Technology Overview

# Environmental Molecular Diagnostics Fact Sheets

November 2011

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

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**EMD-1**

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# Introduction to Environmental Molecular Diagnostics

## New Site Characterization and Remediation Enhancement Tools

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. The following is an introduction to a selection of EMDs applicable to environmental management, including site characterization, remediation, monitoring, and closure. A glossary is included at the end of this fact sheet.*

## What are environmental molecular diagnostics?

"Environmental molecular diagnostics" is a collective term that describes a group of advanced and emerging techniques used to analyze biological and chemical characteristics of soils, sediments, groundwater, and surface water. Many of these tools were originally developed for applications in medicine, defense, and industry. However, over the last decade, great advances have been made in adapting and applying EMDs for site characterization, remediation, monitoring, and closure. EMDs are important and valuable because they can provide key information not available using traditional analytical methods (e.g., groundwater analysis for volatile organic compounds). While they are intended to complement these traditional methods, EMDs can bring a new perspective to all stages in the environmental management decision-making processes. Figure 1 illustrates the connection between the stages of the environmental site management process and the EMDs presented in this and the other fact sheets.

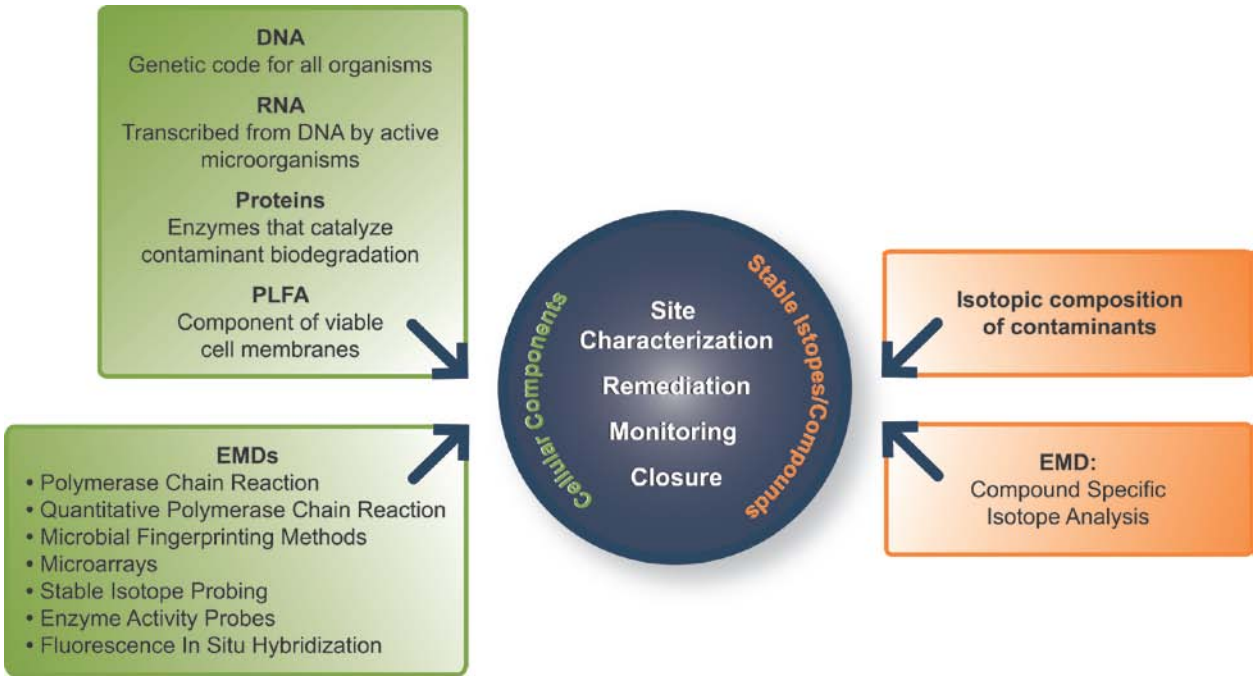


**Figure 1. Environmental molecular diagnostics.**

The EMDs discussed in these fact sheets fall into two distinct categories: compound specific isotope analysis (CSIA), which analyzes the isotopic composition of contaminants, and various methods that analyze biomolecules.

Generally speaking, CSIA measures the amounts of stable isotopes (typically carbon, hydrogen, or chlorine) in contaminants to determine the extent of specific chemical and biochemical reactions impacting the contaminant. As a contaminant degrades through natural or engineered processes, the relative amount of each stable isotope in the contaminant can change. In contrast, the isotopic composition of contaminants is largely unaffected by processes such as dilution that do not result in

degradation of the contaminant. Questions pertaining to a chemical's source, degradation mechanism, and rate of degradation can be answered, supported, and resolved through CSIA.

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

## Why use EMDs?

The environmental management process typically involves site characterization, remediation, monitoring, and closure. A variety of chemical and biological analyses (e.g., analyses for contaminants, geochemical parameters, heterotrophic plate counts) have been developed and are widely accepted as the means to understand environmental and biological conditions at each of these stages. However, characterization and remedial evaluation is best based on converging lines of chemical, geochemical, and microbiological evidence. EMDs provide useful new analytical techniques intended to complement those already in use.

For example, traditional chemical analyses of contaminants by gas chromatography or gas chromatography coupled with mass spectrometry accurately quantify the amount of each contaminant but provide little or no information about the isotopic composition of the contaminants. The ability to measure stable isotopes is important because the relative amounts of each stable isotope in contaminants often change in different and predictable ways in response to biological and physical processes. The isotopic analysis of contaminants using CSIA can therefore answer otherwise irresolvable questions about a contaminant's source, its rate of degradation, and the underlying degradation mechanism. Similar benefits can also be obtained from applying EMDs to biomolecules. Traditionally, cultivation-based methods like heterotrophic plate counts or most-probable-number analyses have been used to estimate numbers of microorganisms and the biodegradation potential of a site (Fredrickson and Balkwill 1998). Unfortunately, the overwhelming majority (>99%) of microorganisms cannot be grown in a laboratory; therefore, cultivation-based methods can dramatically underestimate the size and composition of microbial communities (Amann, Ludwig, and Schleifer 1995 and references therein). The EMDs discussed here can be used to identify and quantify key microorganisms, enzymes, and/or genes involved in specific biodegradation processes without growing microorganisms in the laboratory. These analyses can therefore answer previously unanswerable questions about the potential of sites to support biological remediation processes and the effectiveness of existing remediation approaches.

While traditional techniques are still useful and often appropriate, EMDs can provide an unprecedented level of reliability, efficiency, and precision. Each EMD has advantages and disadvantages that must be considered, and the various EMDs offer more direct, comprehensive, and accurate assessment of contaminant degradation. The EMD results, along with contaminant concentration trends and geochemical information, provide consultants, regulators, and stakeholders with knowledge and information to make informed decisions.

## Example Environmental Remediation Questions EMDs Can Help Answer

- **Site Characterization**
  - Are multiple sources contributing to a groundwater plume?
  - Is biodegradation occurring?
- **Remediation**
  - Is monitored natural attenuation (MNA) feasible?
  - Is biodegradation already occurring? Will it continue in the future?
  - Is complete degradation occurring? Will there be by-products that require remediation?
  - Is biostimulation necessary? Should an electron donor or acceptor be added?
  - Is bioaugmentation necessary? What microorganisms need to be added?
- **Monitoring**
  - Is the chosen remedial technology performing as designed?
  - Did electron donor or acceptor addition promote growth or activity of contaminant-degrading microorganisms?
  - Are bioaugmentation microorganisms surviving in situ?
  - Are contaminant-degrading communities maintained over time under existing site conditions?
  - Will concentrations decrease to remedial objectives?
- **Closure**
  - Is contaminant degradation likely to continue?

The EMDs detailed in the other fact sheets in this series include CSIA, polymerase chain reaction (PCR), quantitative PCR (qPCR), microbial fingerprinting methods, microarrays, stable isotope probing (SIP), enzyme activity probes (EAPs), and fluorescence in situ hybridization (FISH). Lastly, a fact sheet pertaining to EMD sampling methods is also provided. Table 1 summarizes the underlying principle and purpose of each EMD.

**Table 1. Description of EMDs**

| EMD | Principle | Purpose |
|---|---|---|
| Compound specific isotope analysis | Analyzes the relative abundance of various stable isotopes (e.g., $^{13}C/^{12}C$, $^{2}H/^{1}H$) of the component elements of contaminants (e.g., trichloroethene [TCE]). Degradation processes can cause measureable shifts in the isotopic ratios. | • Determine whether contaminant degradation is occurring.<br>• Investigate the degradation mechanism.<br>• Identify contaminant source. |
| Polymerase chain reaction | Amplifies (makes copies of) the genetic material of microorganisms to levels that can be further analyzed using other techniques. | • Detect microorganisms or target genes responsible for contaminant biodegradation.<br>• Process genetic material for use in other EMDs. |
| Quantitative polymerase chain reaction | Quantifies a target gene based on DNA or RNA. | • Quantify the abundance and expression of specific functional genes, microorganisms, or groups of microorganisms responsible for contaminant biodegradation. |
| Microbial fingerprinting methods | Differentiates, and in some cases identifies, microorganisms by unique characteristics of universal biomolecules, including phospholipid fatty acids (PLFA) and nucleic acids (DNA and RNA). | • Provide a profile of the microbial community.<br>• Identify a subset of the microorganisms present.<br>• Quantify living biomass. |
| Microarrays | Detects and estimates the relative abundances of hundreds to tens of thousands of genes simultaneously. | • Provide a comprehensive evaluation of the microbial diversity and community composition. |

| EMD | Principle | Purpose |
|---|---|---|
| Stable isotope probing | Detects the presence of an added synthesized form of the contaminant containing a stable isotope (e.g., $^{13}C$). If contaminant biodegradation is occurring, the isotope will be detected in biomolecules (e.g., phospholipids, DNA) and metabolites (e.g., $CO_2$). | • Determine whether biodegradation of a specific contaminant is occurring.<br>• Identify the microorganisms responsible for this activity. |
| Enzyme activity probes | Detect the transformation of surrogate compounds that resemble specific contaminants. | • Quantify the activity of microorganisms with specific biodegradation capabilities. |
| Fluorescence in situ hybridization | Detects the presence of targeted genetic material in an environmental sample. | • Estimate the number of and/or relative activity of specific microorganisms or groups of microorganisms. |
| EMD sampling methods | Active sampling methods (e.g., low-flow groundwater sampling) and passive microbial sampling devices in which subsurface microorganisms colonize a solid matrix. | • Methods for collection of biomass from environmental media to be used in conjunction with EMDs. |

EMDs differ in the level of information they provide, the type of data they provide, costs, and availability. As of the writing of these fact sheets in 2011, the most often used EMDs are CSIA and qPCR. The level of information provided by EMDs can range from the identity of microorganisms (i.e., genus or species), to the potential activity (i.e., Are the microorganisms present capable of performing a desired task?), to the expressed activity (i.e., Are the microorganisms performing the desired task?). The type of data provided can be qualitative or quantitative. The per sample costs can range $75–$5,000, and analysis may be available commercially or through university or other research laboratories. Table 2 summarizes these differences between the EMDs.

**Table 2. Comparison of EMDs**

| EMD | Identity/potential activity/expressed activity[a] | Quantitative, qualitative (Qa/Ql) | Cost range[b] ($) | Availability[c] |
|---|---|---|---|---|
| Compound specific isotope analysis | E | Qa | 100–2,500 | C/R |
| Polymerase chain reaction | I/P | Ql | 225–350 | WC |
| Quantitative polymerase chain reaction | I/P/E[d] | Qa | 275–425 | WC |
| Microbial fingerprinting methods | I | Qa/Ql | 300–570 | WC |
| Microarrays | I/P/E[d] | Ql | 1,250–5,000 | C/R |
| Stable isotope probing | I/P/E | Qa/Ql | 1,500 and up | C/R |
| Enzyme activity probes | E | Qa | 250–2,500 | C/R |
| Fluorescence in situ hybridization | I/P/E[d] | Qa/Ql | 250–5,000 | C/R |
| EMD sampling methods | I/P | Qa/Ql | 75–200 | WC |

[a] I = identity of microorganisms (i.e., genus or species), P = potential activity (i.e., genetically capable of completing the activity), E = expressed activity (i.e., actually completing the activity at a given time).
[b] Estimated range of per sample costs. The low end of the cost range represents a very restricted analysis and may not be applicable to every site (e.g., for only one compound/target with very limited quality control documentation).
[c] WC = widely commercially available, C = minimally commercially available, R = available through university or other research laboratory.
[d] RNA rather than DNA is used to assess activity.

## When can EMDs be used?

EMDs can be used at environmental cleanup projects affected by a variety of contaminants. These range from volatile organic compounds, such as chlorinated solvents or petroleum constituents, to inorganic metals. Additional applicable contaminants include polycyclic aromatic hydrocarbons, polychlorinated biphenyls, radionuclides, pesticides, explosives, nitrates, perchlorates, and/or chromates. EMDs can be applied to a broad variety of projects because research has been performed to understand the chemical changes and microbial metabolism associated with degradation (Löffler and Edwards 2006, Lovely 2003, Watanabe 2001, Watanabe and Hamamura 2003). Each of the individual fact sheets includes additional information and references.

EMDs can be useful at each stage of environmental management, including site characterization, remediation, monitoring, and closure. Depending on the contaminant, site conditions, and location, different EMDs help answer different management questions. During site characterization, EMDs can determine what microorganisms are present, their relative abundance, what metabolic capabilities and activities are present and occurring, and how contaminants are being degraded. In the case of adjacent contaminant plumes and when combined with other information and analytical results, CSIA can be used to identify sources. During remediation efforts, EMDs are useful in evaluating what approaches are feasible, optimizing remedial strategies, and troubleshooting unsuccessful treatment approaches. EMDs can confirm remediation effectiveness by presenting scientific evidence that biodegradation is occurring, or not. If biodegradation is occurring, some EMDs can be used to estimate the rate of contaminant degradation to determine whether site-specific cleanup goals will be attained within an acceptable time frame. This information may, along with contaminant concentration trends and geochemical information, support closure. It may serve the remedial project well to discuss the use of EMDs with the regulators and the stakeholders, prior to project initiation. This step would provide the opportunity for education and to gain support from involved parties.

### Examples of When EMDs Can Provide New Information

- **Site Characterization**
  - Two neighboring gasoline stations have groundwater releases (plumes) of methyl *tert*-butyl ether (MTBE). In addition to traditional data (e.g., concentration trends, groundwater flow data), CSIA can be used to identify which plume is affecting a downgradient receptor.
- **Remediation**
  - A groundwater plume of chlorinated solvents is being evaluated for MNA. qPCR can be used to quantify whether *Dehalococcoides* microorganisms are present in sufficient number to pursue MNA or active remediation with biostimulation or bioaugmentation will be required.
- **Monitoring**
  - An electron donor was added to stimulate biodegradation of chlorinated solvents (e.g., TCE) in groundwater. qPCR can be used to detect and quantify the genes encoding the enzymes responsible for complete degradation of vinyl chloride to ethene and to monitor the effectiveness of the treatment approach.
- **Closure**
  - A case for MNA of a groundwater gasoline plume is being evaluated for closure. CSIA can be used to estimate the degradation rate of the contaminants and also provide an estimated cleanup time.

## How do EMDs work?

The geochemical and microbiological characteristics of soil, groundwater, or surface water typically change when contaminants are introduced into these environments. As the contaminants degrade either naturally or in response to engineered systems, these geochemical and microbiological characteristics also continue to change, as do the chemical features of the contaminants themselves. The EMDs

discussed in these facts sheets are used to characterize and quantify these changes. For example, CSIA quantifies changes in the natural isotopic composition of contaminants. The molecular biology–based EMDs can quantify changes in the microbial community by determining the numbers of specific microorganisms or specific biochemical reactions at any given time. Specific details pertaining to each of the individual EMDs are found in the applicable fact sheet.

## Who could use EMDs?

Individuals currently using traditional and other analytical techniques as part of environmental management could use EMDs to help create multiple lines of evidence. Typically, this group includes environmental managers (e.g., consultants or regulators) responsible for site characterization and remediation. Samples collected for EMD analysis are sent to a laboratory for analysis. Results are interpreted by the laboratory or the consultant and used to provide a better understanding of environmental conditions. These analyses are documented and presented to regulators and stakeholders via reports and presentations.

There exists an increasing potential for regulators to be called on to review results from EMDs as part of routine reports provided by environmental consultants. One of the primary reasons for developing these fact sheets is to provide regulators with an understanding of EMDs to make more informed decisions. These fact sheets also serve to provide stakeholders with background information, basic technical knowledge, and examples of applicability for EMDs. Community stakeholders may be presented with EMD results as part of public meetings and outreach programs.

## Sampling Protocols

Most sample matrices (soil, sediment, groundwater, in-field filters) can be collected for EMD analyses. Sampling procedures for EMDs are straightforward and, in most cases, can be readily integrated into routine monitoring programs. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile collection containers, (b) shipment of the samples to the laboratory within 24 hours of collection, and (c) maintenance of the samples at an appropriate temperature during handling and transport to the laboratory, usually 4°C. Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood. In addition, specific active and passive sampling methods have been developed to be used in conjunction with EMDs (see the EMD Sampling Methods Fact Sheet). Although methods may vary according to site-specific conditions, it is important that the sampling protocol for a given site be defined, documented, and maintained for the duration of sampling efforts.

Researchers continue to evaluate the implications for collecting soil or sediment versus groundwater samples for EMD analysis (Amos et al. 2009). However, sampling for EMDs currently focuses on groundwater due to the practical limitations of collecting routine soil or sediment samples. This focus on groundwater is justified for the analysis of microorganisms that are found in the aqueous phase (i.e., planktonic nonattached microorganisms vs. microorganisms that are predominantly attached to the aquifer material [e.g., soils, sediments]). Field filtration is also recommended for collecting biomass from groundwater because field filtration increases the likelihood of collecting suspended particles, it decreases shipping costs, and it significantly reduces the costs associated with laboratory extraction procedures. A guidance protocol providing a step-by-step approach for groundwater sampling using field filtration methods was developed as part of the Environmental Security Technology Certification Program (ESTCP) Project ER-0518 and is available in Lebrón et al. (2011) and Ritalahti et al. (2010).

## How are EMD data reported?

Data collected from each of the EMDs presented in these fact sheets are represented differently. Generally speaking, EMD results can be qualitative or quantitative. Qualitative results include information

regarding the type of microorganisms detected and the presence of targeted genes (e.g., PCR) or the interaction between microbial communities (e.g., FISH). Quantitative results typically include the number of detected cells, gene copies, or other measured parameter per gram of soil or milliliter of water (e.g., qPCR). This is comparable to concentration data provided by traditional chemical analyses. More specifically, CSIA data are reported as the isotopic ratio relative to that in an international standard isotopic reference material.

