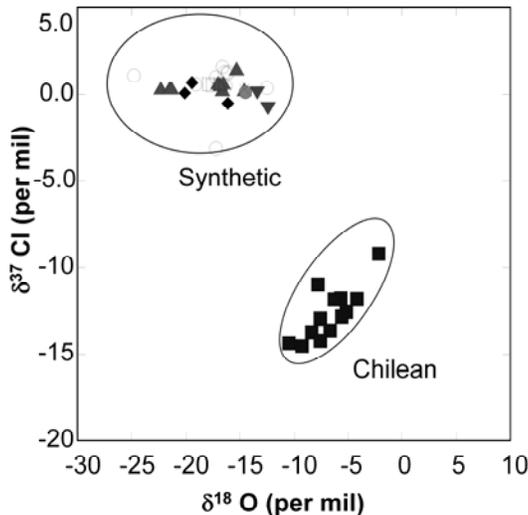


Hydrogen	$^1\text{H}$ , $^2\text{H}$
Oxygen	$^{16}\text{O}$ , $^{17}\text{O}$ , $^{18}\text{O}$
Carbon	$^{12}\text{C}$ , $^{13}\text{C}$
Chlorine	$^{35}\text{Cl}$ , $^{37}\text{Cl}$
Nitrogen	$^{14}\text{N}$ , $^{15}\text{N}$
Sulfur	$^{32}\text{S}$ , $^{34}\text{S}$

Methods have been developed to analyze both Cl and O isotopes in perchlorate with good precision. Oxygen has three stable isotopes ( $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ) and chlorine has two ( $^{35}\text{Cl}$ ,  $^{37}\text{Cl}$ ). The ratios of isotopes in compounds can vary widely based on their mode of formation, which is why stable isotope ratio analysis is sometimes useful for forensic evaluations.

## Forensic Isotopic Analysis: Chilean vs. Synthetic $\delta^{37}\text{Cl}$ and $\delta^{18}\text{O}$

Dual Isotope plot comparing  $\delta^{37}\text{Cl}$  and  $\delta^{18}\text{O}$  in  $\text{ClO}_4^-$  from synthetic and natural Chilean sources



Chlorine markedly "heavier" in synthetic perchlorate

- Laboratory Salts
- ▲ Military
- Herbicides
- Gunpowder
- ◆ Fireworks
- ▼ Road Flares
- × Taiwanese

Oxygen consistently "heavier" in natural (Chilean) perchlorate

- Natural (Chile)

Source: Paul Hatzinger, Ph.D.

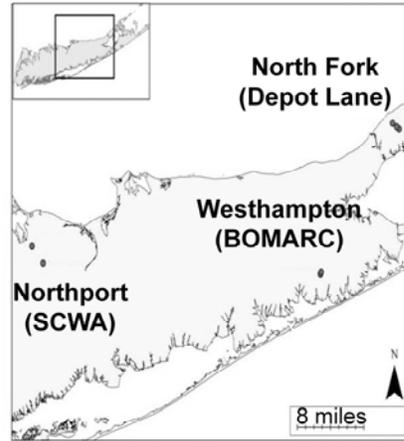
When measuring stable isotope ratios of Cl and O in source materials, all synthetic perchlorate samples (from military sources, flares, fireworks, lab reagents, and other sources) have very similar Cl isotope ratios, with a  $\delta^{37}\text{Cl}$  value very near 0 (similar to the seawater standard used for comparison). The  $\delta^{18}\text{O}$  for synthetic perchlorate varies more widely, presumably based upon the source water used to make perchlorate in an electrochemical cell (water is the source of oxygen in perchlorate manufacture). In contrast, perchlorate derived from Chilean materials (surface salts and imported fertilizers) has a much lower  $\delta^{37}\text{Cl}$  value, and a significantly heavier average  $\delta^{18}\text{O}$  value. When plotted on a dual isotope plot, these sources are clearly distinguishable based on their stable isotope values of Cl and O.

## Case Study: Long Island, NY

**7 Wells**  
**Depot Lane**  
**BOMARC**  
**SCWA**



**Column used to collect perchlorate for CSIA**



Map 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. *Environ. Sci. Technol.* 43: 5619-5625. Copyright 2009 American Chemical Society.

*Photo Source: Paul Hatzinger, Ph.D.*

ITRC EMD Tech Reg, Appendix A.1

Map 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. *Environ. Sci. Technol.* 43: 5619-5625. Copyright 2009 American Chemical Society.

This case study of perchlorate sources in groundwater was conducted on Long Island, NY. There have been a number of perchlorate detections on Long Island in monitoring and public supply wells, with unknown sources. This area has a long agricultural history, as well as potential commercial and military sources. Seven different wells with unknown sources of perchlorate were sampled on Long Island, NY. These included three wells in an agricultural area on the North Fork (Depot Lane), two wells at a former BOMARC missile site (BOMARC; land now used for training by the Suffolk County PD), and two large public supply wells in Northport owned by the Suffolk County Water Authority (SCWA). Perchlorate was collected by passing water through small ion exchange columns, to trap ~5-10 mg per well. A number of other supporting parameters were also collected in each well, including anions and cations, nitrate isotopes, groundwater age dating parameters, dissolved gases, and others. It is always important to utilize CSIA in conjunction with other supporting evidence in forensic investigations.

## Isotope Data, Long Island, NY

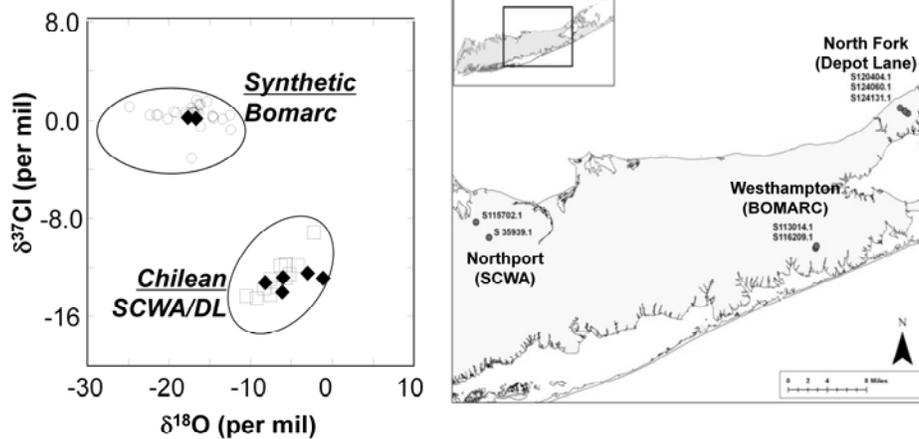


Figure and Map Source: Adapted with permission from Böhlke, J.K.; Hatzinger, P.B.; Sturchio, N.C.; Gu, B.; Abbene, I.; Mroczkowski, S.J. 2009. Atacama perchlorate as an agricultural contaminant in groundwater: Isotopic and chronologic evidence from Long Island, New York. Environ. Sci. Technol. 43: 5619-5625. Copyright 2009 American Chemical Society.

ITRC EMD Tech Reg, Appendix A.1

Figure and Map 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. Environ. Sci. Technol. 43: 5619-5625. Copyright 2009 American Chemical Society.

The isotope data clearly showed two distinct sources in the wells tested. Two of the wells had perchlorate with Cl and O isotope values that were indistinguishable from synthetic sources that has been previously analyzed. These wells, at the former BOMARC site, are believed to have been impacted by disposal of fireworks by the Suffolk County Police Department, who currently use this area as a training facility (and dispose of illegal fireworks there). The other 5 wells tested had perchlorate with isotopic signatures similar to that of perchlorate in Chilean nitrate fertilizers. Thus, the unknown source in this region appears to be past application of Chilean nitrate fertilizers. A large number of wells in California have also been shown in recent years to have perchlorate from this source using CSIA.

## Case Study A3: Distinguishing TCE Sources with CSIA

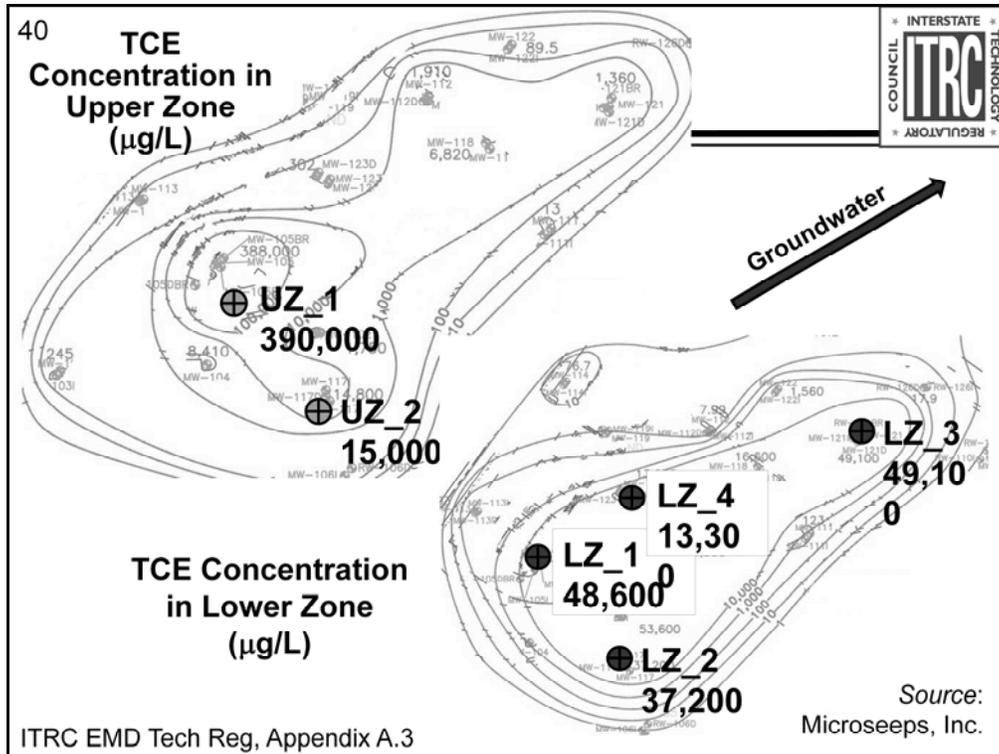


### New Jersey TCE Site

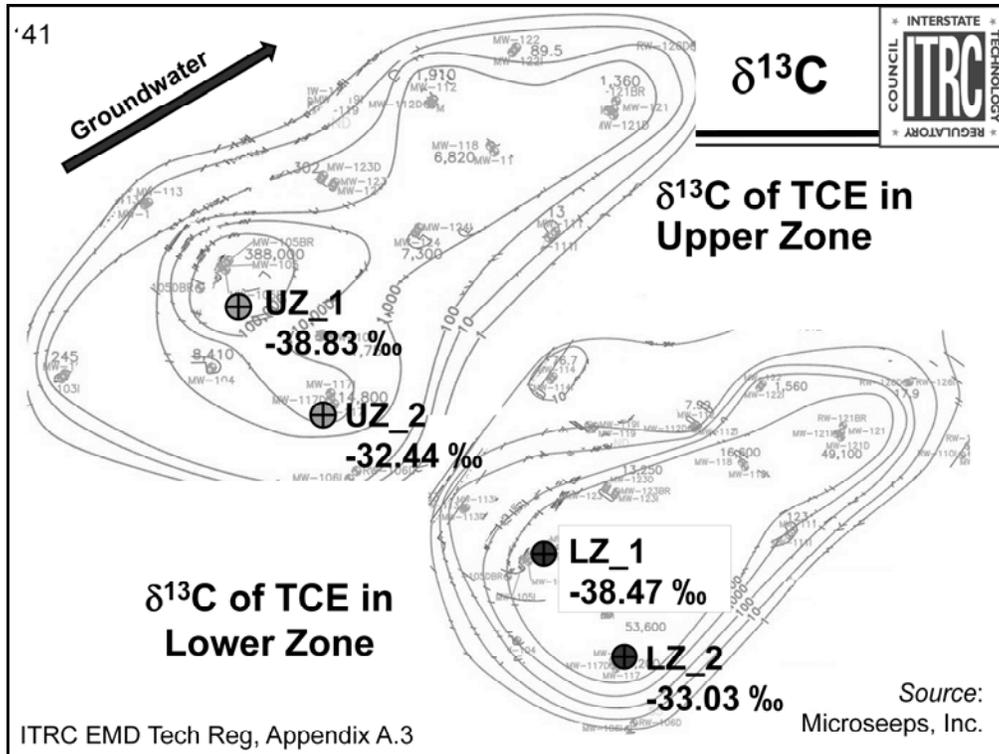
- ▶ Site has two distinct depth zones
  - Upper Zone (UZ) is overburden and mostly unconsolidated
  - Lower Zone (LZ) is bedrock
- ▶ TCE contours suggest two sources:
  - UZ appears to have sources at UZ\_1 and UZ\_2
  - LZ has 3 hot spots
    - LZ\_1
    - LZ\_2
    - LZ\_3

ITRC EMD Tech Reg, Appendix A.3

New Jersey Site. Stable isotope analysis of C in TCE was conducted to evaluate whether one or two sources were present at a NJ site.

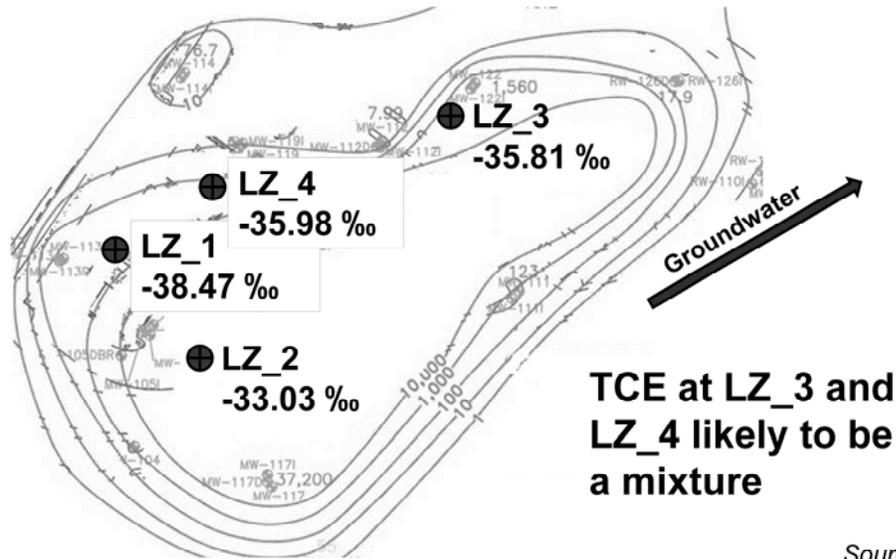


Well UZ\_1 is in an alluvial aquifer and is located above LZ\_1, which is screened in bedrock. Similarly, well UZ\_2 is screened in alluvium above bedrock well LZ\_2. Wells LZ\_3 and LZ\_4 are downgradient bedrock wells.



Stable isotope data are very similar for TCE in UZ\_1 and LZ\_1. The source appears to be the same in this region in alluvium and bedrock. The isotopic signature of C in TCE at UZ\_1 and LZ\_1 is also very negative. The stable isotope value of C in TCE at UZ\_2 and LZ\_2 is similar, but distinctly different than that at UZ\_1 and LZ\_1. The data suggest two distinct sources on this site, one affecting UZ\_1/LZ\_1 and one affecting UZ\_2/LZ\_2.

## TCE in Lower Zone: $\delta^{13}\text{C}$



ITRC EMD Tech Reg, Appendix A.3

Source:  
Microseeps, Inc.

The stable isotope ratios in LZ\_3 and LZ\_4 are between those of the two sources at LZ\_1 and LZ\_2, suggesting that the TCE in these wells is a mixture of the two sources.

Later site assessment work using a membrane interface probe (MIP) supported the CSIA results, showing two distinct sources. Determining whether two sources were present was critical for site planning and management decisions.

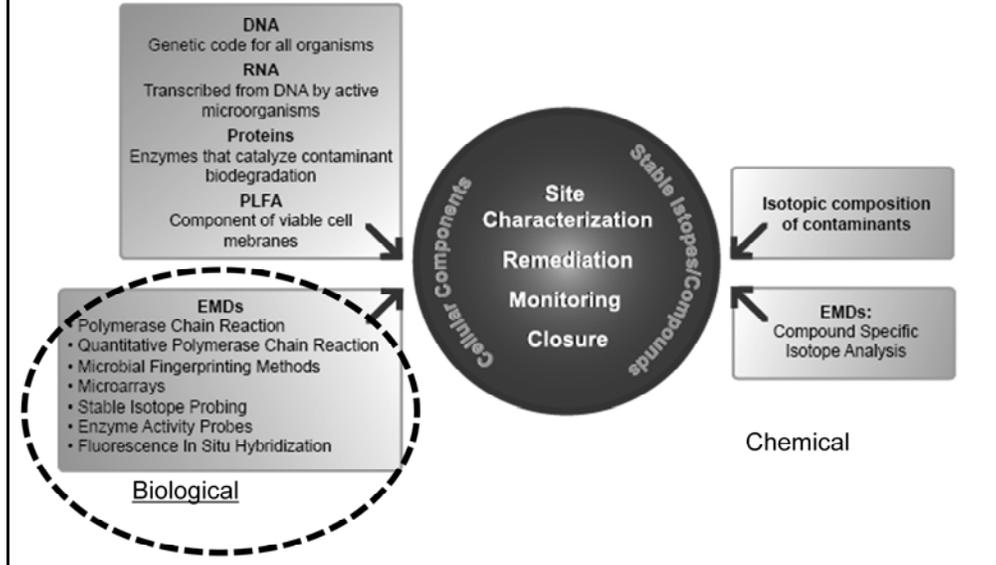
## Today's Training Outline



Training Topics	Tech Reg Sections	Training Progress
Introduction to Training and EMDs	1, 2, 11	
CSIA and Case Studies	3, App. C	
qPCR and Case Study	4, App A	
Question & Answer Break		
Biological EMDs	5-10, App. D	
Case Studies using Multiple EMDs	App. A	
Web-Based Tech Reg Navigation		
Question & Answer Break		

No associated notes.

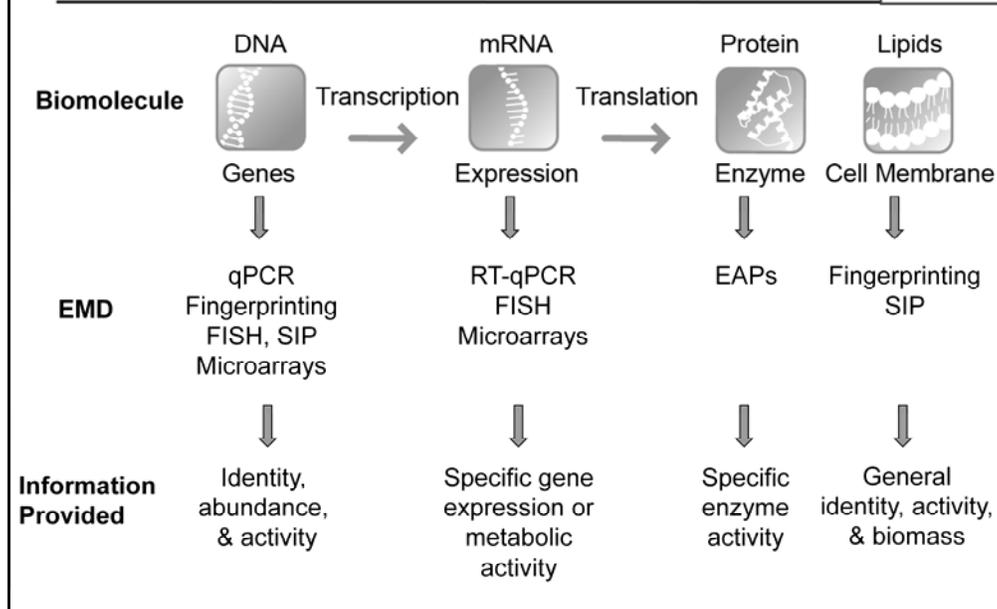
## EMD Descriptions & Use: Biological EMDs



EMDs can be classified into two major categories of analytical techniques: chemical techniques, in particular compound specific isotope analysis (CSIA), and a variety of molecular biological techniques (MBTs).

Biological EMDs provide information about the biodegradation of contaminants

## Cellular Components & Information



This figure depicts the biomolecules used in EMD methods, the EMD that uses that biomolecule, and information gained from the EMD analysis

DNA & RNA are nucleic acids that encode the production of proteins that enzymatically break down contaminants, but they are also biomolecules that can give some specific information

DNA can be used to identify microorganisms, and count their numbers in a sample. The EMDs that use DNA include qPCR, Fingerprinting methods, Stable isotope probing, Fluorescent In situ hybridization, and Microarrays.

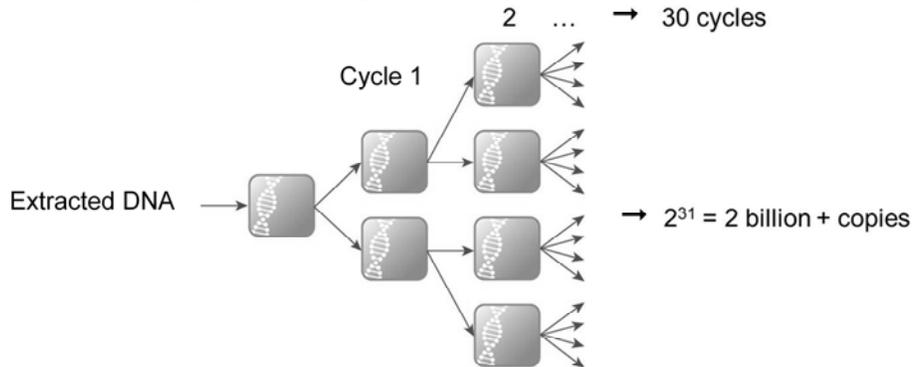
Analysis of mRNA can give information concerning the expression of certain genes, and thus some insight to specific metabolic activity

Proteins and lipids can be assessed to measure activity - proteins are the biological agents that are actually involved in contaminant biodegradation

Lipids can be used to identify major groups of microorganisms and assess the standing biomass of the microbial community at a site.

## Quantitative Polymerase Chain Reaction (qPCR)

### ► DNA amplification process



### ► Used to calculate the number of copies of specific DNA sequence (or gene) in a sample

ITRC EMD Tech Reg, Section 4

The most common biological EMD regulators will likely encounter is the polymerase chain reaction or PCR

PCR is a DNA amplification method; an automated laboratory method to create many copies of a specific fragment of DNA by repeated rounds of DNA replication

Through repeated rounds of DNA replication the target DNA is amplified to eventually produce millions to billions of copies of the target DNA.