## Advantages

- Analysis is typically performed directly on the environmental sample. There is usually no need for microbial cultivation, making EMDs more precise and, in most cases, less time-consuming than traditional microbiological methods.
- CSIA provides accurate and reliable information for many environmental management decisions. In addition, CSIA may be used for environmental forensic investigations (e.g., source and age of contamination).
- Molecular biology–based EMDs can evaluate a broad spectrum of microorganisms and biodegradation processes.
- EMDs are sensitive; some can detect as few as 100 cells or genes in a sample or very low chemical concentrations.
- Analytical results are available in a short turnaround time, typically within a couple of days for most EMDs.
- Molecular biology–based EMDs define microbial characteristics such as community, species, and/or activity which can be used to monitor or improve remedial processes.
- EMDs can be performed on a variety of environmental sample types, such as surface water, groundwater, soil, and sediment.
- EMD data can impart a better understanding of contaminant plume heterogeneity, especially for MNA or in situ remedy selection and monitoring.
- EMDs have been shown to be valuable at each stage of environmental management, including site characterization, remediation, monitoring, and closure.

## Limitations

- While some are still maturing, a number of EMDs are well established. Yet, even as some EMDs have become readily available and applied in the field, widespread understanding and acceptance by consultants, regulators, and stakeholders have not yet been achieved.
- Many molecular biology–based EMDs are limited to currently known biodegradation pathways and gene sequences. However, with ongoing research, EMDs will likely be adapted to emerging contaminants and newly identified biodegradation pathways.
- It is possible for elevated concentrations of minerals, metals, or organic matter to interfere with laboratory analysis.
- Technical expertise is required to evaluate EMD applicability, select the appropriate EMD, and interpret the results.
- Standard EMD protocols for sample collection, storage, analysis, and quality assurance (QA)/quality control (QC) have not yet been established across all laboratories.
- Currently, EMD analyses may be more expensive than traditional site investigation analyses (see Table 2). However, EMDs do not need to be applied with the same frequency; rather, they are typically performed on selected samples, over space and time.

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency (EPA) or other government agencies. However, standards are in development. For example, CSIA and PCR have EPA guidance documents (EPA 2008, 2004, respectively), and standardized methods for qPCR are under development through the Strategic Environmental Research and

Development Program (SERDP) Project ER-1561. SERDP Project ER-1561, titled "Standardized Procedures for Use of Nucleic Acid-Based Tools," investigated through a literature review the challenges associated with standardizing qPCR methods (Lebrón et al. 2008). Preliminary results from SERP Project ER-1561 suggest that even in the absence of a standard methodology, a systematic sampling and analysis program can yield accurate and reproducible results. In addition, each laboratory works under its own standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific QA project plan (QAPP). This plan should include identification of the EMDs being used, the field sampling procedures, the SOPs of the laboratory performing the analysis, and laboratory internal QA/QC information. Development of a QAPP facilitates communication between the user and the laboratory, as well as with regulators and stakeholders. With the QA/QC procedures documented, data collected over time can be reviewed against the same criteria.

For users of EMDs and interpreters of results, some points to review may include the following:

- Was the proper environmental medium sampled (e.g., groundwater or sediments)?
- Are the sampling procedures described?
- Were adequate samples taken, preserved, shipped, received, etc.?
- Was there enough environmental sample collected to give an accurate, repeatable result?
- Was the proper biomolecule targeted for analysis (e.g., DNA or RNA)?
- Were all internal laboratory QA/QC criteria met?
- Were sample results outside QA/QC criteria?
- Were oddities found in the sample results (e.g., detections in a blank sample)?
- Did the sample have to be diluted prior to analysis?

## Underlying Principles of EMDs

The ability of microorganisms to degrade contaminants requires that they have the appropriate genetic information (genes) that confers a particular biochemical activity to the microbial cell. EMDs can detect this "information" in several ways. Some EMDs detect and quantify the presence of genes themselves, while other EMDs detect the products of these genes when they are expressed and lead to biochemical activity. Figure 2 summarizes the flow of information in a microbial cell.

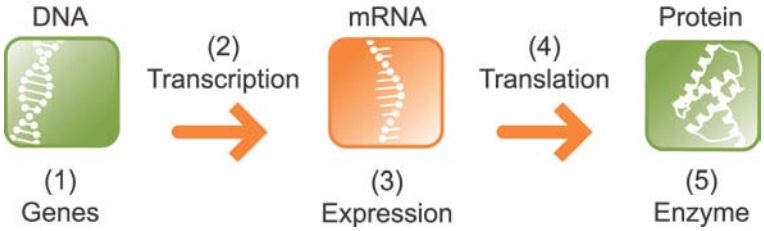


**Figure 2. Flow of information in a microbial cell during gene expression and enzyme production.**

Figure 2 depicts the following steps in the gene expression and enzyme production process:

1. **Genes**—A gene is a segment of DNA within a bacterial cell that contains specialized instructions that direct the cell to make a specific protein. Most proteins (enzymes) act as catalysts that the cell needs in order to metabolize (break down) a contaminant. A typical environmental sample can contain many thousands of different microorganisms. However, only a small fraction may possess the genes necessary to break down a specific contaminant. EMDs, including PCR, qPCR, microarrays, and FISH, are used to detect and/or quantify genes that indicate the presence of specific contaminant-degrading microorganisms (e.g., *Dehalococcoides*) or genes for specific enzymes that biodegrade specific contaminants (e.g., vinyl chloride reductase).

2. **Transcription**—Enzyme production is a two-step process where the gene is first copied (transcribed) into a short-lived, intermediate molecule called messenger RNA (mRNA).
3. **Expression**—A typical cell contains thousands of individual genes. Many of these genes program (encode) production of enzymes responsible for a broad spectrum of different functions. Naturally, a cell does not need all of those enzymes to be produced all of the time. In the interest of efficiency, enzyme production is regulated where transcription of some genes into mRNA is "turned off" and "turned on" only when needed.
4. **Translation**—In the second step of enzyme production, mRNA is translated into the corresponding protein (enzyme).
5. **Enzyme**—The enzyme is the biomolecule that catalyzes the biodegradation of the contaminant. One EMD, EAPs, directly estimates the activity of specific enzymes.

Microorganisms require two types of compounds to gain energy: an electron donor and an electron acceptor (Figure 3). The electron donor is oxidized, and the electrons are transferred to the electron acceptor, producing energy for the cell. In addition to energy, a cell needs carbon to build biomolecules (e.g., DNA, RNA, proteins) and reproduce. For many microorganisms, the electron donor is also the carbon source.

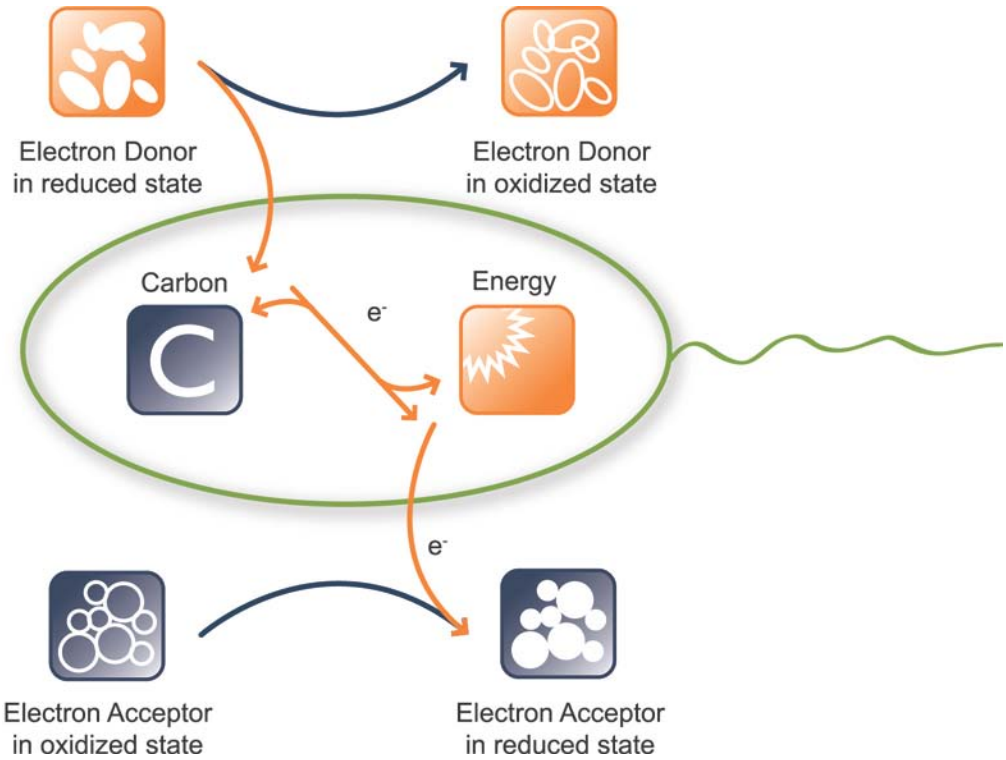


**Figure 3. Microbial metabolism.**

Many common environmental contaminants serve as an electron donor or an electron acceptor. For example, biodegradation of chlorinated ethenes (e.g., TCE) depends on microorganisms that can use them as an electron acceptor. For many other common groundwater contaminants (e.g., benzene, MTBE), biodegradation is a process whereby microorganisms use the contaminant as the electron donor and/or carbon source to produce new cells (biomass).

For contaminants used as carbon and energy sources, SIP is an EMD that can be used to determine whether biodegradation is occurring. Briefly, an environmental sample is exposed to a specially synthesized form of the contaminant containing a "heavy" isotope such as carbon-13 ($^{13}C$) as a "label." After a predetermined time, a sample is recovered for isotopic analysis of the contained biomass and dissolved inorganic carbon. If biodegradation has occurred, some of the $^{13}C$ from the contaminant will

have been completely mineralized to $CO_2$, and some will be incorporated into the biomolecules of the contaminant-degrading microorganisms. In other words, if contaminant biodegradation occurred, elevated levels of the stable isotope "label" will be detected in the products of metabolism: biomolecules and $CO_2$. See the EMD SIP Fact Sheet for additional information.

CSIA is based on quantification of the isotopic ratio (e.g., $^{13}C/^{12}C$, $^{2}H/^{1}H$) of the component elements of the environmental contaminant. Biodegradation and abiotic degradation of a contaminant involve breaking the chemical bonds between the component elements (e.g., C-Cl bond) of the contaminant compound. In general, less energy is required to break a bond between a light isotope and another element (e.g., $^{12}C$-Cl) than is required to break a bond between the heavy isotope and the same atom (e.g., $^{13}C$-Cl). As a result, the ratio of heavy isotopes to light isotopes increases as the contaminant is degraded. Figure 4 illustrates the enrichment of $^{13}C$ during degradation. See the CSIA Fact Sheet for additional information.

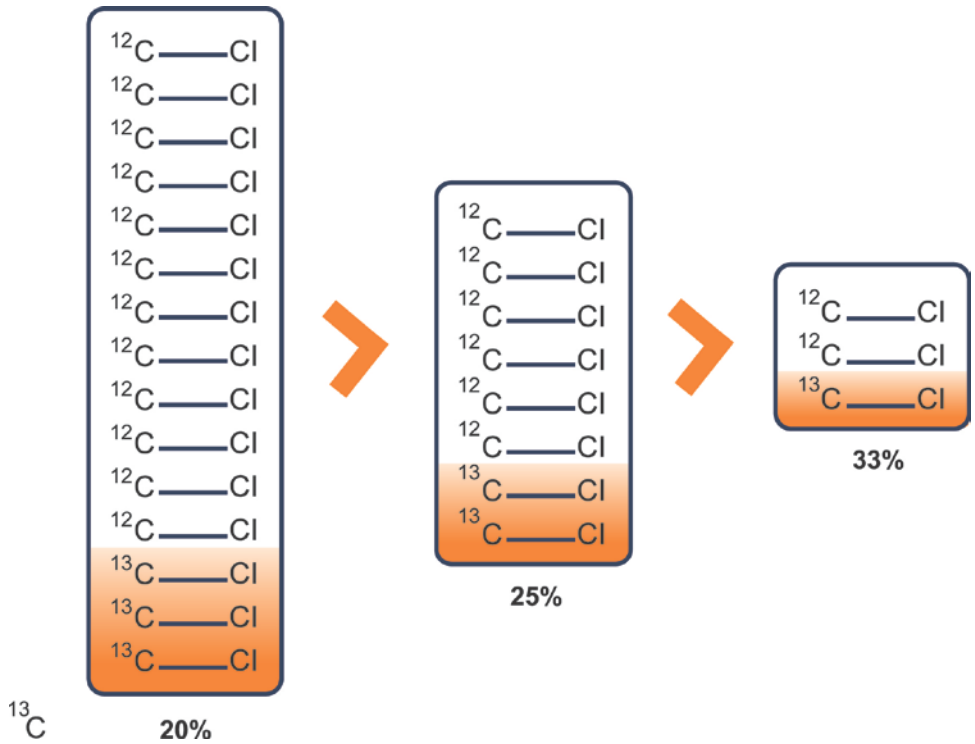


**Figure 4. Illustration of $^{13}C$ enrichment during degradation.**
*Source:* Microseeps, Inc., 2010, used with permission.

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## Glossary

**activity**—Refers to when a microorganism performs a specific function (e.g., sulfate reduction, metabolism of benzene).

**bioaugmentation**—The introduction of cultured microorganisms into the subsurface environment for the purpose of enhancing bioremediation of organic contaminants (EPA 2011).

**biodegradation**—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

**biomolecules**—Classes of compounds produced by or inherent to living cells, including phospholipids, nucleic acids (e.g., DNA, RNA), and proteins.

**bioremediation**—The treatment of environmental contamination through the use of techniques that rely on biodegradation.

**biostimulation**—A remedial technique which provides the electron donor, electron acceptor, and/or nutrients to an existing subsurface microbial community to promote degradation.

**compound specific isotope analysis**—Analyzes the relative abundance of various stable isotopes (e.g., $^{13}C$:$^{12}C$, $^{2}H$:$^{1}H$). Degradation processes can cause shifts in the relative abundance of stable isotopes of the contaminant; changes in isotopic ratios can be measured.

***Dehalococcoides***—A specific group (genus) of bacteria. *Dehalococcoides* species are the only microorganisms that have been isolated to date which are capable of complete reductive dechlorination of perchloroethene and TCE to ethene. Some *Dehalococcoides* species are also capable of reductive dechlorination of chlorinated benzenes, chlorinated phenols, and polychlorinated biphenyls.

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**electron acceptor**—A chemical compound that accepts electrons transferred to it from another compound (based on EPA 2011).

**electron donor**—A chemical compound that donates electrons to another compound (based on EPA 2011).

**environmental forensics**—The process of distinguishing contaminants from different sources.

**enzyme**—Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on EPA 2004).

**enzyme activity probes**—Transformation of surrogate compounds (probes) resembling contaminants produces a fluorescent (or other distinct) signal in cells, which is then detected by microscopy.

**fluorescence in situ hybridization**—Detects the presence of targeted genetic material in an environmental sample and estimates the number of specific microorganisms or groups of microorganisms.

**gene**—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

**genus**—A category of organism classification (taxonomy). A particular genus is a group of related species. For example, *Pseudomonas* is a genus of bacteria.

**heterotrophic plate count**—A test used to estimate the total number of bacteria capable of growing on organic compounds in an environmental sample.

**microarrays**—Detects and estimates the relative abundances of hundreds to thousands of genes simultaneously.

**microbial community**—The microorganisms present in a particular sample.

**microbial diversity**—Microbial diversity can have many definitions but in this context generally refers to the number of different microbial species and their relative abundance in an environmental sample (Nannipieri et al. 2003).

**microbial fingerprinting methods**—A category of related techniques that differentiate microorganisms or groups of microorganisms based on unique characteristics of a universal component or section of a biomolecule.

**phospholipid**—A type of biomolecule that is a primary structural component of the membranes of almost all cells.

**polymerase chain reaction**—Makes copies of a specific DNA sequence within a target gene of microorganisms that can be further analyzed.

**protein**—Large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds (U.S. Navy 2009).

**quantitative polymerase chain reaction**—A laboratory analytical technique for quantification of a target gene based on DNA or RNA.

**RNA (ribonucleic acid)**—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

**species**—The lowest taxonomic rank, and the most basic unit or category of biological classification (Biology Online n.d.).

**stable isotope**—A form of an element that does not undergo radioactive decay at a measureable rate.

**stable isotope probing**—A synthesized form of the contaminant containing a stable isotope (e.g., $^{13}C$) is added. If biodegradation is occurring, the isotope will be detected in biomolecules (phospholipids, DNA).

# Compound Specific Isotope Analysis

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why is compound specific isotope analysis relevant?

Compound specific isotope analysis (CSIA) is an analytical method that measures the ratios of naturally occurring stable isotopes in environmental samples. As described in this fact sheet, CSIA can be used to gain information (e.g., potential contaminant sources, extent of degradation, comingling of contaminant plumes) relevant to environmental remediation decision makers. Stable isotope probing (SIP), which is a separate and distinct EMD method and is discussed in its own fact sheet, uses isotopically enriched (labeled) contaminants and examines the incorporation of stable isotopes into biomolecules and by-products that are generated during biochemical processes associated with contaminant biodegradation.

## What does CSIA do?

CSIA is a laboratory method in which samples collected from the field are analyzed to give information that can be valuable for assessing environmental forensics or contaminant fate. Each element in a compound has a distinct isotopic ratio. For a given element the isotopic ratio is known to within a few percent; however, that ratio can change in systematic ways during the course of biodegradation or other processes. CSIA measures these small changes in isotopic ratios very precisely. Those changes can be exploited to gain important information about the source, transport, and fate of a compound. Table 1 includes information about environmentally relevant elements to which CSIA is often applied.