Quantitative PCR (or qPCR) is a type of PCR that can relate the amplified DNA back to the number of copies in the original sample – thus determining the number of genes – or specific microorganisms – in a sample.

## Some Common qPCR Gene Targets



	Contaminants	Target Gene	Organism or Encoded Enzyme
Chlorinated Solvents	PCE, TCE, DCE, VC	16s rRNA	<i>Dehalococcoides mccartyi (Dhc)</i>
	TCE & DCE	<i>tceA</i>	TCE Reductase
	VC	<i>vcrA, bvcA</i>	Vinyl chloride Reductase
Petroleum	BTEX	<i>tmo</i>	Ring-hydroxylating Toluene Monooxygenases
	B, T, & Chlorobenzene	<i>tod &amp; tol</i>	Toluene Dioxygenase
	Naphthalene	<i>nah</i>	Naphthalene Dioxygenase
	Alkanes	<i>alk</i>	Alkane Monooxygenase
	MTBE	16s rRNA	MTBE-utilizing <i>Methylibium</i> spp
	Toluene & Xylene	<i>bss</i>	Benzylsuccinate Synthase (anaerobic)
	Perchlorate	<i>per</i>	Perchlorate reductase
	PCBs	<i>bph</i>	Biphenyl Dioxygenase

ITRC EMD Tech Reg, Appendix D, Tables D2 & D3

Contaminants are degraded by the actions of enzymes, and the genes that encode for the production of these enzymes are known and reported in the scientific literature.

qPCR tests are available for known gene sequences, and we can use them to screen for degradative genes in environmental samples.

This slide shows some common contaminants regulators may deal with at contaminated sites as well as the target gene that encodes the functional enzymes that's involved in their degradation.

Please Note that some gene targets are phylogenetic genes – typically the 16s rRNA gene of microorganisms used to infer phylogenetic relations and identity – while other targets are functional genes that encode for specific degradative enzymes, such as a reductase or an oxygenase.

## qPCR Benefits and Limitations



### ► Benefits

- **Confirms presence and determines abundance of target microbes and genes**
- Allows monitoring of microbes involved in bioremediation
- Readily commercially available for some key organisms and biodegradation-associated genes

### ► Limitations

- Infers biodegradation (potential or actual):
  - right microorganisms are present and in sufficient quantity for bioremediation to occur, *or*
  - changes in abundance or gene expression through time
- Need to know what you are looking for ahead of time

ITRC EMD Tech Reg, Section 4

qPCR confirms presence, but can also determine the abundance of target microorganisms or their particular genes involved in biodegradation & can be used as a monitoring tool to assess changes in microbial population growth & distribution

qPCR tests for degradative genes are commercially available

The presence of microorganisms – or particular genes – does not always mean they are active in contaminant degradation, but rather the potential is there.

Following changes in abundance of degradative genes through time provides strong inferential evidence that biodegradation is occurring.

Primer sets used in the PCR reaction are necessary to be known ahead of time.

## Case Study A4: DCE Stall Evaluation and Electron Donor Selection



### Site Background:

- ▶ Shallow aquifer impacted with chlorinated solvents
  - ▶ Detection of daughter products suggested reductive dechlorination
  - ▶ High DCE with low VC and ethene suggested cDCE stall
  - ▶ Geochemical parameters suggested mildly anaerobic conditions & high levels of sulfate (842 mg/L)
- ▶ Site Characterization study
    - 1) Control
    - 2) *BioStim A*, and
    - 3) *BioStim B*
  - ▶ qPCR on bead biofilms for: *Dhc*, *methanogens*, & *IRB/SRB*

**BioTrap™  
Sampling  
Device**



ITRC EMD Tech Reg, Appendix A.4

Source: Microbial Insights, Inc.

qPCR used to evaluate cis-DCE stall at a chlorinated solvent site, and help select an appropriate electron donor for groundwater remediation

An *in situ* bioremediation approach was sought to further reduce VOCs in groundwater, and attain site-closure goals

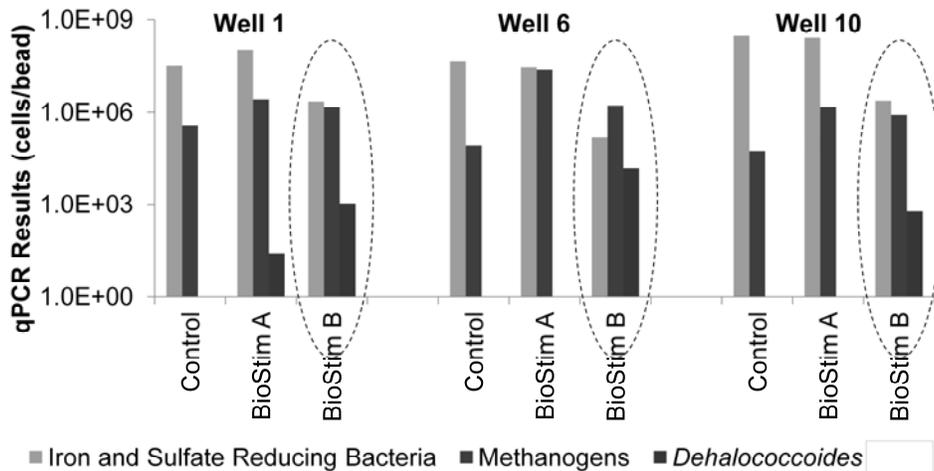
Chlorinated ethene daughter products in groundwater suggested some Reductive Dechlorination was occurring, but accumulation of cDCE with little detection of VC & Ethene suggested a classic cDCE stall.

During site characterization (Phase 1), some additional information was necessary to identify the most promising remediation (either MNA, Biostim A or Biostim B) – qPCR was used to identify microbes and genes involved in Reductive Dechlorination stimulated by amendment additions.

Each application involved the use of a BioTrap sampling device (some amended with biostimulants A or B, or some unamended) followed by qPCR using biofilm recovered from the sampling device beads.

Please see EMD Sampling Methods Fact Sheet (EMD-1) for more information on BioTrap samplers:  
<http://www.itrcweb.org/Guidance/GetDocument?documentID=32>

## Baited Bio-Trap Study Results



ITRC EMD Tech Reg, Appendix A.4

Source: Davis et al., 2008 Integrated Approach to PCE-Impacted Site Characterization, Site Management and Enhanced Bioremediation. Remediation. Autumn. Used with permission

Source: Davis et al., 2008 Integrated Approach to PCE-Impacted Site Characterization, Site Management and Enhanced Bioremediation. Remediation. Autumn. Used with permission

Results from 3 different wells during site characterization BioTrap study for amendment selection

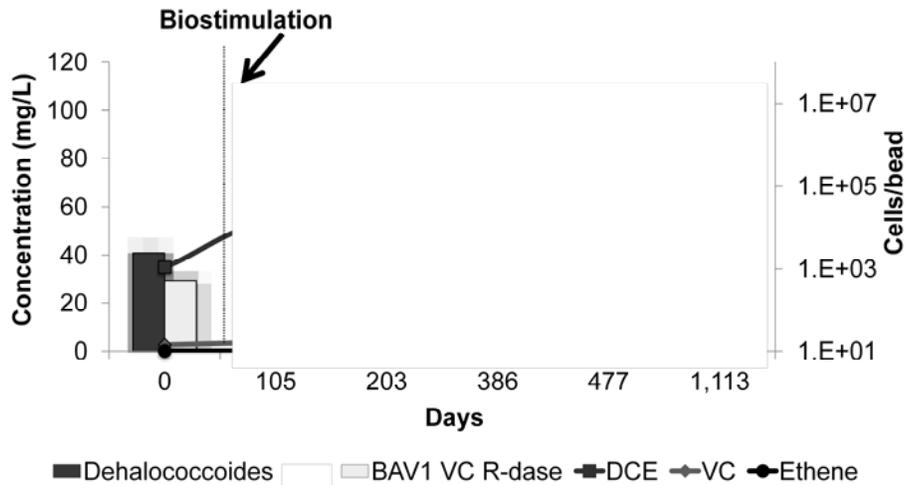
On unamended biotraps, Iron and sulfate reducers (light blue) were typically greater in numbers than methanogens (dk blue), reflecting the mildly reducing geochemical conditions in the aquifer.

Dhc populations were below the laboratory detection limit on control BioTraps, indicating complete reductive dechlorination was unlikely

Dhc responded best with Biostimulant amendment B, and did not respond to amendment A in all wells.

Based on qPCR results, both MNA and biostimulant donor A were eliminated as potential remediation strategies

## qPCR – Biostimulation & Remediation



ITRC EMD Tech Reg, Appendix A.4

Source: modified from Davis et al., 2008 Integrated Approach to PCE-Impacted Site Characterization, Site Management and Enhanced Bioremediation. Remediation. Autumn. Used with permission

Modified from: Davis et al., 2008 Integrated Approach to PCE-Impacted Site Characterization, Site Management and Enhanced Bioremediation. Remediation. Autumn. Used with permission.

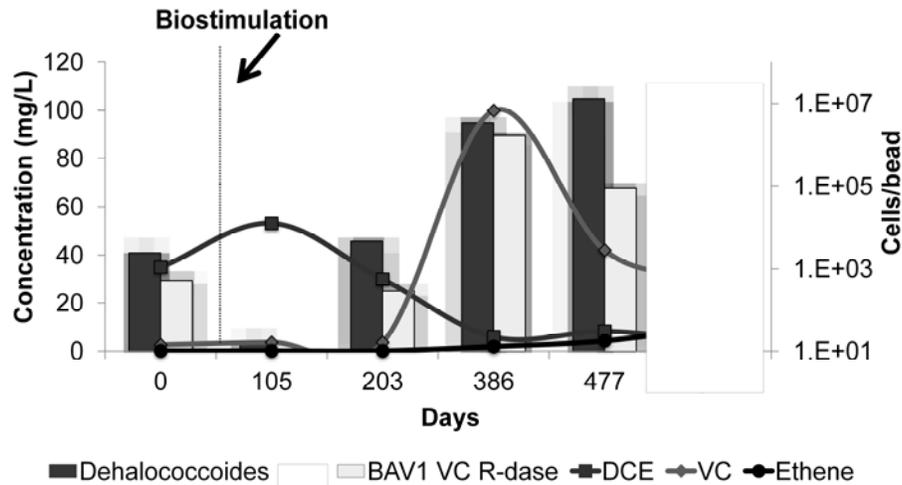
Next, a pilot injection of BioStimulant B was conducted near a single MW, followed by full-scale implementation as a remedy for the site.

The pilot test lasted 492 days, after which full-scale remediation followed

Results:

- Prior to injection (Day 0), *Dhc* populations were on the order of  $10^3$  cells/bead.

## qPCR – Biostimulation & Remediation



ITRC EMD Tech Reg, Appendix A.4

Source: modified from Davis et al., 2008 Integrated Approach to PCE-Impacted Site Characterization, Site Management and Enhanced Bioremediation. Remediation. Autumn. Used with permission

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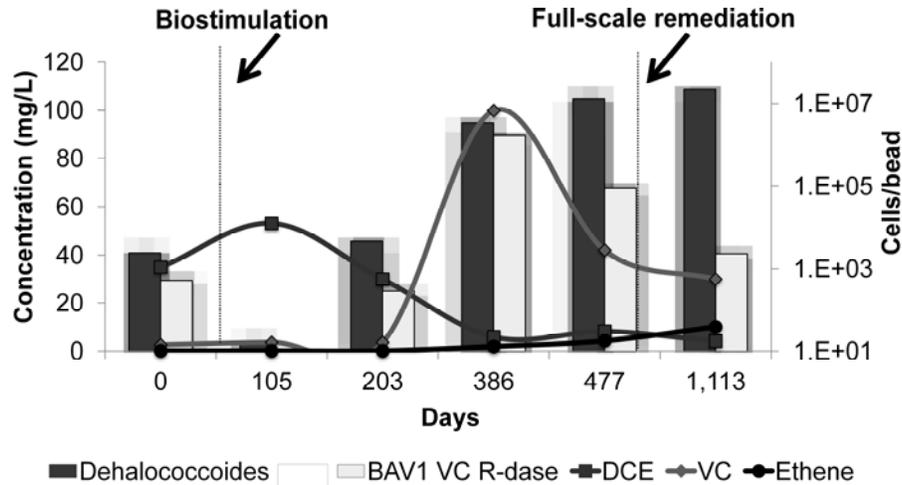
Results:

- Prior to injection (Day 0), *Dhc* populations were on the order of  $10^3$  cells/bead.
- During the first 105 days after injection, *Dhc* decreased to  $10^1$  cells/bead and cis-DCE conc. increased
- After Day 105, *Dhc* abundance returned to  $10^3$  cells/bead, and the cis-DCE conc. decreased by day 203
- After day 203 (at 386), cis-DCE conc. continued to decrease and vinyl chloride conc. temporarily increased due to increased reductive dechlorination of cis-DCE.

During this period, *Dhc* also increased to  $10^6$  to  $10^7$  cells/bead with corresponding increases in vinyl chloride reductase genes (BAV1 vcr)

- *Dhc* numbers maintained after 400 days, even when [VC] decreased (shown at day 477).

## qPCR – Biostimulation & Remediation



ITRC EMD Tech Reg, Appendix A.4

Source: modified from Davis et al., 2008 Integrated Approach to PCE-Impacted Site Characterization, Site Management and Enhanced Bioremediation. Remediation. Autumn. Used with permission

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The pilot test lasted 492 days, after which full-scale remediation followed

Results:

- Prior to injection (Day 0), *Dhc* populations were on the order of  $10^3$  cells/bead.
- During the first 105 days after injection, *Dhc* decreased to  $10^1$  cells/bead and cis-DCE conc. increased
- After Day 105, *Dhc* abundance returned to  $10^3$  cells/bead, and the cis-DCE conc. decreased by day 203
- After day 203 (at 386), cis-DCE conc. continued to decrease and vinyl chloride conc. temporarily increased due to increased reductive dechlorination of cis-DCE.

During this period, *Dhc* also increased to  $10^6$  to  $10^7$  cells/bead with corresponding increases in vinyl chloride reductase genes (BAV1 vcr)

- *Dhc* numbers maintained after 400 days, even when [VC] decreased (shown at day 477).

During full-scale remediation, Biostim B was injected in the source zone and in downgradient areas of the dissolved plume

- *Dhc* numbers were maintained past 1000 days

## Key Results from qPCR

- ▶ Site characterization phase
  - *Dhc* populations ND; complete reductive dechlorination unlikely
- ▶ Baited bio-trap and pilot study
  - electron donor B stimulated growth of *Dhc* & promoted reductive dechlorination
- ▶ Pilot study & remedial monitoring
  - Extended lag prior to reductive dechlorination - temporary increase in methanogens
  - rebound of *Dhc* and vinyl chloride reductase genes (*bvcA*) - complete reductive dechlorination expected
- ▶ **Final Conclusion**
  - qPCR results accepted as a valuable line of evidence
  - site was reclassified to “**No Further Action Required**” status

ITRC EMD Tech Reg, Appendix A.4

Site characterization, qPCR showed *Dhc* was ND and complete Reductive Dechlorination was unlikely; qPCR also showed electron donor B stimulated the growth of *Dhc* and promoted Reductive Dechlorination

Pilot study, qPCR showed an extended lag phase for growth of *Dhc* populations, a temporary increase in MGNs, and a rebound of *Dhc*, its VC-reductase genes, and an increase in Reductive Dechlorination –

Remedial Action: complete Reductive Dechlorination during full scale remediation

qPCR cost-effectively identified the corrective action which led to the site being reclassified to No Further Action required

## Question & Answer Break

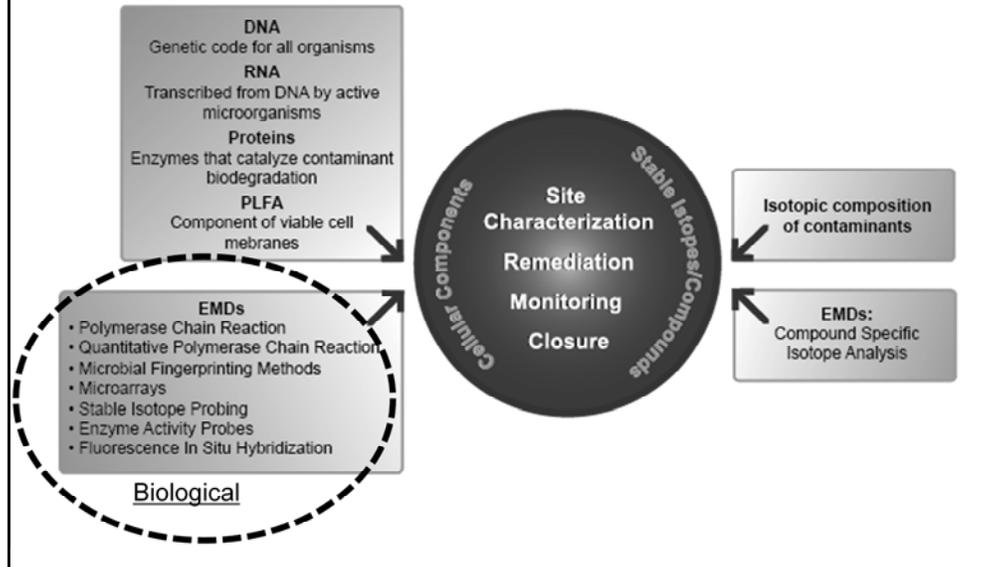
Follow ITRC



Training Topics	Tech Reg Sections	Training Progress
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qPCR and Case Study	4, App A	
Question & Answer Break		
Biological EMDs	5-10, App. D	
Case Studies using Multiple EMDs	App. A	
Web-Based Tech Reg Navigation		
Question & Answer Break		

Question and answer break.

## EMD Descriptions & Use: Biological EMDs

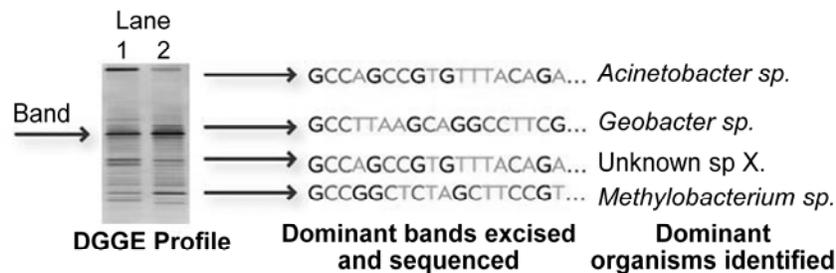


Continuing with descriptions of biological EMDs - or molecular biological tools...

## Microbial Fingerprinting Methods



- ▶ Provides overall “fingerprint” of microbial community composition
- ▶ Variety of laboratory techniques
  - Differentiate microorganisms based on unique characteristics of universal biomolecules
  - DNA or RNA methods – **DGGE, T-RFLP**
  - Phospholipid fatty acids analysis - **PLFA**
- ▶ Some can identify subsets of microorganisms
- ▶ Some can quantify living biomass



ITRC EMD Tech Reg, Section 5

Source: Microbial Insights, Inc.

Microbial Fingerprinting methods understand microbial community make-up without fully to species level

For example, microbial fingerprinting methods can identify a group microorganisms, such as sulfate reducers, that may be involved in biodegradation

Three methods profiled in the Tech Reg include: denaturing gradient gel electrophoresis & terminal restriction fragment length polymorphism, (both using DNA), and Phospholipid fatty acid analysis (using cell membrane lipids).

The figure shows the bands on a denaturing gradient gel (a method to separate DNA sequences) that correspond to DNA of a particular groups of microorganisms, who's identity could be further explored through DNA sequencing.

Fingerprint from restriction enzyme digest

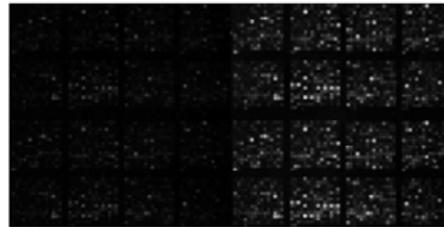
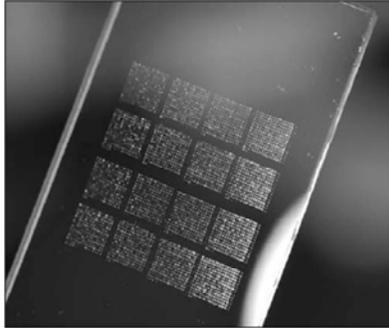
Fingerprint from PLFA of membrane lipids from cells

All generate a community profile - or fingerprint

Use this method as a monitoring tool to follow changes in microbial community profiles

## Microarrays

- ▶ **Comprehensive microbial evaluation**
- ▶ **Simultaneously detects hundreds to thousands of genes**
  - Phylogenetic microarray – who is there?
  - Functional gene microarray – what can they do?
- ▶ Can be performed with RNA to assess microbial activity



Time 1

Time 2

ITRC EMD Tech Reg, Section 6

Source: Frank Loeffler, Ph.D.

Source: Frank Loeffler, Ph.D., University of Tennessee, used with permission

Microarrays allow detection or tracking a large quantity & variety of genes simultaneously – for example hundreds to thousands of genes at a time – allowing for a more comprehensive look at the community all at once.

Basically a slide with ss DNA, that's washed with a sample, & if that sample contains complementary DNA, it will bind and fluoresce & the whole slide can be scanned & read by a computer

A commercially-available microarray contains up to 45,000 genes involved in biogeochemical reactions as well as biodegradation activities. Other commercially available microarrays are also available.

Microarrays can track large numbers of individual genes or organisms within a community over time, and assess changes in community structure (shown in T1 & T2, above)

Can also assess changes in activity - as gene expression - over time, by using RNA instead of DNA in the method

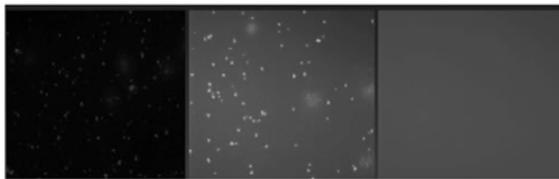
Microarrays are also customizable

## Enzyme Activity Probes (EAPs)



- ▶ **Detects and quantifies specific activities of microorganisms**
- ▶ **Estimates biodegradation activity**
- ▶ **Surrogate compounds**
  - Resemble contaminant of concern
- ▶ **Transformation of surrogate**
  - Signal in cell is detected by microscopy or spectrophotometry
- ▶ **Laboratory test or field-applicable with Push-Pull tests**

Enzyme Activity Probes: Micrographs



DAPI-  
total cells

Enzyme probes  
-positive  
response

Enzyme probes  
-negative  
response

ITRC EMD Tech Reg, Section 8

Source: M. H. Lee

Source: M. H. Lee, PNNL, used with permission.