Since the isotopic ratio in the compound is a function of the starting material and the manufacturing process as well as the degradation of that compound after it was made, CSIA has applications in environmental forensics, biodegradation, and abiotic degradation. Examples include the following:

**Table 1. Environmentally relevant ratios for CSIA**

| Atom | Ratio |
|---|---|
| Hydrogen | $^{2}H/^{1}H$ (D/H) |
| Carbon | $^{13}C/^{12}C$ |
| Nitrogen | $^{15}N/^{14}N$ |
| Oxygen | $^{18}O/^{16}O$ and $^{17}O/^{16}O$ |
| Chlorine | $^{37}Cl/^{35}Cl$ |

- **Biodegradation**
  - Apparent *cis*-dichloroethene (*cis*-DCE) stall: Is the *cis*-DCE biodegrading, or are concentration changes the result of dilution?
  - Dense, nonaqueous-phase liquids: Is there biodegradation?
  - Biodegradation of methyl *tert*-butyl ether (MTBE): Is it occurring?
  - Aerobic *cis*-DCE and vinyl chloride (VC): How do we prove progress without daughter or end products?
  - Cometabolic degradation of chlorinated ethenes: Will the accumulation of VC really be skipped?
- **Environmental Forensics**—Especially when combined with other tests, CSIA can reveal detailed information not just about remedial progress or remedial potential but also about forensic issues such as the potential for multiple sources.
  - Methane: From shallow biodegradation or pipeline gas?
  - Perchlorate: Is it natural or synthetic?
  - Volatile organic compounds (VOCs): Origin from one source or multiple sources?
  - Nitrate: Is it runoff or naturally occurring?
- **Abiotic Degradation**
  - Is biogeochemical transformation occurring?
  - In situ chemical oxidation (ISCO): Was the ISCO successful in destroying contaminant mass and the “rebound” is really just newly desorbed product?
  - In situ chemical reduction, iron wall, nanoscale iron, or other reducing mixtures: Has the contaminant been destroyed or displaced?

## How are the data used?

CSIA can be used to make informed decisions for site characterization, monitoring, remedy selection, and closure. CSIA can differentiate between degradation of a compound (e.g., ISCO) and other processes that can also reduce contaminant concentration but do not reduce contaminant mass (e.g., displacement or dilution). CSIA has also been used to distinguish contaminant sources (e.g., trichloroethene [TCE] from different plumes).

### Example Environmental Remediation Questions CSIA Can Help Answer

- **Site Characterization**
  - Has biological or abiotic degradation occurred? If so, how much and where?
  - Is methane from near-surface biodegradation or natural gas production?
  - Is the TCE a parent from one source or a daughter product of perchloroethene (PCE) from another source?
  - Is there evidence of multiple sources?
  - Is the contaminant in the dissolved or nonaqueous phase?
  - Is there evidence of a rate-limiting step (i.e., accumulation of contaminant intermediates)?
- **Remediation**
  - Is monitored natural attenuation (MNA) feasible?
- **Monitoring**
  - Is remediation progressing as planned?

CSIA has been in use for more than two decades since the merger of two 50-year-old techniques that were each well established: gas chromatography and isotopic analysis. Isotopic analysis has been used extensively in geology and petroleum exploration. Due to the extremely dilute concentrations typically encountered in environmental samples (as compared to those from traditional geology and petroleum exploration applications), the early methods were not suitable for environmental work. However, within the last 10–12 years advances in analytical instrumentation have resulted in the ability to apply CSIA to very dilute samples, and the technique has been broadly applied to evaluate environmental questions.

## How does it work?

Changes in isotopic ratios are caused by the breaking of bonds between atoms. Physical processes such as dilution, diffusion, and volatilization do not change the isotopic ratios in compounds to the same extent as chemical or biochemical processes such as degradation. For VOCs in groundwater, this means that degradation of a compound is, by far, the major cause of significant changes in isotopic ratios. This change in isotopic ratio happens in both biological and abiotic reactions, and CSIA is used to measure those changes. The CSIA laboratory method is implemented using a number of instruments, including a gas chromatograph (GC) and an isotope ratio mass spectrometer (IRMS).

Because compounds are made up of multiple elements, CSIA can be performed on multiple isotopes to gain further insight into origin or mechanisms of degradation. For example, both carbon ($^{13}C/^{12}C$) and hydrogen ($^{2}H/^{1}H$) are often used for MTBE, whereas chlorine ($^{37}Cl/^{35}Cl$) as well as two isotopic ratios of oxygen ($^{18}O/^{16}O$ and $^{17}O/^{16}O$) are often used for perchlorate. Table 1 lists environmentally relevant ratios on which CSIA can be used.

Every contaminant is made of atoms of various elements. The isotopes of a given element (e.g., carbon, hydrogen, chlorine) have the same number of protons and electrons but a different number of neutrons and thus different atomic mass. Each element has a most-abundant isotope (for example, $^{12}C$, or "carbon-twelve," for carbon) and one or more less-abundant isotopes ($^{13}C$, or "carbon-thirteen," for carbon) (Figure 1). The less-abundant isotopes are sometimes heavier (i.e., contain one or more extra neutrons). Some of these heavier, less-abundant isotopes are nonradioactive (i.e., stable), and the only significant difference between these isotopes and their more-abundant counterpart is the increased mass. The

increased mass leads to the process called "isotopic fractionation." Isotopic fractionation is monitored by measuring the isotopic ratio, i.e., by CSIA.

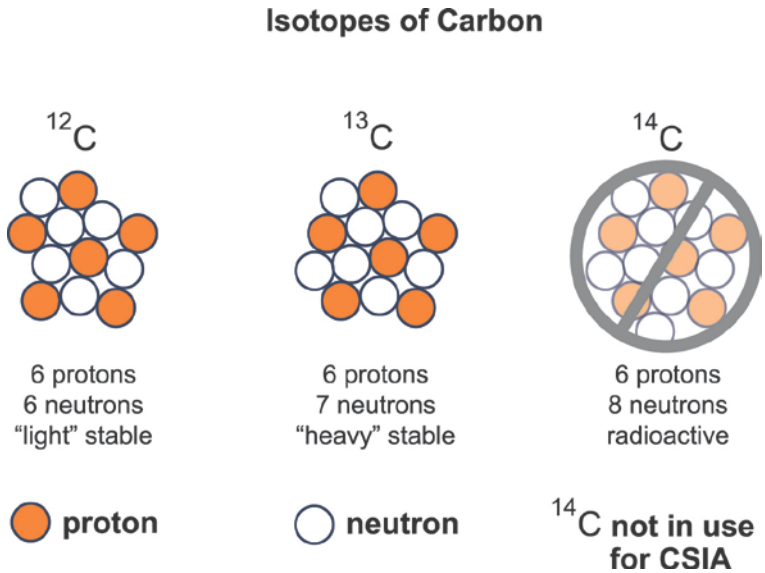


**Figure 1. Schematic of the atomic nuclei of the common carbon isotopes.**
Carbon-fourteen is not used in CSIA. *Source:* Microseeps, Inc., 2010, used with permission.

Isotopic fractionation occurs because it takes slightly less energy to break a bond between a light isotope and another atom than it takes to break a bond between a heavy isotope and that same atom. As a result, the rates of reaction involving the heavier isotopes are slightly slower, so the percentage of heavy isotopes increases in the residual contaminant pool as the contaminant is degraded. Figure 2 shows this "isotopic fractionation" or enrichment for a carbon-chlorine (C-Cl) bond.

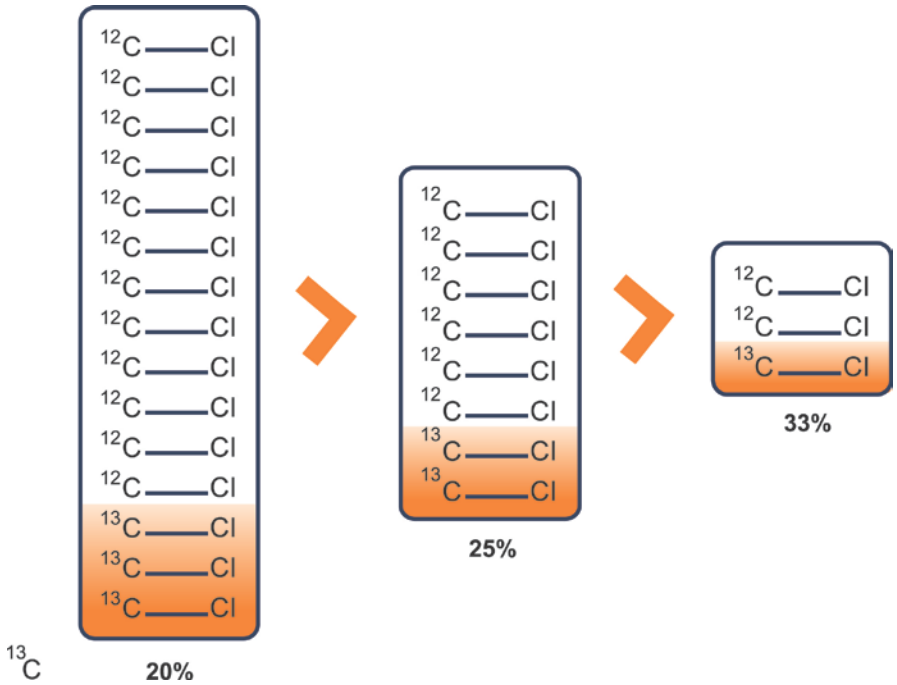


**Figure 2. Illustration of $^{13}C$ enrichment during degradation of a contaminant with a C-Cl bond.**
*Source:* Microseeps, Inc., 2010, used with permission.

## How are the data reported?

CSIA is a very sensitive technique, and because the differences in isotopic ratios are so small, it is more convenient to express the ratios in "per mil" (parts per thousand, or ‰, similar to the usual metric for comparison, parts per hundred, or percent) values, relative to a reference standard. (A variety of standards exist, and it is important that a common standard be used by the laboratory and reported with the analytical results to enable data comparison). This is accomplished by using "delta" notation. The standard is a constant and is the isotopic ratio of an internationally agreed-upon standard (for example, for $^{13}C/^{12}C$ the standard is $R_{std} = 0.01118$). The definition of delta is as follows:

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

where the $R_x$ is the isotopic ratio of sample "x" and $\delta_x$ (called "delta of sample x") is linearly related to the isotopic ratio. Thus, if the $\delta(^{13}C)$ for a TCE sample is "–31 per mil" (a typical value for undegraded TCE), this means that the $^{13}C/^{12}C$ in the sample is 31 per mil, or 3.1 percent, lower than in the internationally agreed-upon standard ($R_{std}$).

## Advantages

- CSIA does not rely on concentration trends or the observation of daughter products.
- Because it isolates the contaminant, CSIA is relevant only to that compound.
- CSIA can detect very small changes in the isotopic ratio. This high level of precision allows for careful assessment across a site to reveal subtle but important differences in the contaminants.
- CSIA is extremely versatile. It can be used for many contaminants in a wide range of applications, as listed on p. 1.
- The advantages of CSIA can be increased by applying it to multiple isotopes in a given molecule (e.g., both $^{13}C/^{12}C$ and $^{37}Cl/^{35}Cl$ in TCE). See Table 1.

## Limitations

- At this time, only a limited number of laboratories provide CSIA services. Though CSIA may theoretically be the way to answer a question at a site, the particular isotopic analyses may not be commercially available. If this is the case, collaboration with academic laboratories and/or application development with commercial laboratory should be investigated.
- Because of the large number of compounds in petroleum products, there is the potential for interference at petroleum-release sites (as well as other sites with many compounds in the groundwater), especially near the contaminant source. SIP is one alternative to assess biodegradation at these sites (see the SIP Fact Sheet for more information). In addition, modifications to CSIA methods can be used to overcome interferences. For example, dual GC column separations have been used effectively to evaluate biodegradation of the lead scavenger 1,2-dibromoethane in groundwater at gasoline release sites (EPA 2008b). The same technique is used for MTBE (Kuder et al. 2005).
- While the method is quite sensitive, there are limitations to that sensitivity. Isotopic fractionation may be so minimal that little or no isotopic enrichment is detected. This effect can occur in molecules with many of the same atom (e.g., several C atoms in one molecule). For example, CSIA of either carbon or hydrogen is unlikely to provide valuable information for many high-molecular-weight polycyclic aromatic hydrocarbons during degradation.

## Developing an Appropriate CSIA Study

The U.S. Environmental Protection Agency's *A Guide for Assessing Biodegradation and Source Identification of Organic Ground Water Contaminants Using Compound Specific Isotope Analysis* (EPA 2008a) discusses many of the technical aspects of this technology, including how to best design a CSIA study to address a particular question.

## Sampling Protocols

The sampling protocols for CSIA depend on what questions are being asked and which compounds are of interest. For example, if CSIA is used to study the isotopic ratios of carbon, hydrogen, or chlorine of VOCs in groundwater, then sampling involves the collection of water samples into standard volatile organic analysis vials. Extra vials are often required due to the need for both concentration/identification and isotopic ratio analyses, as well as potential multiple dilutions and additional quality control (QC) samples. However, if CSIA is being used to study the origin of perchlorate in groundwater, sampling is more complex, often requiring several hundred liters of groundwater to be pumped through special ion exchange columns to trap perchlorate (Bohlke et al. 2009). For all CSIA analyses, the laboratory that will be analyzing the samples should be contacted for technical assistance and to ensure that proper sampling protocols for collecting, handling, and transporting the samples are followed.

## Quality Assurance/Quality Control

The quality assurance (QA)/QC program used for CSIA sampling depends on the application. EPA (2008a) offers QA/QC guidance for the application of CSIA to VOCs in groundwater. Further, the NELAC Institute has general standards for sample handling, data manipulation, training, documentation, and reporting, all of which are important issues in acquiring CSIA services but which are not often covered in technical methods or in method-specific SOPs (NELAC 2011). If NELAC Institute certification is not available from a laboratory, then the user should discuss each of these issues with the laboratory. Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific QA project plan (QAPP).

It should also be noted that the EPA guide (2008a) may not provide sufficient for application of CSIA to perchlorate or other non-VOC contaminants. QA/QC procedures and issues should be developed and discussed with the laboratory performing the analysis, the project manager, the regulators, and stakeholders prior to the collection of samples.

## Additional Information

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NELAC. 2011. “The NELAC Institute.” www.nelac-institute.org/index.php.

# Glossary

***cis*-DCE stall**—In biodegradation through reductive dechlorination, the parent chlorinated ethene is sequentially dechlorinated via the following process: (PCE →) TCE → *cis*-DCE → vinyl chloride → ethene. For a variety of reasons, the slowest step in the process is often the dechlorination of the *cis*-DCE. This phenomenon is known as "*cis*-DCE stall."

**environmental forensics**—The process of distinguishing contaminants from different sources.

**isotopic fractionation**—Some processes (for example, those which involve breaking chemical bonds) have slightly different rates for different isotopes, leading to a more rapid consumption of one isotope over the other. This characteristic is manifested in a change in the isotopic ratio of the residual compound.

**isotopic ratio**—The concentration of the heavy isotope normalized by the concentration of the light isotope.

**stable isotope**—A form of an element that does not undergo radioactive decay at a measureable rate.

# Polymerase Chain Reaction

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why is polymerase chain reaction relevant?

Polymerase chain reaction (PCR) is a technique that can test for the presence of the specific microorganism, family of microorganisms, or expressed genes in environmental samples such as soil, water, or sediment. When combined with traditional monitoring of contaminant concentrations over time, using PCR to identify microorganisms capable of degrading contaminants can provide project managers valuable information for site management and remedy selection. PCR therefore aids project managers (e.g., by providing a survey of specific microorganisms or developing targeted information for specific genes) in site conceptual model development, remedy selection and optimization, and determination of contaminant attenuation rates.

## What does PCR do?

PCR techniques were originally described in the 1960s, were popularized during the late 1980s and early 1990s within the biotechnology industry, and today are routinely used in medical diagnosis and in environmental detection of microorganisms. PCR is a laboratory method that generates multiple copies of a specific (target) DNA sequence, if present in a sample, representing microorganisms or groups of related microorganisms known to biodegrade contaminants. Reverse transcriptase PCR (RT-PCR) is a laboratory method that transforms RNA associated with biodegradation into complementary DNA (cDNA) that is then detected by PCR. Additionally, PCR can be used to amplify DNA sequences for use in further analysis of the sequence by other EMD techniques such as quantitative polymerase chain reaction (qPCR), microarray analysis, and microbial fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE). For more information, see the qPCR, Microarrays, and Microbial Fingerprinting Methods Fact Sheets. PCR has been used to detect microorganisms capable of degrading contaminants such as petroleum hydrocarbons, pentachlorophenol, perchlorate, polychlorinated biphenyls (PCBs), metals, radionuclides, and chlorinated solvents. PCR also has potential applicability in other environmental monitoring efforts, such as tracking of fecal microorganisms, so-called “microbial source tracking,” and identifying and tracking microorganisms potentially related to chemical compounds originating from industrial or energy-related activities.

## How are the data used?

During biodegradation processes, microorganisms break down contaminants using enzymes. PCR can be used to detect the presence of either (a) a specific microorganism or group of microorganisms that are known to be able to biodegrade a specific contaminant or group of contaminants or (b) DNA sequences (genes) that regulate the production of enzymes (proteins) that biodegrade or partially biodegrade these contaminants. The genes a microorganism possesses not only enable identification of the microorganism but also determine which enzymes that microorganism can produce and therefore which contaminants it can biodegrade. Depending on which genes are analyzed, PCR can tell whether either a particular enzyme or a particular microorganism known to biodegrade specific contaminants is present in a sample. Detection of specific genes or microorganisms capable of biodegradation of a contaminant provides a direct line of evidence that bioremediation may be possible at a site. Additionally, this method can be used to analyze the diversity of the microbial community and relative changes in the community diversity in response to remedial activities.

### Example Environmental Remediation Questions PCR Can Help Answer

- **Site Characterization**
  - What known organisms or functional genes are present?
  - How diverse is the microbial community?
- **Remediation**
  - Are the right microorganisms and/or genes present that are capable of degrading the contaminant? If so, how many (qPCR) and where?
  - Are the key microbial pathways active (RT-PCR)?
  - Is monitored natural attenuation (MNA) feasible?
  - Should an amendment be added (biostimulation)?
  - Is bioaugmentation necessary?

## How does it work?

Microorganisms contain deoxyribonucleic acids (DNA) composed of long series of nucleotides represented by the letters A, T, C, and G. Some DNA sequences (the arrangement of the letters one after the other of this four-letter alphabet) are unique to specific organisms and can be used in PCR to determine whether that organism is present in a sample. PCR capitalizes on the ability of DNA polymerase (the enzyme that copies a cell's DNA before it divides in two) to synthesize new strands of DNA complementary to a template DNA strand. The PCR laboratory method selectively amplifies only the genes of interest (if present) in a sample. Sample preparation involves harvesting and concentrating microbial cells from the soil or groundwater sample (e.g., by filtration) and breaking these cells open to release their DNA (Figure 1). A typical reaction mixture contains template DNA (environmental DNA), short DNA primers specific to the target gene, DNA building blocks (e.g., deoxyribonucleotide triphosphates [dNTPs]) and a heat-stable DNA-synthesizing enzyme (DNA polymerase). This mixture is repeatedly cycled through a precise temperature sequence that leads to the exponential increase in the number of copies of the target gene (amplification) (see EPA 2004 for more information).

As shown in Figure 1, there are four steps in the PCR process: (a) In the first step the sample temperature is raised from room temperature (25°C) to 94–97°C, and the double-stranded DNA "unzips" into single strands (denaturation). (b) At 47–60°C the primers attach to the target sequence (annealing). (c) At 72°C DNA polymerase continues attaching dNTPs to each strand (elongation) until there are two double-stranded copies of the target sequence for each double-stranded copy available at the first step. (d) Finally, the sample temperature is raised to 94–97°C again, and the cycle is repeated 30–40 times to achieve the desired amplification of gene copies.

## How are the data reported?

When PCR is used to target a specific gene or microorganism, data from PCR are reported simply as present/absent for the target sequence. However, when the products from PCR are used in another EMD method, more detailed information is available. See the qPCR, Microarrays, and Microbial Fingerprinting Methods Fact Sheets for further information.

### Variations on PCR

**Quantitative PCR**—qPCR estimates the number of specific sequences or genes and by inference the number of microorganisms or groups of microorganisms in a soil, sediment, or groundwater sample. (See the qPCR Fact Sheet for further information.) There are significantly fewer qPCR targets than PCR targets to date.

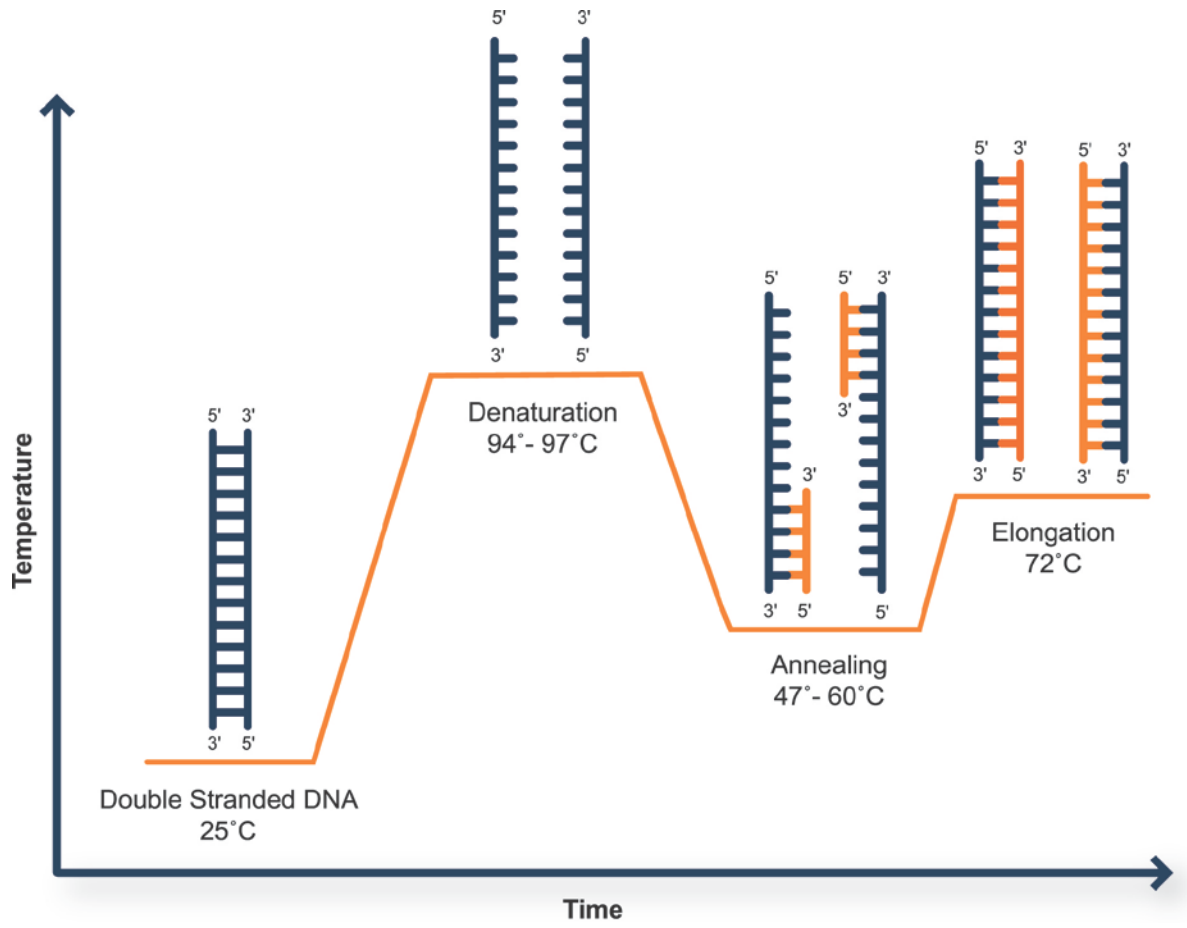


**Figure 1. The PCR process.** *Source:* EPA 2004.

**Reverse transcriptase PCR**—RNA is a nucleic acid, and the production of RNA is a necessary step to "read" genes and produce enzymes. If RNA related to biodegradation is detected in soil or groundwater, it can be inferred that microorganisms are actively making enzymes and thus biodegrading contaminants. This information allows the project manager to discriminate between microorganisms actively degrading contaminants from those microorganisms that may have the capability for biodegradation but may not be degrading contaminants for a variety of reasons. In RT-PCR, the RNA associated with biodegradation is transformed by a chemical reaction in the laboratory (so-called reverse transcription) into cDNA that can then be detected by PCR. When combined with qPCR (RT-qPCR), this method is quantitative (see the qPCR Fact Sheet).

**Nested PCR**—Nested PCR is used to increase the specificity and/or the sensitivity of the PCR method. In nested PCR, two sets of primers are used: the first primer amplifies a specific region/gene in the microorganism's DNA, and the second primer amplifies a sequence within the original sequence.

## Advantages

- PCR analyses are capable of detecting specific microorganisms or target genes within diverse microbial communities such as those present in environmental samples.
- PCR results are available within days, as the technique does not require growing the target microorganisms (which can be difficult, time-consuming, and not always possible).
- PCR analyses are sensitive (can detect as few as 100 cells or gene copies in a sample, e.g., 100 mg of soil or sediment or 1–10 L water).

- PCR analyses can be performed on a variety of sample types (e.g., water, soil, sediment).
- PCR analyses can be used to survey the general microbial community or target specific genes.

## Limitations

- Although not particularly common, PCR results can be affected by the presence of some metals or humic acids in the environmental sample. Samples exhibiting PCR inhibition should be readily identified with basic quality assurance (QA)/quality control (QC) procedures. Furthermore, inhibition can often be eliminated with minor modifications to the DNA extraction procedure.
- Standardization of protocols for sample collection, storage, and extraction between laboratories is currently under way but is not yet complete. There is U.S. Environmental Protection Agency (EPA) guidance for QC for PCR analysis (EPA 2004).
- The development of PCR analysis is based on known biodegradation pathways and gene sequences. With ongoing research, additional PCR analyses will be developed to expand the applicability of the technique to other contaminants and newly identified biodegradation pathways.

## Sampling Protocols

Sample matrices that can be analyzed by PCR include soil, sediment, groundwater, and filters. Sampling for microbiological samples can be easily incorporated into routine environmental monitoring programs. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile sample containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at 4°C during handling and transport to the laboratory. Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by EPA or other government agencies. However, in 2004 the EPA published the *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples*, which is a useful guide for laboratories performing PCR. In addition, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific QA project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal QA/QC information available (such as results for positive and negative controls). PCR analyses include both positive and negative controls to ensure that the PCR reaction occurred properly, i.e., that it was not inhibited by interfering substances in the reaction mixture and that it amplified only the target sequence and not other nontarget DNA. Both positive and negative controls should be included and undergo the exact same PCR protocol as the environmental samples.

## Additional Information

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## Glossary

**bioaugmentation**—The introduction of cultured microorganisms into the subsurface environment for the purpose of enhancing bioremediation of organic contaminants (EPA 2011).

**biodegradation**—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**deoxyribonucleotide triphosphates (dNTPs)**—dNTPs are incorporated into DNA during elongation (EPA 2004).

**enzyme**—Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on EPA 2004).

**gene**—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

**microbial community**—The microorganisms present in a particular sample.

**nucleic acid**—A complex biomolecule consisting of a long "backbone" of organophosphate sugars with nucleotide bases attached.

**primers**—Short strands of DNA that are complementary to the beginning and end of the target gene and thus determine which DNA fragment is amplified during PCR or qPCR.

**protein**—Large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds (U.S. Navy 2009).

**RNA (ribonucleic acid)**—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

**transcription**—The first step in activation of a biochemical pathway where a complementary RNA copy is synthesized from a DNA sequence.

**translation**—The second step of gene expression where messenger RNA (mRNA) produced by transcription is decoded by the cell to produce an active protein.

# Quantitative Polymerase Chain Reaction

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why is quantitative polymerase chain reaction relevant?

Quantitative polymerase chain reaction (qPCR) and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) are used to quantify the abundance and activity of specific microorganisms or expressed genes in pathways capable of biodegradation of contaminants present at a contaminated site. When microorganisms capable of biodegradation of the contaminants are relatively abundant and active under existing subsurface conditions, monitored natural attenuation (MNA) can be an effective site management approach; qPCR provides a means to establish such situations. On the other hand, qPCR results can reveal when contaminant-using microorganisms are present but not thriving, thus providing evidence that enhanced bioremediation options may need to be explored to stimulate the microbial community. Enhanced bioremediation, or biostimulation, involves addition an amendment such as an electron donor (e.g., lactate, emulsified vegetable oil) or acceptor (e.g., oxygen, sulfate) for the specific purpose of stimulating growth and activity of microorganisms capable of biodegradation of the contaminants. Thus, project managers can use qPCR in the characterization phase, remedy selection, monitoring, and site closure to assess microbial growth and activity. Within the environmental restoration industry, qPCR analyses have been offered on a commercial basis since 2002. In total, qPCR has been used to evaluate contaminant biodegradation in all but four states in the United States and countries representing six different continents.

## What does qPCR do?

qPCR is an analytical method used to determine the number of copies of specific genes or DNA targets present in a sample. Depending on which gene is investigated, the qPCR analysis can quantify functional genes (e.g., trichloroethene [TCE] reductase [*tceA*]), specific microorganisms (e.g., *Dehalococcoides*), or groups of related microorganisms (e.g., sulfate-reducing bacteria) in soil, sediment, or groundwater samples. Like polymerase chain reaction (PCR, see the PCR Fact Sheet), qPCR requires specific primers to enable it to selectively amplify (and detect) individual low-abundance genes in samples containing many millions of other genes. As the name suggests, qPCR adds quantitation to the underlying PCR technology.

Since development in the early 1990s, qPCR has been used to study a wide variety of environmental processes, including biodegradation of chlorinated solvents such as perchloroethene (PCE), TCE, etc. (Davis et al. 2008, Hendrickson et al. 2002, Lee et al. 2008); petroleum hydrocarbons such as benzene, toluene, ethylbenzene, and xylenes (Baldwin et al. 2010); polycyclic aromatic hydrocarbons (DeBruyn, Chewning, and Sayler 2007); fuel oxygenates such as methyl *tert*-butyl ether (Hristova et al. 2003); and radionuclides (Amos et al. 2007). qPCR analyses have potential applicability in other environmental monitoring efforts, such as tracking of fecal microorganisms, so-called “microbial source tracking,” and identifying and tracking microorganisms potentially related to chemical compounds originating from industrial or energy-related activities.

## How are the data used?

Data generated from qPCR analyses provide information about the identity and abundance of specific microorganisms that may be present and capable of biodegrading identified contaminants. During biodegradation processes, microorganisms break down contaminants using enzymes. The genes a microorganism possesses not only enable identification of the microorganism but also determine which enzymes that microorganism can produce and therefore which contaminants it can biodegrade. As mentioned, qPCR and RT-qPCR are used to measure the abundance of specific genes in a sample of

soil or groundwater. Depending on which genes are quantified, this information is indicative of the abundance of genes that encode for a particular enzyme (e.g., *tceA*) or the abundance of microorganisms that are known to biodegrade specific contaminants (e.g., *Dehalococcoides*). Quantification of specific genes or microorganisms capable of biodegradation of a contaminant provides a direct line of evidence that helps answer questions about site remediation.

### Example Environmental Remediation Questions qPCR and RT-qPCR Can Help Answer

- **Site Characterization**
  - Assess current conditions and potential for biodegradation
    - Are microorganisms capable of biodegradation of the contaminant present?
    - If so, how many are present?
    - Are key contaminant-degrading microorganisms (qPCR) and biodegradation pathways (RT-qPCR) active?
  - Preliminary identification of remediation alternatives
- **Remediation**
  - Is MNA feasible?
    - Are microorganisms capable of biodegradation of the contaminant present?
    - Are contaminant-degrading microorganisms present in sufficient abundance under existing site conditions?
    - Are contaminant-degrading microorganisms and biodegradation pathways active under existing site conditions (RT-qPCR)?
  - Is biostimulation necessary? Should an amendment be added?
    - Will adding an amendment such as an electron donor (e.g., emulsified vegetable oil) or an electron acceptor (e.g., oxygen) stimulate growth of contaminant using microorganisms?
    - Will amendment addition promote activity of contaminant-degrading microorganisms?
  - Is bioaugmentation necessary?
- **Monitoring**
  - Monitored natural attenuation
    - Are contaminant-degrading populations maintained over time under existing site conditions?
  - Biostimulation
    - Did amendment addition promote growth or activity of contaminant-using microorganisms?
    - Are contaminant-degrading populations maintained over time?
    - Should a second amendment addition be considered?
  - Bioaugmentation
    - Did microbial culture survive in situ?
    - Is the microbial culture maintained over time?
- **Closure**
  - Are contaminant-degrading populations abundant and stable, suggesting that contaminant degradation is likely to continue?

In terms of site characterization and remedy selection, MNA is unlikely to be a feasible site management strategy when qPCR results indicate that contaminant-using populations are not present under existing site conditions. Conversely, when target microbial populations are detected, MNA is at least possible. However, when target microbial populations or activity are low, MNA may not achieve site closure goals in an acceptable time frame, and enhanced bioremediation options may need to be considered. For example, at TCE-impacted sites, the detection of *Dehalococcoides* indicates the potential for complete reductive dechlorination. However, Lu, Wilson, and Kampbell (2006) suggest that a *Dehalococcoides* population of $10^7$ cells per liter is required for “generally useful” rates of reductive dechlorination and effective MNA. With increased use in the industry, empirical relationships between biodegradation rates for different types of contaminants and other target microbial populations will be developed to aid in assessment of MNA. Additionally, RT-qPCR can identify whether or not specific genes are being expressed for contaminant biodegradation.

Enhanced bioremediation, or biostimulation, involves addition of an amendment such as an electron donor (e.g., lactate, emulsified vegetable oil) or acceptor (e.g., oxygen, sulfate) for the specific purpose of stimulating growth and activity of microorganisms capable of biodegradation of the contaminants. Thus, qPCR or RT-qPCR results should reveal an increase in the abundance or activity of contaminant-degrading microorganisms relative to the baseline in response to the amendment. When qPCR analyses, performed as a component of site characterization, indicate that contaminant-degrading microorganisms are not present or present in low abundance, bioaugmentation (i.e., addition of microorganisms) may be a viable remedy to promote bioremediation. Similar to the discussion for biostimulation, qPCR analyses are used to document the in situ maintenance of key members of the commercial culture (e.g., *Dehalococcoides*) or may reveal decreases that suggest that another injection of electron donor or microbial culture may be required to permit continued biodegradation.

## How does it work?

**qPCR**—qPCR is a laboratory analytical method that selectively amplifies and quantifies genes of interest in a sample. Sample preparation involves harvesting and concentrating microbial cells from the soil or groundwater sample (e.g., by filtration) and breaking these cells open to release their deoxyribonucleic acid (DNA). As described in the PCR Fact Sheet, a typical reaction mixture contains template DNA (environmental DNA), short DNA primers specific to the target gene, DNA building blocks (deoxyribonucleotide triphosphate [dNTPs]), and a heat-stable DNA-synthesizing enzyme (DNA polymerase). This mixture is repeatedly cycled through a precise temperature sequence that leads to the exponential increase in the number of copies of the target gene (amplification). For qPCR, fluorescent (light-emitting) dyes or fluorescently labeled “probes” which adhere or attach to the DNA are also added to the reaction mixture. During the amplification process, fluorescence from the dye or released from the probe is measured. As the number of target gene copies increases, the amount of light emitted increases and eventually exceeds a threshold level. The number of cycles required to exceed this threshold level of light emission, the threshold cycle ($C_t$), is proportional to the initial amount of target gene in the sample. The amount of the target gene in an environmental sample is calculated using a calibration curve relating $C_t$ values to known amounts of the target gene (standards).

**RT-qPCR**—In RT-qPCR, ribonucleic acid (RNA) rather than DNA is extracted from the sample and converted (reverse transcribed) into DNA known as “complementary” or “cDNA.” The remainder of the procedure is the same as described for qPCR. RNA is a short-lived type of molecule central to production of proteins including enzymes, with RNA rather than DNA as the basis of the analysis. RNA involvement in enzyme production, including those responsible for contaminant biodegradation, is a two-step process. First, the gene (DNA) is transcribed into a short-lived, intermediate molecule called messenger RNA (mRNA). In the second step, mRNA is translated into the corresponding protein (enzyme). A typical cell contains literally thousands of individual genes, many encoding enzymes responsible for a broad spectrum of different functions. Naturally, a cell does not need all of those enzymes to be produced all of the time. In the interest of efficiency, the microorganism regulates enzyme production where transcription of some genes into mRNA is “turned off” and “turned on” only when needed. Therefore, RT-qPCR with RNA rather than DNA as the basis of the analysis quantifies the expression of target genes and activity of specific microorganisms.

## How are the data reported?

qPCR results are often presented as gene copies per milliliter of groundwater or per gram of soil. In many cases, a cell contains only one copy of the target gene such that gene copies and cell numbers are equal. For instances when a cell contains multiple copies of the target gene, the reported number can be converted based on knowledge of the number of target gene copies per cell. RT-qPCR results are reported as gene copies per milliliter of groundwater or per gram of soil. An active microorganism transcribes many mRNA or rRNA copies even from a single target gene.