EAP method exposes surrogate compounds – or compounds that resemble contaminants of concern – to elicit the actions of degradative enzymes in the same manner as the presence of contaminants.

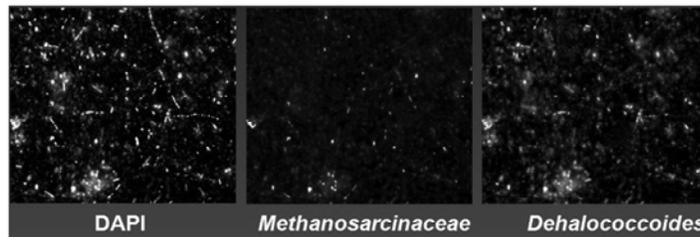
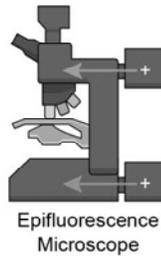
When combined with a fluorescent label, cells become fluorescently labeled when the enzyme reacts with the substrate and we can observe or detect enzyme activity with epifluorescent microscopy or spectrophotometry – **this method Provides direct evidence of the activity of enzymes involved in biodegradation of contaminants.**

When using microscopy (as shown in photomicrographs), this method is a Direct and quantitative measure of enzymatic activity, that can distinguish active cells (cells with active enzymes) from total cells and inactive cells & can be used estimate biodegradation rates.

EAPs can be used in the laboratory, but can also be applied to field sites with a common push-pull test with monitoring wells.

## Fluorescence In Situ Hybridization (FISH)

- ▶ Estimates number and relative activity of specific microorganisms or microbial groups
- ▶ Detects presence of targeted genetic material in whole cells
- ▶ Assesses spatial distributions



ITRC EMD Tech Reg, Section 9

Source: M. H. Lee

Source: M. H. Lee, PNNL, used with permission.

Fluorescence In Situ Hybridization – or FISH uses microscopy to assess the presence and activity of specific microorganisms

This method relies on the hybridization or binding of complementary sequences of DNA or RNA – where a fluorescently-labeled gene sequence (as a marker or PROBE) is hybridized with the genetic material inside a whole cell, and viewed using epifluorescent microscopy.

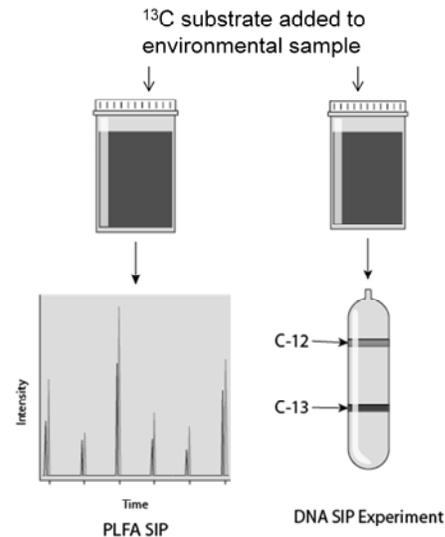
Direct counts of microorganisms carrying specific genes can be measured to determine the presence, abundance and in some case the activity of organisms of interest.

Microscopy also provides visual information of the spatial distribution of microorganisms, and can allow for biomass determinations.

## Stable Isotope Probing (SIP)

### ► Demonstrates occurrence of biodegradation

- Contaminant containing a stable isotope label (e.g.,  $^{13}\text{C}$ )
  - If biodegradation is occurring, the isotope is detected in:
    - biomolecules (DNA, phospholipids)
    - degradation products ( $\text{CO}_2$  or  $\text{CH}_4$ )
- 
- DNA-SIP is capable of identifying microorganisms responsible for biodegradation
  - PLFA-SIP very sensitive, but lacks phylogenetic resolution



ITRC EMD Tech Reg, Section 7

SIP involves the use of stable isotopes as a tracer to follow the uptake or assimilation of the stable isotope into bio-molecules such as DNA or cell membrane lipids.

Stable isotopes that originally came from the contaminant molecules taken up into bio-molecules, **thus demonstrating the occurrence of biodegradation.**

Those stable isotopes can also be manifested in cellular degradation products, such as carbon dioxide and methane.

Our figure is showing an environmental sample being exposed to substrate (or contaminant) that contains a known quantity of stable isotope – typically  $^{13}\text{C}$

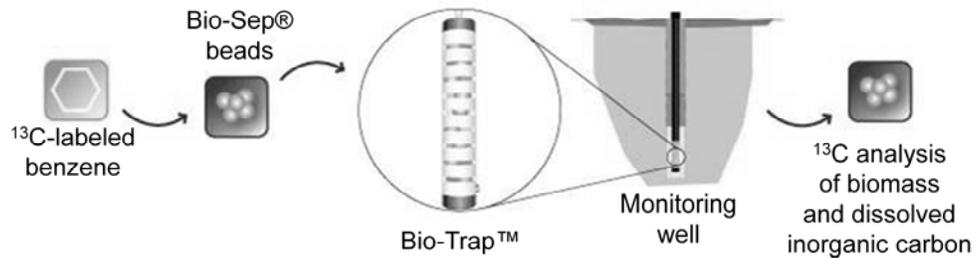
separate the heavy DNA isotopic fraction from the lighter DNA isotopic fraction with density gradient centrifugation – **shown in the figure on the right**) and analyze the labeled DNA by a number of methods to ID microorganisms.

Or extract the PLFAs from cell membranes and analyzing to identify uptake of  $^{13}\text{C}$  into membrane lipids, and to identify the group of microorganisms involved in its uptake (**shown in figure on left**).

PLFA - SIP is limited to identifying only major groups of microorganisms; DNA methods can identify to the species and isolate level.

## Field Application of SIP

- ▶ Involves passive sampling devices with labeled contaminant molecules



ITRC EMD-1, Sampling Devices Fact Sheet

Source: Microbial Insights

Source: Microbial Insights, used with permission

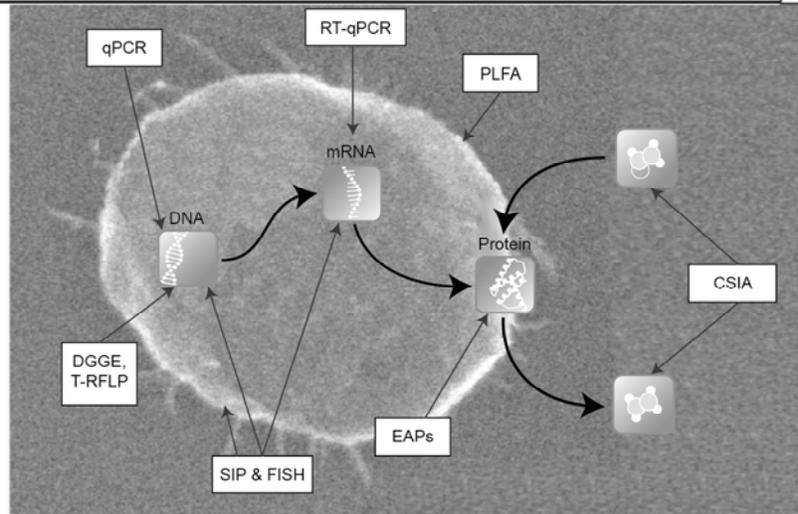
A variation of a stable isotope probing analysis involves the use of passive sampling devices – in this case the BioTrap sampling device mentioned previously – to measure biodegradation in-situ at a field site – in this figure, benzene biodegradation is explored.

This method provides direct evidence of biological degradation of contaminants and requires no prior knowledge of the microorganisms, genes, or enzymes involved in a specific biodegradation process

The analysis at the end of diagram can be DNA-based or involve the analysis of PLFAs.

**This method is very powerful to identify if biodegradation is occurring in-situ at your site.**

## Summary of EMDs and Targeted Cellular Components



*Dehalococcoides mccartyi*

Photomicrograph Source: Reprinted by permission from Macmillian Publishers Ltd: Nature. He et al, 2003. He, J., Ritalahti, K. M., Yang, K.-L., Koenigsberg, S. S. & Loeffler, F. E. (2003). Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 424, 62–65. Copyright 2003. <http://www.nature.com/nature/index.html>

Photomicrograph Source: Reprinted by permission from Macmillian Publishers Ltd: Nature. He et al, 2003. He, J., Ritalahti, K. M., Yang, K.-L., Koenigsberg, S. S. & Loeffler, F. E. (2003). Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 424, 62–65, Copyright 2003. <http://www.nature.com/nature/index.html>

This figure shows *Dehalococcoides mccartyi* – the only microbe known to completely transform PCE into ethene (formerly known as Dhc ethenogenes)

But it also shows, as a summary, where each EMD functions and the cellular components that are targeted by EMD methods (shown with the blue arrows).

## Proper Application of EMDs



- ▶ Traditional site characterization data (contaminant concentrations, geochemistry, etc.) still needed, EMDs complement
- ▶ Laboratory microcosms or cultivation studies may be needed for some sites, EMDs provide additional information
- ▶ Proper EMD selection depends on question being asked (see Table 2.3 in Tech Reg)
- ▶ Some EMDs provide inferential instead of definitive information

Best used to supplement traditional data, as  
**additional lines of evidence**

Some concepts of proper EMD applications

- EMDs should complement (and not replace) traditional SC data
- EMDs provide additional information – but laboratory microcosms or cultivation studies still may be needed to understand biodegradation
- Selecting the proper EMD depends on the question being asked – see Table 2.3
- Some EMDs provide inferential data while some provide more definitive data

EMDs are best used as **additional lines of evidence** to supplement traditional data that's typically collected at a contaminated site

## Today's Training Outline



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qPCR and Case Study	4, App A	
Question & Answer Break		
Biological EMDs	5-10, App. D	
Case Studies using Multiple EMDs	App. A	
Web-Based Tech Reg Navigation		
Question & Answer Break		

No associated notes.

## Case Studies – Multiple EMDs



No.	Env. Medium	Contaminants	Life Cycle	State
<b>CSIA</b>				
✓ A.1	Groundwater	Perchlorate	Site Characterization	NY
A.2	Vapor Intrusion	PCE, TCE, DCE	Site Characterization	CA
✓ A.3	Groundwater	TCE	Site Characterization	NJ
<b>qPCR</b>				
✓ A.4	Groundwater	PCE, TCE, DCE	Remediation	NY
→ A.5	Groundwater	PCE, TCE, DCE	Remediation	CA
<b>RT-qPCR</b>				
A.6	Groundwater	BTEX and MTBE	Remediation	CA
<b>EAP</b>				
A.7	Groundwater	TCE	Remediation	KY
<b>SIP</b>				
→ A.8	Groundwater	TCE, 1,4-dioxane	Remediation	AZ
A.9	Groundwater	Fuel oil compounds	Remediation	NJ
<b>Microarrays</b>				
A.10	Groundwater	Uranium	Remediation	CO

ITRC EMD Tech Reg Appendix A

This table in Appendix A shows all of the case studies and includes the complementary EMDs used at case studies.

Each method section in the Tech Reg includes short application summaries with paper citations for more case studies.

67 **Case Study A5: qPCR and CSIA to Evaluate Culture Distribution and Confirm Degradation**



- ▶ Seal Beach Naval Weapons Station
- ▶ ESTCP Project ER-0513
- ▶ Demonstration area had TCE concentrations as high as 190 mg/L
- ▶ Compared passive and active bioaugmentation



ITRC EMD Tech Reg, Appendix A.5

Site photo courtesy of J. Trotsky, US Navy.

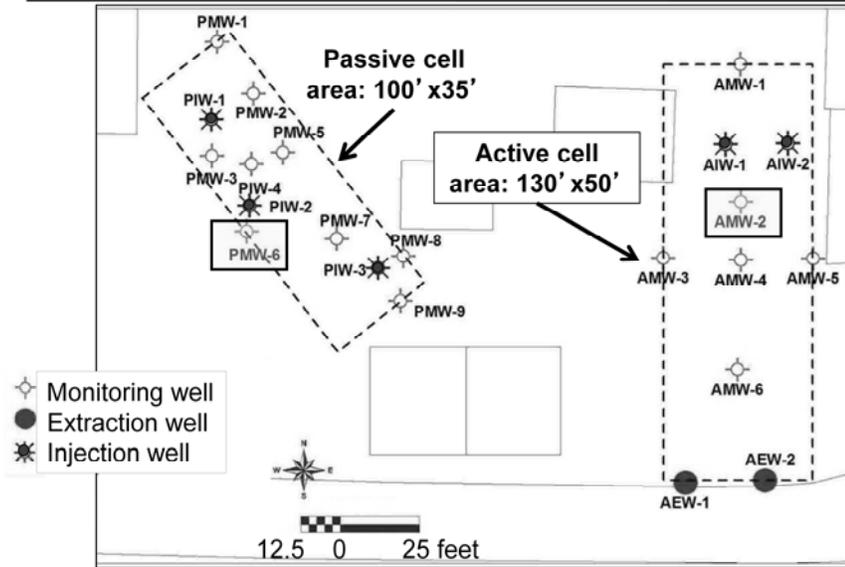
Site photo courtesy of J. Trotsky, US Navy.

Web location for Environmental Security Technology Certification Program (ESTCP) project reports:  
[www.serdp.org](http://www.serdp.org)

Passive = Injection wells located throughout treatment area and microbial culture is distributed via ambient groundwater flow

Active = Recirculation system and culture is distributed via forced advection

# Demonstration Layout



ITRC EMD Tech Reg, Appendix A.5

Source: ESTCP Project ER-200513

Source: Figure 5-1 of the Final Report for A Low-Cost, Passive Approach for Bacterial Growth and Distribution for Large-Scale Implementation of Bioaugmentation (ESTCP Project ER-200513)

## Use of EMDs

---



- ▶ qPCR was used to
  - Determine if *Dhc* existed at the site and select an appropriate bioaugmentation culture
  - Evaluate bacterial growth and distribution throughout both treatment cells
- ▶ CSIA was used to
  - Determine the extent of dechlorination in both treatment cells during the demonstration period

ITRC EMD Tech Reg, Appendix A.5

No associated notes.

## Project Approach

- ▶ Aquifer pre-conditioned
- ▶ Pre-bioaugmentation monitoring
  - *Dhc* detected, but *Dhc* functional gene *vcrA* was not detected
- ▶ Performed bioaugmentation using microbial culture containing the *vcrA* gene
- ▶ *vcrA* was used as a “tracer”
- ▶ Continued electron donor addition
  - Active cell: Weekly injections
  - Passive cell: Monthly injections

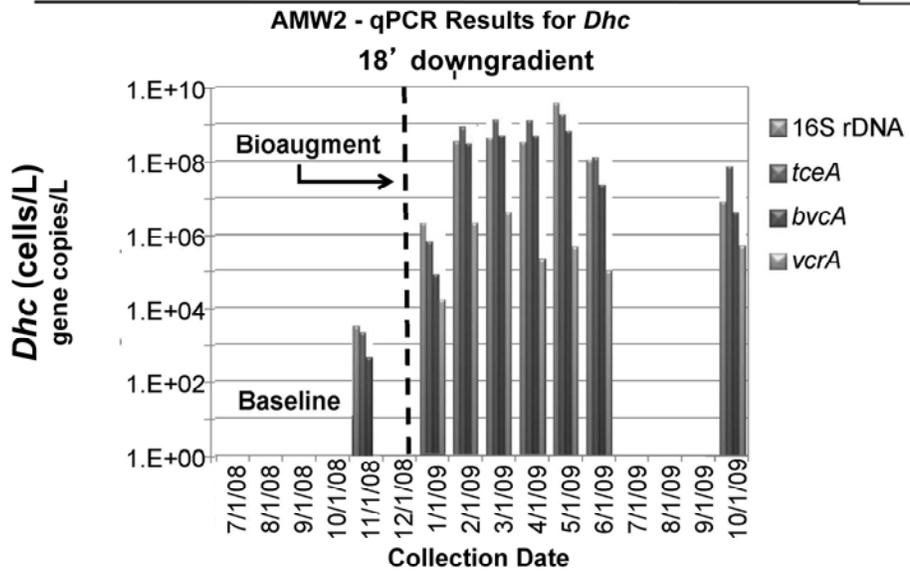


ITRC EMD Tech Reg, Appendix A.5

Source: Final Report for  
ESTCP Project ER-200513

Source: Final Report for ESTCP Project ER-200513,

# qPCR Results Show Culture Distribution in Active Cell

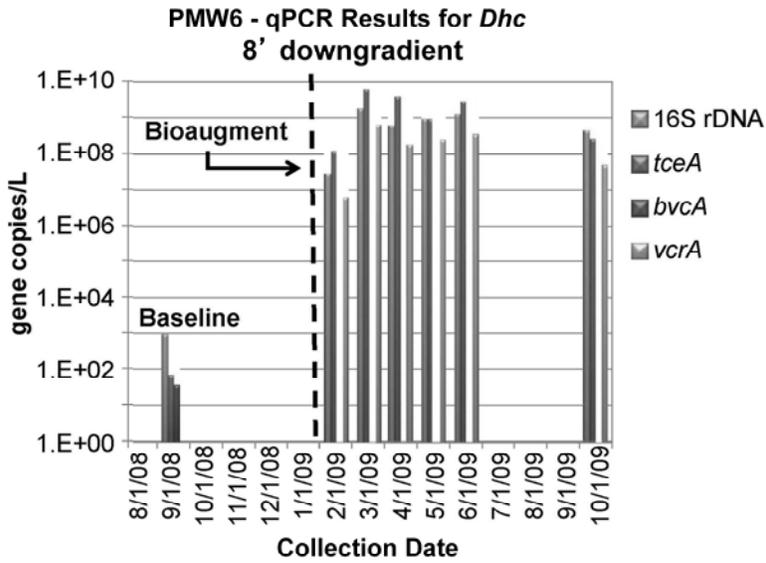


ITRC EMD Tech Reg, Appendix A.5

Source: ESTCP Project ER-200513

Source: Final Report for ESTCP Project ER-200513

# qPCR Results Show Culture Distribution in Passive Cell



ITRC EMD Tech Reg, Appendix A.5

Source: ESTCP Project ER-200513

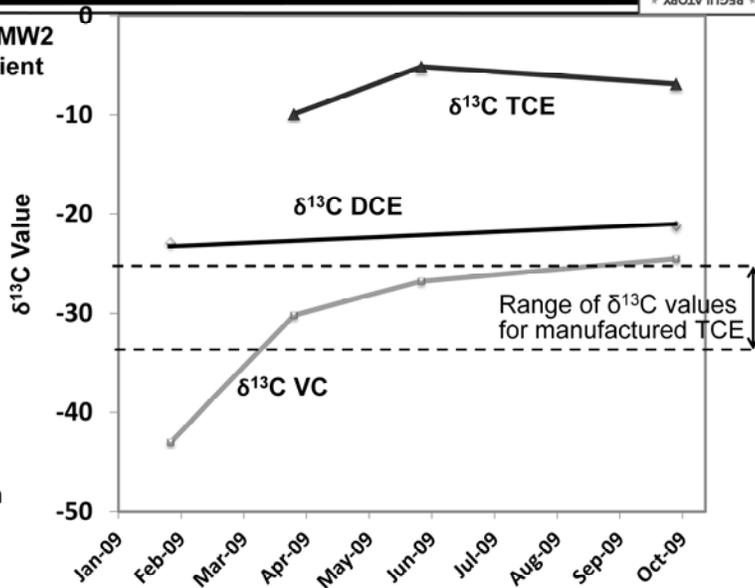
Source: Final Report for ESTCP Project ER-200513

# CSIA Results Indicate Degradation



**Active Cell - AMW2  
18' downgradient**

- ▶ Data indicate TCE is substantially degraded
- ▶ *cis*-DCE and VC values becoming heavier indicating they are being degraded
- ▶ CSIA data consistent with VOC and ethene data



ITRC EMD Tech Reg, Appendix A.5

Source: ESTCP Project ER-200513

Source: Final Report for ESTCP Project ER-200513

[www.serdp.org](http://www.serdp.org)

## Conclusions

---



- ▶ qPCR results showed
  - *Dhc* was distributed similar distances from injection points in both treatment cells, and populations were sustained
  - Better overall distribution throughout the treatment cell was achieved using the passive approach
- ▶ CSIA results
  - Were consistent with VOC trends
  - Indicated TCE, c-DCE, and VC were degrading

ITRC EMD Tech Reg, Appendix A.5

No associated notes.

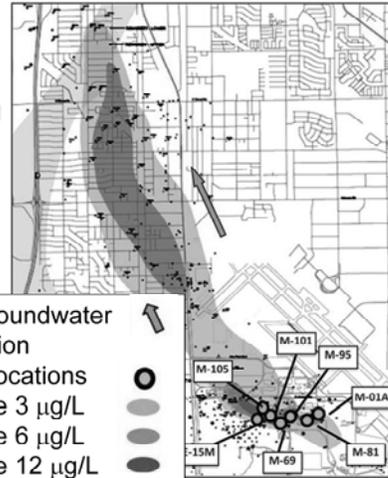
## Case Study A8: Use of Multiple EMDs to Evaluate MNA Potential for TCE and 1,4-Dioxane

- ▶ Air Force Plant 44, Tucson, AZ
- ▶ 6-mile long plume
  - TCE
  - 1,4-dioxane
- ▶ Plumes appear to be shrinking/attenuating
- ▶ 1987 – Present: Pump and Treat
- ▶ Operation for >50 more years
- ▶ MNA & bioremediation are being explored



Photo: Courtesy of the US Air Force

### 1,4-Dioxane Plume



General groundwater  
flow direction

Sampling locations

1,4-dioxane 3 µg/L

1,4-dioxane 6 µg/L

1,4-dioxane 12 µg/L

Source: Chiang, S.D., R. Mora, W. H. Diguseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." *Journal of Environmental Monitoring*, 14, 2317-2326, 2012. Reproduced by permission of The Royal Society of Chemistry. <http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b>

ITRC EMD Tech Reg, Appendix A.9

Photo: Courtesy of the United States Air Force.

Source: Chiang, S.D., R. Mora, W. H. Diguseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." *Journal of Environmental Monitoring*, 14, 2317-2326, 2012. Reproduced by permission of The Royal Society of Chemistry.