## Advantages

- Cultivation-dependent techniques like plate counts are laborious, time-consuming, and most importantly under-representative, because the overwhelming majority (>99%) of microorganisms present in the environment cannot be grown in the laboratory (Amann, Ludwig, and Schleifer 1995 and references therein). Cultivation-independent methods like qPCR and RT-qPCR do not require growing the target microorganisms and provide more accurate quantification than traditional methods.
- A qPCR analysis is capable of detecting specific microorganisms or target genes in the complex mixture of other (nontarget) microorganisms present in environmental samples.
- Results are typically available within days.
- Typical method detection limits (100 target gene copies) are several orders of magnitude lower than the target population required to achieve a "reasonable" rate of contaminant biodegradation.
- Can be performed with DNA obtained from a variety of sample types (e.g., water, soil, sediment, passive microbial sampling devices).
- qPCR analyses have been developed to monitor key microorganisms and processes responsible for biodegradation of a broad spectrum of common environmental contaminants (see Table 1).
- RT-qPCR is used to assess target gene expression and estimate microbial activity.

## Limitations

- The development of qPCR analyses is based on known biodegradation pathways and gene sequences. With ongoing research, additional qPCR analyses will be developed to expand the applicability of the technique to other contaminants and newly identified biodegradation pathways.
- Although not particularly common, qPCR results can be affected by the presence of some metals or humic acids in the environmental sample. Samples exhibiting PCR inhibition should be readily identified with basic quality assurance (QA)/quality control (QC) procedures. Furthermore, inhibition can often be eliminated with minor modifications to the DNA extraction procedure.
- RT-qPCR must be used to distinguish between dead cells containing the target gene and live cells.
- Though each laboratory employs its own methodology, prescribed standardized protocols for sample collection, storage, preservation, DNA extraction, and nucleic acid targets do not currently exist. Efforts to generate standard operating procedures (SOPs) are currently under way (Lebrón et al. 2008, Hatt et al. 2011).

## Available qPCR Targets

Table 1 shows the gene targets for qPCR analyses that are currently (2011) commercially available for assessing biodegradation pathways for a broad spectrum of contaminants. Additional qPCR and RT-qPCR analyses may be available at academic and research laboratories; others will be developed as new biochemical pathways are discovered and corresponding genes are identified. Selecting an appropriate qPCR analysis depends on the contaminant and the most likely biodegradation pathway, given the current redox conditions.

## Sampling Protocols

Sample matrices that can be analyzed by qPCR include soil, sediment, groundwater, and filters. Sampling for microbiological samples can be easily incorporated into routine environmental monitoring programs. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at 4°C during handling and transport to the laboratory. Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

## Table 1. Current qPCR gene targets for assessing biodegradation pathways

| Contaminant Group | Contaminants | Target | Redox Conditions | Environmental Relevance / Data Interpretation |
|---|---|---|---|---|
| Chlorinated Solvents | PCE, TCE, DCE, VC | ***Dehalococcoides*** | Anaerobic | • Only known group of bacteria capable of complete dechlorination of tetrachloroethene (PCE) and/or trichloroethene (TCE) to ethene. |
| Chlorinated Solvents | TCE and DCE | **TCE Reductase** | Anaerobic | • *Dehalococcoides* functional genes encoding reductive dehalogenases for trichloroethene (TCE) and *cis*-dichloroethene (*cis*-DCE). |
| Chlorinated Solvents | VC | **Vinyl Chloride Reductase** | Anaerobic | • *Dehalococcoides* functional genes encoding reductive dehalogenases for vinyl chloride (VC). |
| Chlorinated Solvents | TCA, PCE, TCE | ***Dehalobacter*** | Anaerobic | • Capable of dechlorination of PCE and TCE to *cis*-DCE.<br>• Some strains capble of converting TCA, a common co-contaminant at PCE/TCE-impacted sites, to chloroethane. |
| Chlorinated Solvents | PCE, TCE | ***Desulfuromonas*** | Anaerobic | • Capable of dechlorination of PCE and TCE to *cis*-DCE using acetate as an electron donor. |
| Chlorinated Solvents | PCE, TCE, DCA, DCP | ***Desulfitobacterium*** | Anaerobic | • Capable of dechlorination of PCE and TCE to *cis*-DCE.<br>• Some strains capable of dechlorination of chlorophenols, 1,2-dichloroethane and 1,2-dichloropropane. |
| Chlorinated Solvents | Chlorobenzene | **Toluene Dioxygenase** | Aerobic | • Catalyzes biodegradation of chlorobenzene by incorporation of oxygen into the aromatic ring. |
| | | | | |
| Petroleum Hydrocarbons | BTEX | **Ring-hydroxylating Toluene Monooxygenases** | Aerobic | • Catalyzes the initial (and sometimes second) hydroxylation of BTEX compounds. |
| Petroleum Hydrocarbons | BTEX | **Phenol Hydroxylase** | Aerobic | • Catalyzes further oxidation of BTEX compounds. |
| Petroleum Hydrocarbons | T, X | **Toluene/Xylene Monoxygenase** | Aerobic | • Attacks toluene and xylenes at the methyl group. |
| Petroleum Hydrocarbons | B, T, chlorobenzene | **Toluene Dioxygenase** | Aerobic | • Catalyzes biodegradation of benzene and toluene by incorporation of oxygen into the aromatic ring. |
| Petroleum Hydrocarbons | Naphthalene | **Naphthalene Dioxygenase** | Aerobic | • Catalyzes aerobic biodegradation of naphthalene and other PAHs by incorporation of oxygen into the aromatic ring. |
| Petroleum Hydrocarbons | Naphthalene and PAHs | **Naphthalene-Inducible Dioxygenase** | Aerobic | • Catalyzes aerobic biodegradation of naphthalene and other PAHs by incorporation of oxygen into the aromatic ring. |
| Petroleum Hydrocarbons | Alkanes | **Alkane Monooxygenase** | Aerobic | • Catalyzes biodegradation of straight chain petroleum hydrocarbons. |
| Petroleum Hydrocarbons | MTBE | **MTBE utilizing PM1** | Aerobic | • Targets *Methylibium petroleiphilum* PM1, one of the few bacteria isolated that is capable of growth on MTBE |
| Petroleum Hydrocarbons | T, X | **Benzylsuccinate Synthase** | Anaerobic | • Targets gene encoding enzyme in anaerobic biodegradation of toluene. |
| | | | | |
| Cometabolism Chlorinated Solvents | TCE | **Methanotrophs** | Aerobic | • Targets two types of methane oxidizing bacteria (methanotrophs)<br>• Indicates the potential for cometabolic oxidation of TCE |
| Cometabolism Chlorinated Solvents | TCE | **Soluble Methane Monooxygenase** | Aerobic | • Targets the soluble methane monooxygenase gene<br>• Soluble methane monooxygenases are generally believed to support faster cometabolism of TCE |
| | | | | |
| Perchlorate | Perchlorate, chlorate | **Perchlorate Reductase** | Anoxic | • Catalyzes reduction of perchlorate and in most isolates reduction of chlorate to chlorite<br>• Presence indicates the potential for aerobic BTEX biodegradation. |
| | | | | |
| Polychlorinated biphenyls | PCBs (lightly chlorinated) | **Biphenyl Dioxygenase** | Aerobic | • Catalyzes catalyzes initial oxidation of lightly chlorinated PCB congeners. |

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency (EPA) or other government agencies. However, most laboratories work under SOPs and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Data quality should be evaluated by detailing the laboratory requirements in a site-specific QA project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal QA/QC information available (such as results for positive and negative controls). Data reports include a lower quantification limit, a practical quantification limit, and data quality "flags" such as estimated value (J) similar to those of more routine chemical analyses. In 2004 EPA published *Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples*, which contains sections relevant to qPCR.

Under the Strategic Environmental Research and Development Program (SERDP) Project ER-1561, standard protocols are being developed, with guidance for collecting, preserving, storing, transporting, and processing samples for analyses, as well as evaluating results under various conditions (e.g., potential inhibitors and the impacts, if any, caused by monitoring well structure). Additionally, standardized *Dehalococcoides* reference materials and internal microbial controls are being designed to facilitate comparison of qPCR results between laboratories and to monitor the efficiency of each step from sample collection to quantification for qPCR methods. An assessment of the biases affecting the accuracy, precision, and reproducibility of qPCR analyses has been reported (Lebrón et al. 2008, Hatt et al. 2011).

## Additional Information

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## Glossary

**contaminant-degrading population**—The group of organisms that are capable of degrading a particular contaminant.

***Dehalococcoides***—A specific group (genus) of bacteria. *Dehalococcoides* species are the only microorganisms that have been isolated to date which are capable of complete reductive dechlorination of PCE and TCE to ethene. Some *Dehalococcoides* species are also capable of reductive dechlorination of chlorinated benzenes, chlorinated phenols, and polychlorinated biphenyls.

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**dNTPs (deoxyribonucleotide triphosphates)**—dNTPs are incorporated into DNA during elongation (EPA 2004).

**electron acceptor**—A chemical compound that accepts electrons transferred to it from another compound (based on EPA 2011).

**electron donor**—A chemical compound that donates electrons to another compound (based on EPA 2011).

**enzyme**—Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on EPA 2004).

**functional gene**—A segment of DNA that encodes an enzyme or other protein that performs a known biochemical reaction. For example, the functional gene *tceA* encodes the reductive dehalogenase enzyme that initiates reductive dechlorination of TCE. Other genes can code for RNA entities which can regulate the activity of other DNA target sequences.

**gene**—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

**microbial community**—The microorganisms present in a particular sample.

**nucleic acid**—A complex biomolecule consisting of a long "backbone" of organophosphate sugars with nucleotide bases attached.

**primers**—Short strands of DNA that are complementary to the beginning and end of the target gene and thus determine which DNA fragment is amplified during PCR or qPCR.

**probes**—(1) short DNA strands (see *microarray probes*, Microarray Fact Sheet; *FISH probes*, FISH Fact Sheet; *qPCR probes*); (2) surrogate compounds (see *enzyme activity probes*, EAP Fact Sheet).

**qPCR probes**—Short, defined segments of DNA or RNA, that may or may not be labeled and that are designed to bind with the target gene if found in the environmental sample.

**qPCR target (target gene)**—The specific gene quantified by a particular qPCR analysis. For example, vinyl chloride reductase genes are the target genes in qPCR analyses performed to assess reductive dechlorination of vinyl chloride to ethene. Similarly, a qPCR analysis targeting the toluene dioxygenase gene is used to evaluate aerobic biodegradation of toluene and benzene.

**RNA (ribonucleic acid)**—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

**transcription**—The first step in activation of a biochemical pathway where a complementary RNA copy is synthesized from a DNA sequence.

**translation**—The second step of gene expression where messenger RNA (mRNA) produced by transcription is decoded by the cell to produce an active protein.

# Microbial Fingerprinting Methods

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why are microbial fingerprinting methods relevant?

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

## What does microbial fingerprinting do?

Microbial fingerprinting methods are a category of techniques that differentiate microorganisms or groups of microorganisms based on unique characteristics of a universal component or section of a biomolecule (e.g., phospholipids, DNA, or RNA). Microbial fingerprinting methods provide an overall profile of the microbial community, and some can be used to identify subsets of the microorganisms present. The types of microbial fingerprinting methods described below include phospholipid fatty acid (PLFA) analysis, denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (T-RFLP). DGGE and T-RFLP are also known as genetic fingerprinting methods. Microbial fingerprinting methods have been used to investigate microbial communities at many different environmental remediation sites, ranging from metal-contaminated sites (EPA 2009) to retail gasoline stations (Nales, Butler, and Edwards 1998) to Superfund sites (EPA 2006). Microbial fingerprinting methods also have potential applicability in other environmental monitoring efforts, such as tracking of fecal microorganisms, so-called “microbial source tracking,” and identifying and tracking microorganisms potentially related to chemical compounds originating from industrial or energy-related activities.

## How are the data used?

Data generated from microbial fingerprinting methods are used to understand which microorganisms are present and how they are intrinsically coupled to their environmental conditions. For example, geochemical conditions (such as the availability of electron acceptors) influence which microorganisms are present and active at a site, while the microbial activities (such as electron acceptor consumption) can strongly impact the site geochemistry. A microbial fingerprinting method therefore can provide valuable information as to whether subsurface conditions are conducive to bioremediation and in evaluating the effectiveness of monitored natural attenuation (MNA). Most engineered bioremediation strategies involve the addition of an amendment to stimulate the growth and activity of specific groups of microorganisms capable of performing desired processes. Microbial fingerprinting methods can also be used to track the overall changes in the microbial community over time or in response to remediation activities. Data gathered from the microbial fingerprinting methods then can be used to evaluate the performance of the bioremediation strategy.

### Example Environmental Remediation Questions Microbial Fingerprinting Methods Can Help Answer

- **Site Characterization**
  - Assess current conditions and potential for biodegradation
    - Are conditions conducive to microbial activity?
    - How diverse is the microbial community?
    - What are the dominant microorganisms present?
  - Emerging contaminants
    - What types of microorganisms are present in impacted wells?
- **Remediation**
  - Is MNA feasible?
    - What is the microbial biomass?
    - Are conditions conducive to microbial activity?
    - What are the dominant microorganisms present under existing conditions?
    - What microorganisms are detected in impacted versus nonimpacted wells?
  - Is biostimulation necessary? Should an amendment be added?
    - Will adding an amendment such as an electron donor (e.g., emulsified vegetable oil) or an electron acceptor (e.g., oxygen) increase total biomass?
    - What kind of microorganisms will respond to the amendment?
- **Monitoring**
  - Physical/chemical treatment
    - Did biomass decrease after physical/chemical treatment?
    - Was the microbial community adversely impacted?
    - Did the microbial community recover?
    - Was there a shift in the dominant members of the microbial community?
    - Is biodegradation feasible as a subsequent polishing step?
  - MNA
    - What is the microbial biomass?
    - Are conditions conducive to microbial activity?
    - What are the dominant microorganisms present under existing conditions?
    - What microorganisms are detected in impacted versus nonimpacted wells?
  - Biostimulation
    - Did microbial biomass increase in response to amendment?
    - Is biomass maintained over time?
    - What kinds of microorganisms responded to the amendment?
    - Is the shift in the microbial community consistent with the biostimulation strategy?
- **Closure**
  - Do formerly impacted wells have a diverse microbial community?
  - How do these microbial communities compare to background?

## What are the different microbial fingerprinting methods, and how do they work?

### PLFA Analysis

Phospholipids are a primary structural component of the membranes of all living cells and break down rapidly upon cell death. Therefore, the mass of PLFAs in a sample is a direct measure of the viable biomass in the sample. While all cell membranes contain phospholipids, not all organisms or groups of organisms contain the same PLFA types in the same proportions. Some classes of organisms produce unique or “signature” types of PLFA (Hedrick et al. 2000). Quantifying these PLFA groups therefore creates a profile or fingerprint of the viable microbial community and provides insight into several important microbial functional groups (e.g., iron- and sulfate-reducing bacteria).

PLFA analysis is similar to quantification of other chemical compounds present as mixtures (e.g., volatile organic compounds) in environmental samples: (1) extraction, (2) separation by gas chromatography with flame ionization detection, and if necessary, (3) confirmation of identification by mass spectroscopy. PLFA analysis can also be combined with stable isotope probing (SIP) to demonstrate that biodegradation is occurring by quantifying incorporation of the stable isotope label into biomass (see the SIP Fact Sheet for more information). PLFA analysis is commercially available.

## DGGE Analysis

DGGE is a nucleic acid (DNA or RNA)–based technique used to generate a genetic fingerprint of the microbial community and potentially identify dominant microorganisms. DGGE profiles are most often used to compare differences or changes in microbial community diversity and structure between samples, over time or space or in response to treatment. DGGE usually encompasses a four-step process: (1) DNA or RNA extraction, (2) amplification, (3) separation and visualization, and (4) sequence identification. The amplification step uses polymerase chain reaction (PCR) to generate a multitude of copies of a variable region within a target gene (see the PCR Fact Sheet for more information). The DNA sequence of this variable region is different for each type of bacteria. Thus, the PCR step generates a mixture of the gene segments each representing a species present in the original sample. The third step of DGGE uses an electric current (electrophoresis) and a denaturing process to separate this mixture based on the DNA sequence, producing a profile, or fingerprint, of the microbial community. Figure 1 shows a typical acrylamide gel image: a subset of the individual "bands" are excised (physically cut) from the gel, the DNA sequence is determined for each excised band, and the resulting DNA sequence is compared to a database to identify the microbial population corresponding to each band (Muyzer, de Waal, and Uitterlinden 1993). Further interpretation is based largely on linking site conditions and activities to general characteristics of the microorganisms that were identified in the sample. DGGE is commercially available.

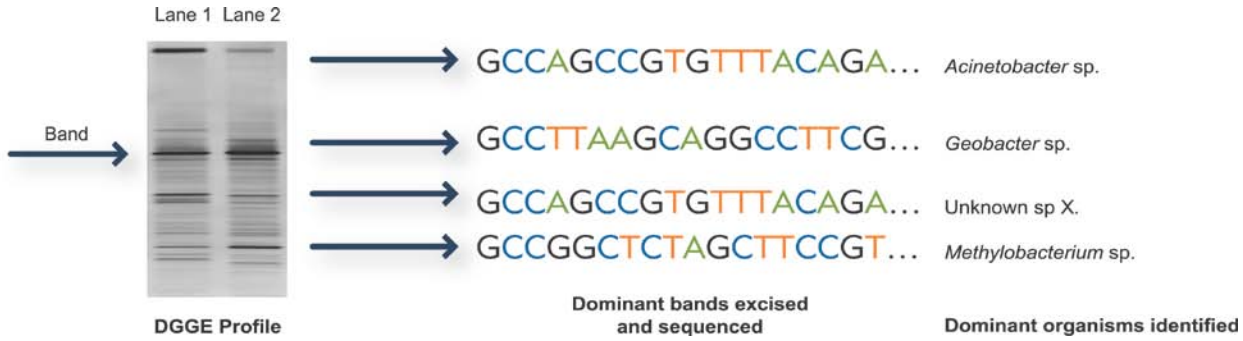


**Figure 1. DGGE bands, with diagnostic sequences and identifications of the microorganisms responsible for a given band.** The figure illustrates the separation and visualization (step 3) and sequence identification (step 4) aspects of the DGGE fingerprinting method.
*Source:* Microbial Insights, Inc., 2010, used with permission.

## T-RFLP Analysis

T-RFLP has also been employed to characterize microbial communities (Osborn, Moore, and Timmis 2001). Similar to DGGE, T-RFLP is a nucleic acid (DNA or RNA)–based technique that provides a fingerprint of the microbial community and can be used to identify specific microbial populations. T-RFLP is a four-step process: (1) DNA or RNA extraction, (2) PCR amplification, (3) enzyme digestion, and (4) fragment identification. Following isolation of the total community DNA or RNA, PCR amplification with a fluorescent PCR primer is used to make multiple copies of a target gene (see the PCR Fact Sheet for additional information), and the PCR products are then digested with restriction enzymes that cut the DNA molecule at known sequences. The size of each resulting terminal restriction fragment is indicative of a specific microorganism. T-RFLP offers greater sensitivity than DGGE (i.e., it may detect microorganisms that are present at lower numbers in a sample). T-RFLP is commercially available.

Table 1 presents a comparison of the microbial fingerprinting methods PLFA, DGGE, and T-RFLP.

**Table 1. Comparison of microbial fingerprinting methods**

| Method | Biomolecule | Quantitative | Identification | Level of prior knowledge required | Commercially available |
|---|---|---|---|---|---|
| PLFA | Phospholipids | Yes | No | None | Yes |
| DGGE | DNA or RNA | No | Yes (genus)[a] | Must choose target kingdom (Bacteria, Fungi, Archaea) | Yes |
| T-RFLP | DNA or RNA | No | Yes (genus)[a] | Must choose target kingdom (Bacteria, Fungi, Archaea) | Yes |

[a] Number of microorganisms that can be identified depends on the complexity of the sample.

## How are the data reported?

For PLFA, the total biomass in the sample is presented as the total number of cells per milliliter of groundwater or per gram of soil. Community structure is presented in the percentage of the different functional groups (e.g., iron reducers, sulfate reducers, or fermenters). The physiological responses of Proteobacteria (organisms which change their PLFA in response to different environmental stresses) are reported as decreased permeability and slowed growth ratios. These ratios are best used in long-term monitoring projects where multiple measurements are taken over time and trends are evaluated over time (Hedrick et al. 2000).

For DGGE and T-RFLP, the identities of the dominant genera within the community are presented (Muyzer, de Waal, and Uitterlinden 1993). A DGGE report typically includes a photograph of an acrylamide gel similar to that shown in Figure 1, the family or genus of the microorganisms identified, and the similarity index to gauge how well the DNA sequence recovered from the sample matches that found in the comparison database. However, since individual “bands” are excised from the gel for sequencing, typically only 3–10 microorganisms are identified by DGGE analysis. The number of microorganisms that can be identified by T-RLFP can be 10 times greater, providing more comprehensive examination of the microbial community composition (Osborn, Moore, and Timmis 2001).

## Advantages

- The microbial fingerprinting methods discussed are cultivation independent, meaning that they do not require growth of the microorganisms in the laboratory. Laboratory cultivation is difficult, time-consuming, and not always possible for several important microorganisms.
- In general, microbial fingerprinting methods require little prior knowledge about which microorganisms are of interest. So these methods may be useful for emerging contaminants (i.e., contaminants for which little information is currently available).
- Microbial fingerprinting methods can capture the presence and activity of uncultured and previously unidentified microorganisms.
- PLFA analysis provides a direct measure of viable biomass in addition to a biochemical profile of the microbial community.
- PLFA analysis can be used in conjunction with SIP to document that biodegradation is occurring (see the SIP Fact Sheet for more information). Fingerprinting techniques based on DNA can also be used with SIP but often require greater quantities of the labeled compound.
- The genetic fingerprinting methods allow identification of some members of the microbial community to the family or genus level.

## Limitations

- PLFA analysis cannot be used to identify specific microorganisms.
- Genetic fingerprinting methods (e.g., DGGE, T-RFLP) can be used to identify specific microorganisms. However, the number of microorganisms that can be identified depends on the complexity of the microbial community.

- The genetic fingerprinting methods are not quantitative. See the Quantitative Polymerase Chain Reaction (qPCR) Fact Sheet for quantification of a specific functional gene or group of microorganisms.
- Important microbial processes may be performed by a numerically small portion of the total community (<1%) that is not detected in a DGGE profile.
- Interpretation of microbial community fingerprints is somewhat subjective and less straightforward than for other EMDs.

## Choosing between PLFA Analysis, DGGE, and Other EMDs

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

PLFA analysis provides a measure of total viable biomass and a broad-based profile of the microbial community composition grouped into general categories. Other than in combination with SIP, PLFA analysis is best suited for addressing general questions such as whether a treatment increased (or decreased) total biomass or substantially altered redox conditions.

DGGE and T-RFLP provide a DNA-based profile of the microbial community and allow identification of the predominant organisms generally to the family or genus level but cannot quantify specific organisms or microbial functions. DGGE profiles are used to visually display differences or shifts in microbial community composition over time or in response to treatment. Subsequent sequence analysis is somewhat exploratory, seeking to answer the question, “Who is there?” Most often, DGGE analysis is performed when identification of the predominant organisms is required but little is known about the microbial community of the sample prior to analysis.

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

## Sampling Protocols

Almost any type of sample matrix (e.g., soil, sediment, groundwater, in-field filters) can be submitted for microbial fingerprinting analysis. Sampling usually involves collecting small amounts of the soil or groundwater in a container, sealing it and storing at 4$^{o}$C until time of analysis. Sampling for microbiological samples can be easily incorporated into routine environmental monitoring programs. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at 4$^{o}$C during handling and transport to the laboratory. Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency or other government agencies. However, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific quality assurance project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal quality assurance/quality control information available (such as results for positive and negative controls). For microbial fingerprinting methods, data reports include a lower quantification limit, a practical quantification limit, and data quality "flags" such as estimated value (J), similar to those of more routine chemical analyses. Positive and negative controls are typically included with each analysis.

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## Glossary

**biodegradation**—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

**biomolecules**—Classes of compounds produced by or inherent to living cells including phospholipids, nucleic acids (e.g., DNA, RNA), and proteins.

**biostimulation**—A remedial technique which provides the electron donor, electron acceptor, and/or nutrients to an existing subsurface microbial community to promote degradation.

**denaturing gradient gel electrophoresis (DGGE)**—Type of gel electrophoresis used to separate mixtures of PCR products based on the melting point, which is reflective of the DNA sequence. DGGE is used to generate a genetic fingerprint of the microbial community and potentially identify dominant microorganisms.

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**genus**—A category of organism classification (taxonomy). A particular genus is a group of related species. For example, *Pseudomonas* is a genus of bacteria.

**microbial community composition**—Description of the types or identities of microorganisms present in a sample.

**microbial fingerprinting methods**—A category of techniques that differentiate microorganisms or groups of microorganisms based on unique characteristics of a universal component or section of a biomolecule.

**phospholipid**—A type of biomolecule that is a primary structural component of the membranes of almost all cells.

**PLFA**—Phospholipid fatty acids derived from the two hydrocarbon tails of phospholipids.

**primers**—Short strands of DNA that are complementary to the beginning and end of the target gene and thus determine which DNA fragment is amplified during PCR or qPCR.

**Proteobacteria**—A specific phylum of bacteria. Some proteobacteria modify specific phospholipids in their cell membranes in response to environmental stresses.

**redox conditions**—Description of the oxidation/reduction potential of the subsurface (e.g., aerobic, anaerobic, sulfate-reducing, or methanogenic conditions).

**RNA (ribonucleic acid)**—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

**terminal electron acceptors**—Compounds used by microorganisms to support their respiration. In aerobic organisms the terminal electron acceptor is oxygen ($O_2$). In anaerobic organisms compounds other than $O_2$ are used. These include common naturally occurring compounds such as nitrate ($NO_3^-$) or sulfate ($SO_4^{2-}$) or anthropogenic contaminants such as chlorinated ethenes (e.g., perchloroethene). Atoms from electron acceptors are typically not incorporated into biomolecules made by organisms that reduce these compounds during respiration.

**terminal restriction fragment length polymorphism (T-RFLP)**—A nucleic acid (DNA or RNA)–based technique used to generate a genetic fingerprint of the microbial community and potentially identify dominant microorganisms.

**viable biomass**—In this context, living microorganisms (capable of metabolism and/or reproduction).

# Microarrays

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why are microarrays relevant?

Microarrays offer the ability to simultaneously detect and semiquantitatively assess the relative abundance of thousands of different microbial biomarker genes as a comprehensive evaluation of the microbial community composition and its potential activity within an environmental sample. Microarray analysis offers advantages at sites that require a comprehensive view of the microbial community and where a larger number of biomarker gene targets need to be monitored to assess biodegradation. Microarray analysis may provide valuable insight into biodegradation of emerging contaminants, for which little is known regarding the microorganisms and degradation pathways involved. Microarrays have been used in research settings since 1996 but have only recently become commercially available for environmental applications. Microarrays have been used to document microbial diversity in a number of environments, including the petroleum release in the Gulf of Mexico (Hazen et al. 2010) and sites impacted by radionuclides like uranium (Chandler et al. 2010, Rastogi et al. 2010).

## What do microarrays do?

Environmental samples can contain thousands of different microorganisms and many different functional genes, some of which can serve as process-specific biomarkers. Phylogenetic microarrays evaluate community composition based on the presence/absence of microbial 16S rRNA genes present in a sample and answer the question, “Who is there?” A functional gene microarray targets genes involved in specific processes, for example a gene encoding a key enzyme involved in a degradation pathway, and can help answer questions about “potential activity.” For example, functional gene microarray analysis can provide information on the capabilities of the microbial population to transform contaminants (e.g., degrade organic compounds, reduce metals such as Cr[VI]). Phylogenetic and functional microarrays can also be interrogated with RNA extracted from environmental samples and provide information about general activity (phylogenetic arrays) or about the activity of specific functional genes and pathways (functional array). Thus, microarrays can provide information about activity and determine, “Who is active?” and, “What pathway is active?”

## How are the data used?

The strength of the microarray approach is that many species or genes can be monitored simultaneously, and the overall responses of a microbial community to perturbations such as implementation of a remedy can be monitored over time or compared within impacted and background zones. A gram of soil or a liter of groundwater can contain billions of microorganisms, representing thousands of unique species that carry out different processes. Biodegradation of a contaminant of interest may require a single microbial population, a group of microorganisms, or a diverse community. In other words, a process of interest may be sufficiently monitored by looking at the dynamics of a couple of genes (e.g., genes encoding oxygenases involved in aerobic benzene biodegradation) in a couple of candidate species, while monitoring of more complex processes (e.g., nitrogen cycle, sulfur cycle, heavy-metals reduction) may be substantially improved by the analysis of hundreds or thousands of genes or assessment of flux of species present in a diverse community. Thus, microarrays can provide valuable insights for environmental remediation and monitoring.

### Example Environmental Remediation Questions Microarrays Can Help Answer

- **Site Characterization**
  - Assess current conditions and potential for biodegradation
    - What known microorganisms or functional genes are present?
    - How diverse is the microbial community?
  - Emerging contaminants
    - What types of microorganisms are present in impacted wells versus background wells?
- **Remediation**
  - Is monitored natural attenuation feasible?
    - Are supporting and contaminant-degrading microorganisms present in impacted zones?
    - Are competing microorganisms present?
  - Is biostimulation necessary? Should an amendment be added?
    - What kind of microorganisms will respond to the amendment?
- **Monitoring**
  - Monitored natural attenuation
    - What known microorganisms or functional genes are present?
    - Are competing microorganisms present?
    - How diverse is the microbial community?
    - Does microbial community structure (e.g., diversity) change over time?
    - What microorganisms are detected in impacted versus nonimpacted wells, and do these microorganisms support contaminant biodegradation?
  - Biostimulation
    - What changes were evident in the overall microbial community composition following amendment?
    - Did amendment addition promote growth of a specific group of microorganisms or increase of functional genes?
    - Is the shift in the microbial community consistent with the biostimulation strategy?
- **Closure**
  - How does the diversity of the microbial community in formerly impacted wells compare to the diversity of the microbial community in background wells?

## How do they work?

Microarrays are a collection of many short DNA strands, called “probes,” that are attached to a solid surface (e.g., a glass slide). The probes are selected for their specific, known DNA sequence, to which only complementary pieces of DNA (target) will bind (hybridize). After DNA is extracted from an environmental sample, it is fragmented and labeled with fluorescent chemicals and applied to the microarray. When hybridization (i.e., specific binding) occurs, the labeled DNA that complements its respective microarray probe is bound in place, producing characteristic fluorescent signals. DNA that does not have a complementary probe on the microarray slide is removed in a washing step. Detection and relative quantification are based on the fluorescent signal remaining after the washing step. This approach can also be applied to RNA obtained from the environmental sample. In this case, the RNA is transformed to complementary DNA (cDNA) in a step called “reverse transcription.” Hybridization of the cDNA to the array can provide information about activity. The strengths of the microarray approach are that many genes or species can be monitored simultaneously and the overall responses of a microbial community in response to remedial action can be monitored over temporal and spatial scales.

Figure 1 illustrates the results from a microarray analysis of DNA extracted from two individual environmental samples. For example, Sample A could be a groundwater sample obtained from a monitoring well in the contaminant source area, whereas Sample B could have been collected from a background well located upgradient of the site. The DNA from Sample A is labeled with a green fluorescent dye, and DNA from Sample B is labeled with a fluorescent red dye. Each position or “spot” in the microarray grid contains a specific gene probe. If Sample A contains complementary DNA target

sequences, the labeled DNA fragments will bind to the corresponding gene probes, producing a green signal at each of these positions. If Sample B DNA binds to the gene probes, the signals will be red. If DNA from both samples binds to the gene probes, a combination of both colors will appear (i.e., yellow). In the end, genes detected in Sample A only appear green, genes detected in Sample B only appear red, and genes detected in both samples appear yellow (a mix of green and red). Thus, in this example, the microarray results illustrate which microorganisms are unique to the contaminant source area (green), which are detected only in the background area (red), and which are present in both areas (yellow).

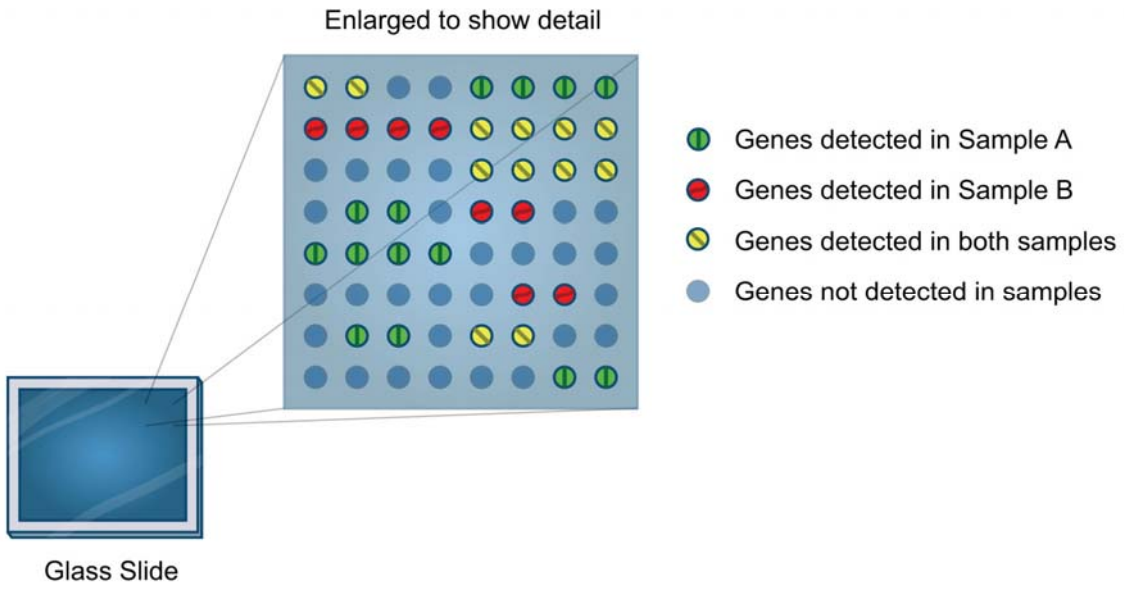


**Figure 1. Example of results from a two-dye microarray.**

## How are the data reported?

Phylogenetic microarray results are usually reported as a list of probable microorganisms (genus and species) detected in the sample. Similarly, functional gene microarray results include a list of the specific genes detected (e.g., a gene encoding nitrite reductase) and the gene type based on the biological process involved (e.g., denitrification). Statistical procedures have been developed that can aid in the interpretation of the results; however, microarray data interpretation requires expertise.

## Advantages

- Detection and relative quantification of thousands of organisms or functional genes in a single analysis.
- Information about gene expression (i.e., activity) can be obtained.
- Databases of known microorganisms and functional genes are becoming more comprehensive, making interpretation of results more meaningful and thus microarray analysis more applicable to environmental remediation. Microarrays provide a large quantity of information, which can be used to develop an understanding of the site that may not be possible using conventional environmental sampling and analytical testing. The microarray results may provide project managers with better information to use in the selection of remedial action alternatives or guide the selection of specific EMDs for efficient site monitoring. For example, microarrays can identify site-specific biomarker genes that provide meaningful information, and qPCR can then be applied to specifically monitor these genes.
- Gives an indication of the microbial diversity and possibly identifies the presence of microbes implicated in the biodegradation of the target contaminants. Microarrays can be based on both DNA and RNA, providing information on microbial community structure and metabolic activities, respectively.

## Limitations

- At the present time, few microarrays are commercially available that are relevant to environmental remediation.
- Careful design and thorough optimization and testing are needed to eliminate false positive signals (unspecific hybridization). Users should be sure to request documentation from the laboratory about the testing and validation of the microarrays.
- Quantification of the results can be difficult. Although recent studies have demonstrated relationships between signal intensity and target gene abundance, the dynamic range of the signal (i.e., the difference between the maximum and minimum signal) is limited and can hinder accurate quantification.
- Standardization of performance testing across different microarray platforms and guidelines for application and data interpretation are not readily available.
- The interpretation of data typically requires significant expertise, including knowledge of advanced statistical analyses.
- Microarray probes are based on genetic sequences of known microorganisms and biodegradation pathways cataloged in public databases—novel or as-yet undiscovered genes cannot be detected with microarrays. However, as new microorganisms and biodegradation pathways are identified, corresponding probes can be readily added to existing microarrays to expand the applicability of the technique to other contaminants and newly identified biodegradation pathways.