<http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b>

TCE historic max = 1,100 ug/L

Dioxane historic max = 7,000 ug/L

P&T = 2,400 gpm system

## Approach Using EMDs

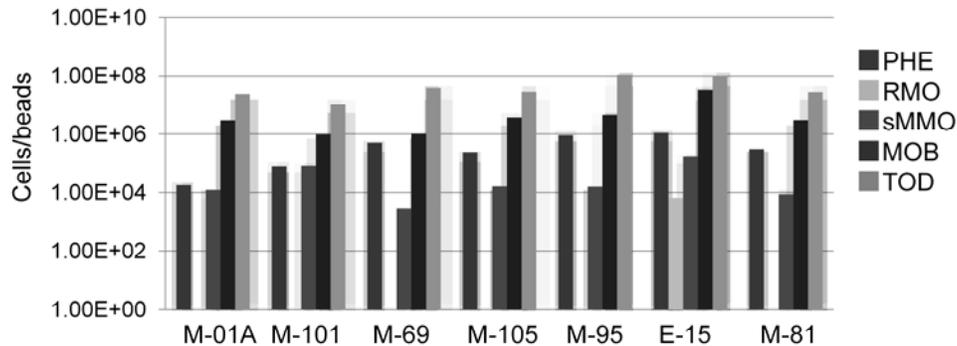


Step	Objective/Question Answered	Measurements
1	Geochemical conditions?	Field measurements Geochemical parameter analyses
2	Contaminant-degrading bacteria and enzymes <u>present</u> ?	<b>qPCR :</b> <ul style="list-style-type: none"> <li>• Methanotrophs (MOB)</li> <li>• Soluble methane monooxygenase (sMMO)</li> <li>• Toluene monooxygenases (PHE and RMO)</li> <li>• Toluene dioxygenase (TOD for TCE only)</li> </ul>
3	TCE and/or 1,4-dioxane being aerobically degraded?	<b>Compound Specific Isotope Analysis (CSIA)</b> for TCE  <b>Stable Isotope Probing (SIP)</b>
4	Contaminant-degrading enzymes metabolically <u>active</u> ?	<b>Enzyme activity probes (EAPs)</b>

ITRC EMD Tech Reg, Appendix A.9

No associated notes.

## qPCR Results Indicate Presence



- ▶ Targets were present and abundant in all wells
- ▶ sMMO and MOB were abundant even though methane <math><0.5 \mu\text{g/L}</math>
- ▶ PHE, RMO, and TOD were present and abundant even though toluene was not present

Source: Chiang, S.D., R. Mora, W. H. Diguseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." *Journal of Environmental Monitoring*, 14, 2317-2326, 2012. Reproduced by permission of The Royal Society of Chemistry. <http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b>

ITRC EMD Tech Reg, Appendix A.9

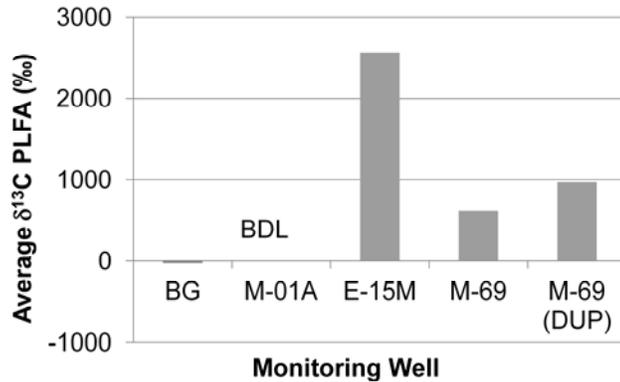
Source: Chiang, S.D., R. Mora, W. H. Diguseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." *Journal of Environmental Monitoring*, 14, 2317-2326, 2012. Reproduced by permission of The Royal Society of Chemistry.

<http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b>

## SIP Results Show Incorporation into Biomass



### 1,4-Dioxane SIP Traps



- ▶  $^{13}\text{C}$  incorporation into biomass occurred in three out of four traps
- ▶ High  $^{13}\text{C}$  dioxane incorporation into biomass at E-15M suggests the use of dioxane as a growth supporting substrate

Source: Chiang, S.D., R. Mora, W. H. Diguseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." *Journal of Environmental Monitoring*, 14, 2317-2326, 2012. Reproduced by permission of The Royal Society of Chemistry. <http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b>

ITRC EMD Tech Reg, Appendix A.9

Source: Chiang, S.D., R. Mora, W. H. Diguseppi, G. Davis, K. Sublette, P. Gedalanga, and S. Mahendra. "Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools." *Journal of Environmental Monitoring*, 14, 2317-2326, 2012. Reproduced by permission of The Royal Society of Chemistry.

<http://pubs.rsc.org/en/content/articlelanding/2012/em/c2em30358b>

## CSIA Results Serve as Baseline



- ▶ CSIA groundwater sampling before SIP trap deployment
- ▶ CSIA data used to establish baseline isotopic fractionation and evaluate whether TCE degradation has occurred
- ▶  $\delta^{13}\text{C}$  of undegraded TCE ranges from  $\sim -24$  to  $-34$  (EPA, 2008)
- ▶ Comparison between wells shows a difference of up to 3 ‰
- ▶ Even robust aerobic co-metabolism of TCE results in small shifts in  $\delta^{13}\text{C}$

Loc ID	M-101	E-15M	E-15M (DUP)	M-69	M-69 (DUP)
Conc ( $\mu\text{g/L}$ ) Feb 2009	330	110	110	390	390
$\delta^{13}\text{C}$ (‰)	-25.12	-25.22	-25.55	-28.02	-27.92

ITRC EMD Tech Reg, Appendix A.9

CSIA results do not indicate degradation, but are useful as a baseline.

## EAP Results Indicate Enzymes Are Active

Units = cells per milliliter



Sample	Probes for Toluene Oxygenases (PHE, RMO, TOL, TOD)				Probe for sMMO
	PA	3-HPA	CINN	3EB	Coumarin
M-69	-	1.05E+04	-	-	15.22
M-69	8.21E+03	1.25E+04	-	-	-
M-01A	2.54E+04	-	2.14E+04	8.12E+03	-
M-81	2.15E+04	2.04E+04	-	-	42.11
M-105	2.68E+04	2.21E+04	1.12E+04	-	-
M-101	3.54E+04	-	-	-	-
M-95	2.45E+04	-	1.42E+04	-	-

- ▶ At least one positive result in all wells
- ▶ Enzymes are active and intrinsic aerobic biodegradation is occurring

ITRC EMD Tech Reg, Appendix A.9

No associated notes.

## Conclusions

---



- ▶ qPCR results showed presence and abundance
- ▶ CSIA results did not confirm TCE degradation, but did serve as a baseline
- ▶ Incorporation of  $^{13}\text{C}$  into biomass suggests 1,4-dioxane may serve as a growth supporting substrate
- ▶ EAP results confirmed that enzymes are active

Multiple EMDs and lines of evidence showed that MNA can be considered as part of the site remedial strategy

No associated notes.

## Today's Training Outline



Training Topics	Tech Reg Sections	Training Progress
Introduction to Training and EMDs	1, 2, 11	
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qPCR and Case Study	4, App A	
Question & Answer Break		
Biological EMDs	5-10, App. D	
Case Studies using Multiple EMDs	App. A	
Web-Based Tech Reg Navigation		
Question & Answer Break		

No associated notes.

# EMD Technical Regulatory Guidance Document (Tech Reg)

**Environmental Molecular Diagnostics**  
New Tools for Better Decisions

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**Welcome**

Environmental molecular diagnostics (EMDs) are a group of advanced and emerging techniques used to analyze biological and chemical characteristics of environmental samples. Many of these techniques were originally developed for applications in medicine, defense, and industry. Over the last decade, however, EMDs have proven effective in environmental site management. EMDs have applications in each phase of environmental site management and provide additional lines of evidence for making better decisions.

This online guidance document will help you to understand how EMDs might benefit your site management decisions. If you are reading this document for the first time, please review the following FAQs.

You can review this guidance following the navigation at the left (beginning with the Executive Summary), or you can use the **Questions** tab at the top of the navigation window to directly access specific questions that EMDs can help to answer. The **Topics** tab summarizes parts of this document that are of particular interest to specific audiences.

If you are trying to determine if EMDs can benefit your site, then you may wish to start with Figure 2-2.

You can also click on one of the project stages below to begin:

```

graph LR
    A[Site Characterization] --> B[Remediation]
    B --> C[Monitoring]
    C --> D[Closure]
  
```

If you are interested in specific EMD methods, you can start here:

- Compound Specific Isotope Analysis
- Quantitative Polymerase Chain Reaction
- Microbial Fingerprinting Methods
- Microarrays
- Stable Isotope Probing (SIP)

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- Electronic EMD Technical and Regulatory Guidance
- streamlined navigation based on table of contents
- search feature
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# EMD Tech Reg Contents



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2 Using EMDs in Site Management

EMDs can provide historical context, make decisions, and provide official regulatory data.

This section

- using EMDs
- clarify
- identify
- decision

While the extent of one of four site-specific questions the included in Figure 2-1 shows overall characterization.

EMDs do not provide biological mechanisms (biogeochemical data) and is used to understand the remediation process.

- Using EMDs in site management
  - Comparison of different methods
  - Strengths
  - Limitations
- Summary of EMDs
  - Summary of method
  - Applications
  - Data Interpretation
- Data quality considerations
- Regulatory/stakeholder acceptance
- Case studies
- FAQ, glossary, more information

EMD provides) data (including current and life cycle. Often, decision remediation of equal or that supplements

ams, most activities fall into le). Figure 2-1 introduces conventional data. The questions supplemental information to the simplicity. Often at sites the remediation phase and some

to explain the degradation r it is necessary to implement attering and interpretation. A g, geochemistry, and EMD jid reflect a current station of the EMD results can

No associated notes.

# EMD Tech Reg Topics: Crib Notes!

**Environmental Molecular Diagnostics**  
New Tools for Better Decisions

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Suggested Reading for Regulators

Various portions of this guidance can be helpful to state or federal regulators as they review site investigation, remediation, monitoring, and closure reports submitted by an outside environmental consultant or other manager. The recommended reviewing strategy for state or federal regulators is shown below. For those not familiar with isotopic chemistry, microbiology or molecular techniques, highlighted terms open glossary definitions and further details can be found in Appendix C: Isotopic Chemistry and Appendix

**Recommended Review Approach for State and Federal Regulators**

1. Begin with EMD Definitions for an overview of the methods.
2. Section 2: Using EMDs in Site Management explains how these methods relate to the project life cycle.
3. Review the EMD method-specific descriptions (see Section 3 through Section 9).
4. Case studies for each EMD are included in Appendix A.
5. Section 11 Regulatory Acceptance and Issues describes issues of concern for regulators.
6. Section 12 Public and Tribal Stakeholder Acceptance and Issues provides insight into stakeholder concerns.
7. For information related to data quality considerations, review Section 10: Data Quality, Sampling, QA/QC, and Procedures for Biological EMDs or Section 3.3: Data Reporting, Validation, and Interpretation.

**Example Scenario for CSIA**

Specifically, the following sections may benefit regulators who are reviewing documents in which compound specific isotope analysis (CSIA) is used for characterization of a plume that may have more than one contributing source (see Section 3.2.2).

The following sections include a review of CSIA and guidance on how it can appropriately be applied, how to interpret the results, and what data quality considerations should be made. This sequence of topics provides regulators with a working knowledge of the analysis, correct application to the field, interpretation of results, and potential points of clarification.

- EMD Definitions
- Comparison of EMDs in Answering Primary Questions
- Compound Specific Isotope Analysis (CSIA)
- Data Quality Issues for CSIA

**Suggested Reading for:**

- Regulators
- Consultants
- Public and Stakeholders

**Quick links to key sections specific to reader interests**

No associated notes.

# Tech Reg Using EMDs in Site Management (Table 2-1)



Environmental Molecular Diagnostics  
New Tools for Better Decisions

Table 2-1. How EMDs can complement conventional data.

	Potential data gaps	Complementary EMD data
<b>Remediation</b>	<ul style="list-style-type: none"> <li>Weak assessment of biodegradation compounds.</li> <li>Does not differentiate various source contaminants.</li> <li>Little information on degradation mechanisms.</li> </ul>	<ul style="list-style-type: none"> <li>Update CSM with EMD results</li> </ul>
<b>Monitoring</b>	<ul style="list-style-type: none"> <li>Limited ability to confirm that the targeted degradation mechanism is occurring.</li> <li>Limited ability to confirm anticipated degradation rates of abiotic and biotic processes.</li> </ul>	<ul style="list-style-type: none"> <li>Provides details related to the presence and rate of abiotic and biotic degradation.</li> <li>Supports lines of evidence of abiotic and biotic treatment.</li> <li>Provides strong lines of evidence to support a switch in remedy.</li> </ul>

**With Conventional Data:**  
Evaluate concentration trends

**EMDs allow for:**  
More accurate evaluation of microbial response to remedy

**Potential Data Gaps:**  
Limited ability to optimize remedy

Conventional data do not allow you to determine the microbial response to remediation activities --e.g. no information on growth versus decay of key organisms, are key organisms actively degrading contaminants or are they just hanging out?  
--can use microbial information for optimal placement of remediation amendments, timing of amendment additions, evaluating dechlorination stall

# 87 Tech Reg Using EMDs in Site Management (Table 2-2)



Environmental Molecular Diagnostics

Table 2-2. Benefits, Limitations, and Complementary EMDs.

EMD	Description	Potential benefits	Potential limitations	Complementary EMDs
CSIA	Analytical method that quantifies the relative abundance of stable isotopes (such as <sup>13</sup> C/ <sup>12</sup> C) in contaminants.	<ul style="list-style-type: none"> <li>Commercially available.</li> <li>Provides direct</li> </ul>	<ul style="list-style-type: none"> <li>Need fractionation factors for key contaminants and degrading organisms.</li> </ul>	qPCR, SIP
qPCR	Chemical process that generates copies of specific DNA sequences in a quantifiable and repeatable manner. Used to <u>quantify</u> the number of copies of a specific DNA sequence that are present in a sample.	<ul style="list-style-type: none"> <li>Provides information to identify multiple sources</li> <li>Readily commercially available for some key organisms and biodegradation associated genes.</li> <li>Confirms</li> </ul>	<ul style="list-style-type: none"> <li>Does not confirm bioremediation is actually occurring, but only that the right microorganisms are present in sufficient quantity for bioremediation potentially to occur.</li> </ul>	

**Limitations:** Does not confirm biodegradation activity. Consider also using CSIA

**Advantages & Benefits:** Quantifies number of gene copies, confirming presence of target microbes or degradation gene in sample

No associated notes.

# Tech Reg Using EMDs to Answer Primary Questions (Table 2-3)

**Environmental Molecular Diagnostics**  
New Tools for Better Decisions

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

Questions	Figure	CSIA	qPCR	RT-qPCR	Finger-printing	Microarrays	SIP	EAP	FISH
<b>Site Characterization</b>									
A) Are contaminant-degrading microorganisms present?	2-3	X	X	X	X	X	X	X	X
B) Are contaminant-degrading microorganisms present?	2-4	X	X	X		X	X	X	X
C) Are the microorganisms capable of complete degradation?	2-5		X	X		X			X
D) Is biodegradation occurring?	2-6	X					X	X	
E) Is the contaminant being abiotically degraded?	-	X							
F) Are there multiple sources contributing to the contamination?	-	X							
G) If there is a potential for multiple sources, can the sources be distinguished?	-	X							

Are contaminant degrading organisms present?

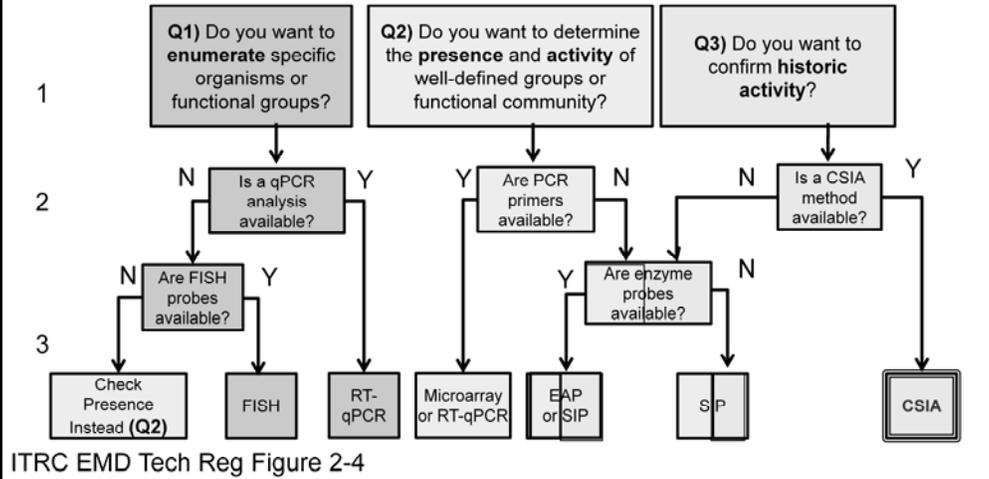
Is biodegradation occurring?

No associated notes.

## Decision Diagrams

TABLE 2.3 Questions:

- B) Site Characterization - Are contaminant-degrading microorganisms active?  
Q) Monitoring - Are contaminant-degrading microorganisms remaining active?



- 1- Supplemental Questions
- 2- Decision Steps
- 3 – EMD to Consider

CSIA is widely commercially available.

Questions are discussed on the Questions Tab.

## EMD Method Sections

---



- ▶ Summary
- ▶ Applications
- ▶ Data interpretation
  - How are the data reported?
  - How are the data interpreted?
- ▶ Practical considerations
- ▶ Additional information

ITRC EMD Tech Reg, Sections 3 - 9

No associated notes.

# EMD Method Sections: qPCR Example



**Environmental Molecular Diagnostics**  
*New Tools for Better Decisions*





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## 4 Quantitative Polymerase Chain Reaction

### 4.1 Summary of qPCR

The quantitative polymerase chain reaction (qPCR) also called quantitative real-time polymerase chain reaction, or (qPCR) 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 (Armann 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 includes a list of current qPCR targets.

[Read more](#)

### 4.2 Applications

Site characterization, remediation, monitoring, and site closure require thorough examination of available chemical, geochemical, and microbiological data. 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](#)

[Read more](#)

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).

No associated notes.

# EMD Method Sections: Example Applications (Table 4-1)

Environmental Molecular Diagnostics  
New Tools for Better Decisions

Table 4-1. Example Applications of qPCR

Title and location	General description	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
Weapons Station, CA (see description below)	Monitor biodegradation at Seal Beach Naval Weapons Station	TCE, cis-DCE, VC	qPCR, CSIA	Monitoring
RT-qPCR Case Study, Northern California (Appendix A.6)	Evaluate oxygen transport 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 cometabolism targeting aerobic oxygenases	TCE	EAP, CSIA, qPCR	Site characterization and remediation
SIP Case Study - Air Force Plant 44, AZ (Appendix A.9)	Evaluated aerobic cometabolism targeting aerobic oxygenases	TCE; 1,4-dioxane	PLFA, SIP, qPCR, EAP	Remediation Selection (Natural attenuation)
SIP Case Study - Fuel Compounds, NJ (Appendix A.10)	Evaluated presence and activity of naphthalene-degrading bacteria	Naphthalene	SIP	Monitoring

Annotations:

- Site: Case Study A5, CA (points to the Weapons Station, CA row)
- COCs: chlorinated solvents (points to the contaminants TCE, cis-DCE, VC in the Weapons Station, CA row)
- EMDs used: qPCR & CSIA (points to the EMDs used qPCR, CSIA in the Weapons Station, CA row)
- Project Life Cycle: Monitoring (points to the Project life cycle stage Monitoring in the Weapons Station, CA row)

No associated notes.



# EMD Method Sections: Data Reports



## ► Recommended and Desirable Information for Lab Reports

Typical reporting limits  
 • 100 gene copies per L or g soil

Laboratory Control Sample Results  
 • DNA extraction blank  
 • laboratory qPCR positive and negative

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

Report information	Typical information or acceptable ranges
<b>Common Laboratory Report Information</b>	
Site Identifier	Location, name, monitoring well
Sample type or matrix	Water, sediment, soil, Bio-Trap®
<b>Specific qPCR Information</b>	
<i>Recommended</i>	
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 <i>Dhc</i> .
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
<i>Desirable</i>	
Analysis Method	
Volume Extracted	0.5 to 2 L (see Note 1)
Processing time after sampling	Preferably within 24 hours

ITRC EMD Tech Reg Table 4-2

No associated notes.

## Biological EMD Data Quality



- ▶ Basic concepts
- ▶ Project life cycle sample requirements
  - Number, frequency and location of samples
  - Active versus passive sampling
  - Aseptic sampling and sterility
  - Trends and assessment of remedial progress and optimization
- ▶ QA/QC
  - Laboratory procedures and controls
  - Sample collection and handling
- ▶ Known biases
- ▶ Reporting

ITRC EMD Tech Reg, Section 10

No associated notes.

# Wrap Up: Ready to...



- ▶ Describe the utility of EMDs during remediation activities
- ▶ Define when EMDs can cost effectively augment traditional data sets
- ▶ Determine when and how to utilize the EMD Tech Reg

EMD Tech Reg web site [www.itrcweb.org/EMD-2](http://www.itrcweb.org/EMD-2)

## Thank You

Follow ITRC



- ▶ **2nd question and answer break**
- ▶ **Links to additional resources**
  - <http://www.clu-in.org/conf/itrc/EMD/resource.cfm>
- ▶ **Feedback form – *please complete***
  - <http://www.clu-in.org/conf/itrc/EMD/feedback.cfm>



Need confirmation of your participation today?

Fill out the feedback form and check box for confirmation email and certificate.

Links to additional resources:

<http://www.clu-in.org/conf/itrc/EMD/resource.cfm>

Your feedback is important – please fill out the form at:

<http://www.clu-in.org/conf/itrc/EMD/feedback.cfm>

### **The benefits that ITRC offers to state regulators and technology developers, vendors, and consultants include:**

- ✓ Helping regulators build their knowledge base and raise their confidence about new environmental technologies
- ✓ Helping regulators save time and money when evaluating environmental technologies
- ✓ Guiding technology developers in the collection of performance data to satisfy the requirements of multiple states
- ✓ Helping technology vendors avoid the time and expense of conducting duplicative and costly demonstrations
- ✓ Providing a reliable network among members of the environmental community to focus on innovative environmental technologies

### **How you can get involved with ITRC:**

- ✓ Join an ITRC Team – with just 10% of your time you can have a positive impact on the regulatory process and acceptance of innovative technologies and approaches
- ✓ Sponsor ITRC's technical team and other activities
- ✓ Use ITRC products and attend training courses
- ✓ Submit proposals for new technical teams and projects