## Sampling Protocols

Almost any type of sample matrix (e.g., soil, sediment, groundwater) can be submitted for microarray analysis. Sampling usually involves collecting 10–20 g of soil or 1–2 L of groundwater and placement in sealed containers. Microarrays need a minimum of 2–5 µg of DNA; otherwise, it is necessary to amplify the sample prior to microarray analysis. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at 4°C during handling and transport to the laboratory. Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency (EPA) or other government agencies. However, EPA (2007) has an interim guidance for microarray analysis. In addition, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific quality assurance project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal quality assurance/quality control information available (such as results for positive and negative controls). Specifically for microarrays, the arrays typically contain control probes and internal controls for analytical and technical performance of the system, as well as controls for normalization of signal. Standards currently exist for reporting data from microarray analysis (Brazma et al. 2001).

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# Glossary

**16S rRNA**—A subunit of the ribosome composed of ribonucleic acid (RNA). The RNA sequence is used to classify and identify microorganisms (e.g., genus and species).

**biodegradation**—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

**biomarker**—A distinctive (unique) characteristic of a biomolecule that can be measured and used as an indicator of a target microorganism or biological process. For example, a specific DNA sequence (used as a probe on a microarray) could be a biomarker for a particular microorganism (e.g., *Desulfotomaculum*).

**functional gene**—A segment of DNA that encodes an enzyme or other protein that performs a known biochemical reaction. For example, the functional gene *tceA* encodes the reductive dehalogenase enzyme that initiates reductive dechlorination of trichloroethene. Other genes can code for RNA entities which can regulate the activity of other DNA target sequences.

**genus**—A category of organism classification (taxonomy). A particular genus is a group of related species. For example, *Pseudomonas* is a genus of bacteria.

**microarray probe**—A short, defined segment of DNA designed to bind with the target gene if found in the environmental sample. The probe is attached to the solid surface of the microarray.

**microbial community**—The microorganisms present in a particular sample.

**microbial diversity**—Microbial diversity can have many definitions but in this context generally refers to the number of different microbial species and their relative abundance in an environmental sample (Nannipieri et al. 2003).

**nitrite reductase gene**—Functional genes encoding the enzymes that catalyze nitrite reduction. Nitrite reductase genes are commonly used as the target gene to detect microorganisms capable of denitrification.

**phylogeny (phylogenetic analysis)**—Classification of microorganisms into groups (e.g., genus and species) based in part on the rRNA sequences.

**ribosome**—A multicomponent biological molecule which is part of the protein-synthesizing machinery of the cell.

**species**—The lowest taxonomic rank and the most basic unit or category of biological classification (Biology Online n.d.).

# Stable Isotope Probing

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why is stable isotope probing relevant?

Stable isotope probing (SIP) techniques are used to determine whether biodegradation of a specific contaminant can or does occur at a contaminated site. If a specific biodegradation process is detected, SIP approaches can also be used to identify the microorganisms responsible for this activity. A unique feature of SIP approaches that distinguishes them from virtually all other EMDs is that they do not require any prior knowledge of the microorganisms, genes, or enzymes involved in a specific biodegradation processes and can therefore be applied to novel and otherwise uncharacterized contaminants.

## What does SIP do?

Collectively, SIP approaches all use isotopically labeled contaminants to detect and quantify biodegradation processes and to characterize the microorganisms responsible for these activities. These approaches are "culture-independent" methods that minimize biases inherent in other techniques that identify and quantify microorganisms after they have been extracted from environmental samples and cultivated in laboratories as either individual pure strains or mixed microbial communities. SIP is also substantially different from compound specific isotope analysis (CSIA, see the CSIA Fact Sheet), in two key respects. First, SIP approaches require contaminants that have been artificially enriched with high levels of stable isotopes such as $^{13}C$ and $^{15}N$. In contrast, CSIA examines only changes in the naturally occurring low levels of stable isotopes found in contaminants. Second, SIP focuses on analyzing changes in the isotopic composition of biomolecules (e.g., lipids, nucleic acids, proteins) derived from microorganisms. In contrast, CSIA focuses on analyzing the isotopic composition of contaminants themselves. Most SIP approaches have been developed over the last decade, and their inherent simplicity and versatility has led to the introduction of several commercial applications of these powerful techniques in the environmental field. SIP approaches have now been used to characterize the biodegradation of many contaminants including polycyclic aromatic hydrocarbons (PAHs), fuel oxygenates, pesticides, and gasoline constituents, including benzene, toluene, ethylbenzene, and xylenes (see Table 1).

## How are the data used?

Data generated from SIP approaches can be used to establish whether biodegradation of a specific contaminant can or does occur at a site. Depending on which approach is used, SIP techniques can also provide additional information that ranges from identification of broad groups of microorganisms through to identification of specific organisms, genes, and enzymes involved in a particular biodegradation process. These data can be used to simply demonstrate the occurrence of a particular biodegradation process. Data from SIP analyses can also be used to confirm the effectiveness of existing remediation processes or aid in the design of remediation approaches.

### Example Environmental Remediation Questions SIP Can Help Answer

- **Site Characterization**
  - Are microorganisms present that are capable of degrading the contaminant?
- **Remediation**
  - Can biodegradation of a contaminant occur within a particular set of environmental conditions?
- **Monitoring**
  - Is biodegradation of a contaminant occurring?

**Table 1. Examples of recent applications of SIP to important contaminants**

| Contaminant | Biomolecule | Redox conditions | Isotope | Lab or field | Reference | PMID[a] |
|---|---|---|---|---|---|---|
| Benzene | DNA | Aerobic | $^{13}C$ | lab | Xie et al. 2010 | 20549308 |
| Benzene | DNA | Sulfate reducing | $^{13}C$ | lab | Herrmann et al. 2010 | 19840104 |
| Benzene | DNA | Methanogenic | $^{13}C$ | lab | Sakai et al. 2009 | 19914583 |
| Benzene | DNA | Aerobic and anaerobic | $^{13}C$ | both | Liou et al. 2008 | 18430012 |
| Benzene | DNA | Iron reducing | $^{13}C$ | lab | Kanapuli et al. 2007 | 18043671 |
| Benzene | RNA | Aerobic and anaerobic | $^{13}C$ | lab | Kasai et al. 2006 | 16672506 |
| Benzene and toluene | PLFA | Anaerobic | $^{13}C$ | field | Geyer et al. 2005 | 16053100 |
| Benzoate | DNA | Aerobic | $^{13}C$ | field | Pumphrey and Madsen 2008 | 18469130 |
| Biphenyl | DNA | Aerobic | $^{13}C$ | lab | Uhlik et al. 2009 | 19700551 |
| Methane | PLFA | Aerobic | $^{13}C$ | lab | Bodelier et al. 2009 | 19194481 |
| Methane | PLFA and RNA | Aerobic | $^{13}C$ | field | Qiu et al. 2008 | 18385771 |
| Methyl chloride | DNA | Aerobic | $^{13}C$ | lab | Borodina et al. 2005 | 16104855 |
| Pentachlorophenol | RNA | Aerobic | $^{13}C$ | lab | Mahmood et al. 2005 | 16104858 |
| Perchloroethylene | DNA | Dehalorespiring | $^{13}C$ | lab | Kittelmann and Friedrich 2008 | 18211265 |
| Phenol | RNA | Nitrate reducing | $^{13}C$ | lab | Sueoka et al. 2009 | 19146573 |
| Phenol | DNA | Aerobic | $^{13}C$ | field | DeRito et al. 2005 | 16332760 |
| Polyaromatic hydrocarbons | DNA | Aerobic | $^{13}C$ | lab | Singleton et al. 2005 | 15746319 |
| Polychlorinated biphenyls | PLFA and DNA | Aerobic | $^{13}C$ | lab | Tillmann et al. 2005 | 16329907 |
| Pyrene | DNA | Aerobic | $^{13}C$ | lab | Jones et al. 2008 | 18165874 |
| Toluene | DNA | Sulfate reducing | $^{13}C$ | lab | Winderl et al. 2010 | 20428224 |
| Toluene | DNA | Aerobic | $^{13}C$ | lab | Sun et al. 2010 | 20008173 |
| Toluene | DNA | Sulfate reducing | $^{13}C$ | lab | Bombach et al. 2010 | 19951369 |
| 2,4,6-Trinitrotoluene | DNA | Sulfate reducing | $^{15}N$ and $^{13}C$ | lab | Gallagher et al. 2010 | 20081008 |
| Uranium | PFLA and DNA | Anaerobic | $^{13}C$ | field | Chang et al. 2005 | 16382923 |

[a] PMID = PubMed reference number. Abstracts of the referenced studies can be obtained at the PubMed web page (www.ncbi.nlm.nih.gov/pubmed) by entering the PMID number in the search box.

## How does it work?

All living organisms are made of four key carbon-containing biomolecules (e.g., lipids, sugars, proteins, and nucleic acids). Microorganisms synthesize these biomolecules from simple metabolites they generate during the degradation of chemicals present in their environment. When a microorganism grows and divides, the isotopic composition of the biomolecules in the new cells reflects the isotopic composition of the chemicals on which the microorganism was grown. For instance, if a microorganism is grown on sugars consisting entirely of carbon-12 ($^{12}C$, the most abundant stable form of carbon), every carbon atom in every biomolecule in the newly formed cells is $^{12}C$. However, if the microorganism is grown on sugars enriched in $^{13}C$ (the other but more rare stable isotope of carbon), the new biomolecules are significantly enriched in $^{13}C$. This effect is exploited in SIP, and consequently all SIP approaches involve exposing samples to isotopically enriched contaminants. After a predetermined period of time, all forms of the specific biomolecule under investigation are extracted from the sample and analyzed. If biodegradation of the isotopically enriched contaminant has occurred, some of the extracted biomolecules will contain elevated levels of the stable isotope originally present in the contaminant. As there are no significant alternative routes that allow the stable isotope to migrate from the contaminant to biomolecules, detection of elevated levels of stable isotopes in biomolecules provides compelling evidence for biodegradation.

Most SIP studies use $^{13}C$-labeled compounds, although compounds enriched with nitrogen ($^{15}N$) and oxygen ($^{18}O$) can also be used. The most frequently used and commercially available form of SIP involves analysis of fatty acids from microbial phospholipids. This technique, known as phospholipid fatty acid (PLFA)-SIP, uses contaminants that contain a relatively low level of isotopic enrichment (e.g., 10% $^{13}C$). After exposure of samples to $^{13}C$-labeled contaminants, all forms of phospholipid (labeled and unlabeled) are extracted using solvents. A subsequent analysis of the solvent extracted phospholipids by mass spectrometry (gas chromatography [GC]/mass spectrometry [MS] or GC/isotope ratio mass spectrometry [IRMS]) can quantify total $^{13}C$ incorporation into PLFAs. This measurement can establish that biodegradation has occurred and can estimate the amount of contaminant that has been degraded. A further identification of specific PLFAs that have elevated levels of $^{13}C$ can help identify broad groups of microorganisms responsible for the biodegradation process. The analysis of $^{13}C$ incorporation into PLFAs also can be supplemented by data showing the detection of elevated $^{13}C$ levels in terminal metabolites such as $CO_2$ and $CH_4$.

Other forms of SIP such as DNA-SIP or protein-SIP are currently mainly research and discovery tools and require contaminants with very high levels of isotopic enrichment (ideally 100% $^{13}C$). Although not routinely available as a commercial service, the analysis of $^{13}C$-labeled DNA using molecular approaches (e.g., quantitative polymerase chain reaction [qPCR], microarrays, DNA sequencing) or $^{13}C$-labeled proteins using MS can provide precise information about which species of microorganisms are responsible for a biodegradation reaction and even the specific enzymes and pathways involved in the biodegradation process. These analyses can be particularly useful when little is known about either the microorganisms or the pathways involved in a particular biodegradation process.

## How are the data reported?

Positive PLFA-SIP data typically report the level of $^{13}C$ enrichment in solvent-extracted bulk or individual fatty acids in the form of change in $^{13}C$ abundance. These data are obtained from either GC/MS or GC/IRMS measurements. Positive DNA/RNA-SIP data simply state that $^{13}C$-labeled DNA or RNA was detected based on separation from unlabeled $^{12}C$-DNA or -RNA by ultracentrifugation. Additional supporting lines of evidence in all $^{13}C$ SIP analyses can be expected to include measurements of $^{13}CO_2$ or $^{13}CH_4$ production.

## Advantages

- Can be used without any prior knowledge of the organisms responsible for biodegradation of the contaminant.
- Applicable to different environmental media (water, soil, sediment). Although often a laboratory technique, PLFA-SIP approaches have been used with in situ passive sampling devices such as Bio-Traps™.
- Can be conducted on any contaminant as long as isotopically enriched forms are available. SIP approaches are most appropriate for compounds that are used as carbon and energy sources (e.g., hydrocarbons, gasoline oxygenates, PAHs) for microorganisms, as carbon from these compounds is incorporated into biomolecules during biodegradation.
- Different SIP approaches can provide different information, ranging from whether or not biodegradation has actually occurred to which microbial species are degrading the molecule and which biodegradation pathways and enzymes they are using.

## Limitations

- Not appropriate for contaminants used as terminal electron acceptors, such as chlorinated ethenes or perchlorate. Atoms from these compounds are typically not incorporated into biomolecules by organisms that reduce these compounds as electron acceptors.
- Some isotopically labeled compounds can be expensive (especially if they have to be custom-made).
- Different SIP methods can require different lengths of time to implement. In the field, using passive microbial sampling devices such as Bio-Traps, the sampling devices may be deployed for 30–90 days. In the laboratory the time to completion depends on the rate of disappearance of the contaminant in the specific SIP study.
- Care has to be taken in extrapolating the results of SIP studies to field conditions, even when using passive (field) sampling devices. The conditions required to obtain a detectable SIP signal (e.g., high localized concentrations of isotopically labeled contaminant) may be substantially different from the prevailing conditions in the subsurface.
- Permits may be required for the use of passive microbial sampling devices for use in in situ SIP studies although the amounts of isotopically labeled contaminants used in these analyses are typically very small.
- Cross feeding can occur when isotopically labeled metabolites are excreted by one organism and then used by other microorganisms. In DNA-SIP, this effect can potentially lead to identification of organisms that are not directly responsible for the biodegradation process of interest. The extent and significance of cross feeding can be estimated by conducting several SIP analyses over time. Cross feeding would be expected to be the least significant in the earliest time points.

## Sampling Protocols

Passive microbial sampling devices are generally used in groundwater wells to conduct in situ SIP studies. The isotopically enriched contaminant (e.g., $^{13}$C-methyl *tert*-butyl ether) is applied to media in the passive microbial sampling device, and the sampling device is incubated in a well for a given period of time. The biomolecules of interest (e.g., DNA, PLFA) are then extracted from the media and analyzed. Additional details on passive microbial sampling devices are provided in the EMD Sampling Methods Fact Sheet. An alternative to these studies is laboratory incubations in which the isotopically enriched contaminant is added to field samples (e.g., microcosms with groundwater and aquifer sediment from the site of interest) and these samples are incubated under relevant conditions (e.g., similar temperature, oxidation-reduction potential to the field site). Subsamples can then be collected from the laboratory incubations over time for analysis of stable isotope incorporation into biomolecules. In all cases biomolecules such as DNA and lipids are extracted from samples using standard laboratory techniques used in other analyses described in other EMD fact sheets (e.g., qPCR, Microbial Fingerprinting). Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting samples are in place and understood.

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols approved by the U.S. Environmental Protection Agency or other government agencies. However, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific quality assurance project plan (QAPP). This plan includes identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal quality assurance (QA)/quality control information available (such as results for positive and negative controls). SIP approaches all detect incorporation of isotopes of specific elements into new biomolecules. While the absolute degree of isotopic enrichment of a pure chemical is not a major QA issue (i.e., the same organisms/biomolecules are labeled in samples that are incubated with a pure compound that has 10% $^{13}C$ enrichment as with the same pure compound with 100% $^{13}C$ enrichment), the purity of the parent chemical is of critical importance. Ambiguous results can be obtained in a SIP study using a $^{13}C$-enriched chemical that is impure, even if the chemical of interest is 100% enriched with $^{13}C$. For example, a SIP study evaluating benzene degradation that uses 99% pure benzene that is enriched 100% with $^{13}C$ may yield inaccurate results if the 1% impurity is also $^{13}C$ enriched (e.g., $^{13}C$-toluene). In this case, the organisms detected may actually be degrading toluene rather than benzene, even though the toluene is present as a minor constituent. Consequently, the use of highly purified isotopically labeled compounds is of paramount importance in SIP studies.

Whenever possible, SIP analyses should involve use of control incubations in which isotopically labeled compounds are added to sterilized or inhibited samples as well as active samples. Although no standards currently exist, isotopically labeled biomolecules should also be extracted from samples either using protocols described by commercial kit manufacturers or individual laboratory SOPs.

## Additional Information

See Table 1 for current references for SIP applications. The table includes PubMed reference numbers (PMID). The abstract of a published study can be obtained at the PubMed web page (www.ncbi.nlm.nih.gov/pubmed) by entering the PMID number in the search box.

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## Glossary

**biomolecules**—Classes of compounds produced by or inherent to living cells including phospholipids, nucleic acids (e.g., DNA, RNA), and proteins.

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**enzyme**—Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on EPA 2004).

**gene**—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

**isotopically labeled contaminant**—A contaminant that has been specially synthesized to deliberately contain specific isotopes at elevated levels above those found in either natural or commercial bulk forms of the same chemical.

**lipids**—A diverse range of organic compounds that are defined as being insoluble in water but soluble in nonaqueous solvents. Lipids include oils, waxes, and sterols.

**microcosm**—A sample that is regarded as a small but representative portion of something larger. In environmental studies microcosm are typically small samples of soil, sediment, or water incubated in enclosed containers under laboratory conditions.

**phospholipid**—A type of biomolecule that is a primary structural component of the membranes of almost all cells.

**PLFA—**Phospholipid fatty acids derived from the two hydrocarbon tails of phospholipids.

**stable isotope**—A form of an element that does not undergo radioactive decay at a measureable rate.

**terminal electron acceptors**—Compounds used by microorganisms to support respiration. In aerobic organisms the terminal electron acceptor is oxygen ($O_2$). Anaerobic organisms use compounds other than $O_2$. These include common naturally occurring compounds such as nitrate ($NO_3^-$) or sulfate ($SO_4^{2-}$) or anthropogenic contaminants such as chlorinated ethenes (e.g., perchloroethene). Atoms from electron acceptors are typically not incorporated into biomolecules made by organisms that reduce these compounds during respiration.

# Enzyme Activity Probes

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why are enzyme activity probes relevant?

Enzyme activity probes (EAPs) are chemicals used to detect and quantify specific activities of microorganisms in environmental samples (e.g., soil, water, or sediment). A unique feature of EAPs is that they are the only EMD that directly estimates the activities of microorganisms involved in biodegrading specific contaminants. EAP analyses are also conducted without prior cultivation of microorganisms or extensive sample preparation. These analyses are therefore simple to conduct and can provide a direct estimate of specific microbial activities at the time of sampling. When combined with traditional monitoring of contaminant concentrations over time, EAP analyses can provide project managers valuable information for site characterization, site management, and remedy selection.

## What do EAPs do?

EAPs are compounds that serve as alternative or surrogate substrates for the protein catalysts (enzymes) responsible for the metabolic activities of microorganisms. These surrogate compounds are transformed by target enzymes into distinct and readily detectable products. As most enzymes are not functional outside cells due to rapid degradation or inactivation, there is often a strong relationship between the rate of transformation of an EAP and the number of active microbial cells that possess an active form of the enzyme of interest.

The simplest EAPs, such as fluorescein diacetate (FDA), are transformed by common enzymes found in all microorganisms. Enzymatic hydrolysis of FDA can therefore be used to detect and estimate the total number of currently active (living) organisms in a sample. Other more sophisticated EAPs are transformed only by specific enzymes responsible for the transformation of specific contaminants. These EAPs can therefore be used to detect and estimate the numbers of organisms in a sample that are currently capable of biodegrading that contaminant. In many cases EAP analyses are conducted in the laboratory using unmodified environmental samples. These analyses can detect and quantify the numbers of organisms with specific capabilities in relatively small samples. In some cases EAPs have also been used in field-scale applications to determine in situ rates of biodegradation of specific contaminants such as chlorinated solvents.

Various forms of EAPs have been used by microbiologists for decades prior to their development and application for detecting contaminant degrading microorganisms. For example, some bacteria have the ability to convert atmospheric nitrogen gas into ammonia. This activity is of central importance to the biological nitrogen cycle. The activity of nitrogenase, the key enzyme responsible for this activity, can be determined by its additional ability to transform acetylene to ethylene. This analysis is known as the acetylene reduction assay and has been used in literally hundreds of published studies about the environmental distribution of nitrogen-fixing microorganisms.

A wide range of EAPs has been developed over the last 30 years that target enzymes involved in both anaerobic (without oxygen) and aerobic (with oxygen) contaminant biodegradation processes (see Table 1). EAPs have been used to evaluate sites contaminated with chlorinated solvents, petroleum hydrocarbons, pesticides, and numerous other contaminants.

## How are the data used?

EAPs can estimate the number of microorganisms in an environmental sample that contain an active form of the enzyme of interest. A single EAP analysis can therefore provide direct evidence that the

microorganisms responsible for biodegradation are present and active at the time of sampling. Likewise, a time series of EAP analyses can quantify changes in these activities in response to natural or engineered changes in environmental conditions.

### Example Environmental Remediation Questions EAPs Can Help Answer

- **Site Characterization**
  - Are the key microbial pathways active?
  - Which known organisms or enzymes are present and active?
  - Are the enzymes capable of degrading the contaminant present and active? If so, how many and where?
- **Remediation**
  - Should an amendment be added (biostimulation)?
  - Should appropriate microorganisms be added (e.g., degradative enzymes through bioaugmentation)?
- **Monitoring**
  - What is the rate of contaminant degradation?

## How does it work?

In microorganisms virtually all transformations of organic chemicals are achieved through the activity of protein catalysts known as enzymes. In intact microbial cells the activities of enzymes are often coordinated in the form of pathways. In a pathway a sequential series of biochemical transformations occurs, with each step being catalyzed by an individual and often unique enzyme. The product of the first enzyme-catalyzed reaction produces the substrate for the second enzyme in the pathway, and so forth. In some cases specific enzymes initiate, or are intimately involved in, the pathway that enables a bacterium to grow on a contaminant. For instance, toluene monooxygenases initiate toluene biodegradation in bacteria that can grow on toluene. In other cases, bacteria can fortuitously transform contaminants through the activity of key enzymes otherwise used by the bacterium to grow on more innocuous compounds. An example would be trichloroethene (TCE) transformation by methane monooxygenase, an enzyme that normally initiates the pathway of methane oxidation in bacteria that can grow on methane. Irrespective of the type of biodegradation process involved, all EAPs capitalize on the fact that the key enzymes in contaminant biodegradation processes are rarely absolutely specific and can often transform many other compounds. If appropriately designed and characterized, these alternative or surrogate substrates (the EAPs) can therefore be used as a “reporters” for the enzyme responsible for this activity.

The design of EAPs requires that the alternative or surrogate substrate be transformed by the target enzyme into a stable product that is readily detectable. Some EAPs are initially colorless compounds that are transformed to strongly fluorescent products. As these products diffuse only slowly out of cells, they accumulate internally and “color” the organism. The organisms that contain the active enzyme can then be detected, discriminated, and quantified using an epifluorescent microscope and cell counting (see Figure 1 for an example of one class of EAP). Other EAPs contain unusual chemical signatures, such as fluorine atoms, that can be monitored and more precisely measured in the presence of high concentrations of contaminants, such as chlorinated solvents.

As EAPs require enzymes that are active for a quantifiable product to be generated, no EAP signal is detected if the appropriate enzyme is not present or it is present but not active in a given sample. Table 1 lists a number of the currently validated EAPs, which have been developed for a wide range of enzymes. These include EAPs for ubiquitous cellular enzymes, including esterases, lipases, and proteases, as well as EAPs for specific aromatic oxygenases (toluene, phenol, benzene), methane monooxygenase, naphthalene dioxygenase, and reductive dehalogenases.

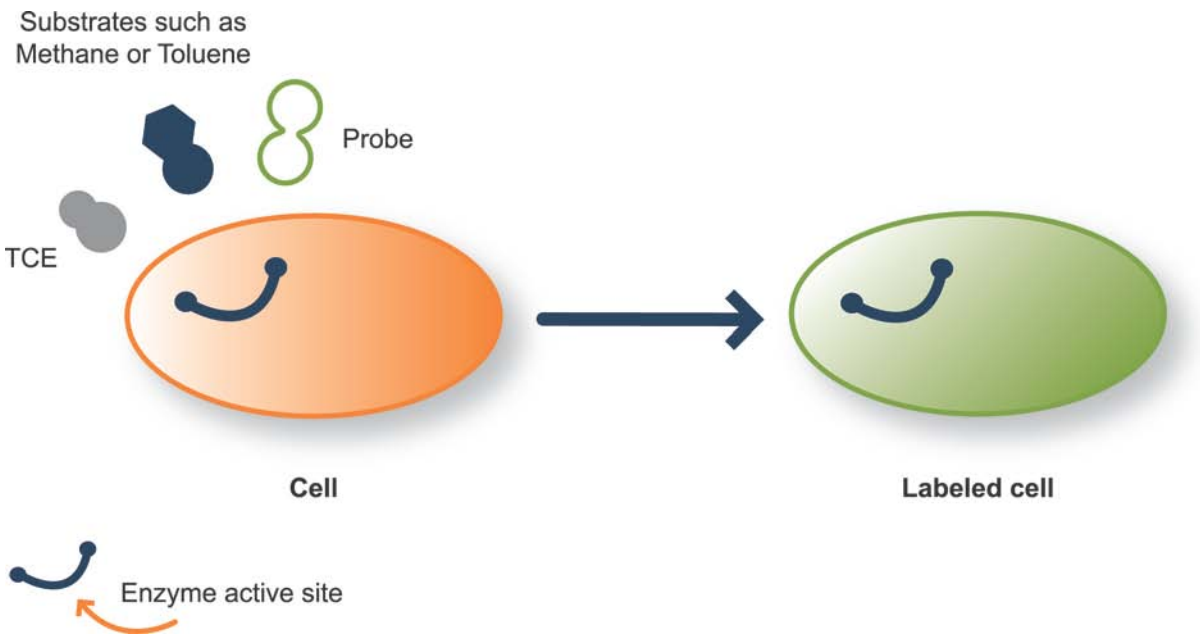


**Figure 1. In this example of one class of EAPs, the target enzyme is an oxygenase.** These enzymes are found in bacteria that can grow on substrates such as methane, toluene, propane, or ammonia. These enzymes can also often attack and degrade contaminants such as chlorinated solvents (e.g., TCE). The activity of the oxygenase enzyme enables the cell to substantially oxidize either TCE or the growth-supporting substrates to $CO_2$ and other simple metabolites. In contrast, the EAP is a colorless compound which is transformed by oxygenase enzymes into a stable and strongly fluorescent product that accumulates inside the cells. Consequently, the cells are stained only when the target oxygenase enzyme is actively functioning. *Source:* M. H. Lee, 2010, used with permission.

## How are the data reported?

Many of the EAPs used in laboratory analyses are highly fluorescent, and positive and negative results can be determined by observation with the naked eye. In more quantitative analyses, "colored" cells can be manually counted using an epifluorescent microscope and compared to the total number of cells stained with DNA-reactive stains such as acridine orange. The fraction of the total cells that are active can then be determined (active cells/total cells) and recorded as the percent of total. Most EAP data are presented as total active cells per volume of groundwater and/or per weight of soil analyzed. Alternatively, if EAPs are used in microcosm or field-scale analyses, absolute rates of EAP transformation can be provided.

## Advantages

- EAPs provide the most direct evidence that a microbial enzyme of interest is present in a sample and that the enzyme and organism are active at the time of sampling.
- EAPs can examine whole cells (i.e., cells that are intact) filtered directly onto flat filters and therefore do not have intrinsic biases associated with extracting and/or amplifying biomolecules such as DNA, RNA, or proteins.
- EAPs are capable of detecting very low levels of activity (≤100 cells) in complex microbial community without the need to cultivate the active bacteria.
- A wide variety of sample types can be analyzed with EAPs.
- A wide variety of EAPs are available for both anaerobic and aerobic degradation pathways.
- EAPs can also be used to measure rates of degradation in laboratory microcosms and field sites.

**Table 1. Current EAP targets for assessing biodegradation**

| EAPs | Contaminants | Target enzymes | Conditions | References |
|---|---|---|---|---|
| Fluorescein diacetate | Overall microbial activity | Lipase(s), esterase(s), and protease(s) | Anaerobic, aerobic | Schnurer and Rosswall 1982; Jones and Senft 1985; Fontvielle, Outaguerouine, and Thevenot 1992; Battin 1997; Adam and Duncan 2001 |
| Trans-cinnamonitrile | Chlorobenzene, TCE, dichloroethene (DCE), petroleum hydrocarbons | Toluene dioxygenase | Aerobic | Keener, Watwood, and Apel 1998; Keener et al. 2001; Watwood, Keener, and Smith 2002; Lee et al. 2008 |
| Trans-2-furanacrylonitrile | Toluene, xylene | Toluene-2-monooxygenase | Aerobic | Keener, Watwood, and Apel 1998; Keener et al. 2001; Watwood, Keener, and Smith 2002; Lee et al. 2008 |
| 3-Hydroxyphenylacetylene; phenylacetylene | Perchloroethene (PCE); TCE; DCE; benzene, toluene, ethylbenzene, and xylenes (BTEX) | Toluene-2-, 3-, and 4-monooxygenase | Aerobic | Keener, Watwood, and Apel 1998; Keener et al. 2001; Watwood, Keener, and Smith 2002; Kauffman et al. 2003; Lee et al. 2008 |
| 3-Ethynyl-benzoate | Toluene, benzene, phenol | Toluene-side chain monooxygenase | Aerobic | Clingenpeel et al. 2005 |
| 3-Hydroxyphenylacetylene | Toluene, xylene | Xylene monooxygenase | Aerobic | Keener, Watwood, and Apel 1998; Keener et al. 2001; Watwood, Keener, and Smith 2002; Lee et al. 2008 |
| Coumarin | TCE, DCE, vinyl chloride (VC) | Methane monooxygenase | Aerobic | Miller et al. 2002, Wymore et al. 2007 |
| Indole | Naphthalene, methylbenzenes, benzene, toluene, biphenyl | Naphthalene dioxygenase | Aerobic | Ensley et al. 1983 |
| Vinyl bromide | VC | VC reductase | Anaerobic | Gu et al. 2003 |
| Trichlorofluoroethene | TCE, PCE | Reductive dehalogenase(s) | Anaerobic | Vancheeswaran, Hyman, and Semprini 1999; Hageman et al. 2001, 2004; Field et al. 2005 |
| 1-Chloro-1-fluoroethene | VC | VC reductase | Anaerobic | Pon and Semprini 2004, Ennis et al. 2005, Taylor et al. 2007 |

## Limitations

- Not all EAPs are commercially available, and some may available only through universities or research laboratories.
- The specificity of EAPs is typically validated only for known for organisms with known, well-characterized enzymes. Uncharacterized enzymes may also react with EAPs and contribute to the signal.
- Protocols for sample collection, storage, and analysis have not yet been standardized for all EAPs.
- EAP analyses involving direct visualization can be time-consuming.
- Environmental conditions such as pH, temperature, the presence of metals or other chemicals in the subsurface may cause inhibition of the microbial metabolic activity and should be considered and accounted for when evaluating environmental samples.
- Autofluorescence and other background fluorescence that occur naturally in groundwater, surface water, and in soils and sediments can be problematic with some EAPs and should be taken into

consideration when planning sample analysis and appropriate quality assurance (QA)/quality control (QC) measures.

- Permits may be required to use EAPs in in situ analyses of microbial activities.

## Sampling Protocols

For laboratory-based EAP analyses a wide variety of sample matrices (soil, sediment, and groundwater) can be submitted. Samples collected using in-line filters (e.g., Supor®, Sterivex™) are not appropriate for several reasons; filters are enclosed within a casing and typically frozen for shipping. Thus, cells entrapped on the membranes will be damaged or killed during transport, but activity assessment requires live cells for detection. Additionally cells on filter membranes within the unit are often artificially exposed to saturated conditions of oxygen (in situ conditions are altered during transport), thereby inactivating anaerobic enzymes, activating aerobic enzymes, and making the analysis of activity inaccurate.

Sampling procedures for EAP analysis can be readily integrated into existing monitoring programs. However, as EAPs rely entirely on microbial activity, due care must be taken to preserve this activity and to also avoid microbial contamination. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile sample containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at 4°C during handling and transport to the laboratory. Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency or other government agencies. However, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific QA project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal QA/QC information available (such as results for positive and negative controls). Specific for direct-visualization EAPs, QA/QC metrics should include accuracy, precision, and reproducibility, which can be addressed through positive, negative, and blank controls and duplicate or triplicate analyses, as well as studies to inhibit the targeted enzymatic activity. Generally, for these EAPs a minimum of 200 total cells is counted on three separate slide preparations such that statistical analyses can be completed. Duplicate or triplicate analyses of a single sample then results in six or nine slides being prepared and counted, respectively. In addition, supporting data can be provided through quantitative polymerase chain reaction (qPCR) or polymerase chain reaction (PCR) targeting the gene of the enzyme of interest (see PCR and qPCR Fact Sheets for details on these methods).

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## Glossary

**active site**—Part of an enzyme where catalysis of the substrate occurs.

**bioaugmentation**—The introduction of cultured microorganisms into the subsurface environment for the purpose of enhancing bioremediation of organic contaminants (EPA 2011).

**biodegradation**—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

**biostimulation**—A remedial technique which provides the electron donor, electron acceptor, and/or nutrients to an existing subsurface microbial community to promote degradation.

**dehalogenase**—An enzyme that catalyzes the removal of a halogen atom from an organic compound.

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**enzyme**—Any of numerous proteins or conjugated proteins produced by living organisms and facilitating biochemical reactions (based on EPA 2004).

**enzyme activity probes**—Transformation of surrogate compounds (probes) resembling contaminants produces a fluorescent (or other distinct) signal in cells, which is then detected by microscopy.

**epifluorescent microscope**—A type of microscope that uses a high-energy light source (e.g., ultraviolet light) and specialized filters to visualize fluorescently stained specimens. Epifluorescent microscopy procedures can be used to determine both the total number of cells and total number of viable or active cells in a sample.

**gene**—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

**microcosm**—A sample that is regarded as a small but representative portion of something larger. In environmental studies microcosm are typically small samples of soil, sediment, or water incubated in enclosed containers under laboratory conditions.

**oxygenase**—An enzyme that catalyzes the incorporation of molecular oxygen into a compound (based on Madigan et al. 2010).

**probes**—(1) short DNA strands (see *microarray probes*, Microarray Fact Sheet; *FISH probes*, FISH Fact Sheet; *qPCR probes*, qPCR Fact Sheet); (2) surrogate compounds (see *enzyme activity probes*).

**protein**—Large organic compounds made of amino acids arranged in a linear chain and joined together by peptide bonds (U.S. Navy 2009).

**RNA (ribonucleic acid)**—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

**substrate**—Any substance that is acted on by an enzyme.

**whole cell**—The entirety of a microbial cell, without extraction of DNA, RNA, etc. A whole-cell preparation does not modify the cell but evaluates it as unit.

# Fluorescence In Situ Hybridization

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## Why is fluorescence in situ hybridization relevant?

Fluorescence in situ hybridization (FISH) is a molecular biology technique that can be used to detect microorganisms known to biodegrade contaminants. When combined with traditional measuring of changes in contaminant concentration over time, FISH provides project managers valuable information for site management, including site conceptual model development, remedy selection, and optimization and determination of contaminant attenuation rates.

## What does FISH do?

To implement FISH, environmental samples (soil, water, sediment) are taken to a laboratory and in a series of steps a fluorescent dye is attached to a particular gene of interest in microorganisms or families of microorganisms. These targeted microorganisms can then be observed and their abundance and spatial distribution determined under a microscope by detecting the fluorescent light emitted. For the purposes of environmental investigation, the targeted microorganisms are typically ones capable of degrading specific contaminants.

Originally developed in the 1990s, FISH is routinely used in medical fields such as genetic counselling, disease identification, and microorganism species identification. More recently, it has been applied to the study of environmental processes, mostly in the wastewater treatment field, but also for contaminant biodegradation at sites with coal tar, herbicides, or chlorinated solvents, as well as at wetlands used to treat acid mine drainage. The FISH technique also has potential applicability in other environmental monitoring efforts, such as tracking of fecal microorganisms, so-called “microbial source tracking,” and identifying and tracking microorganisms potentially related to chemical compounds originating from industrial or energy-related activities.

## How are the data used?

FISH can provide valuable insights for environmental remediation alone and in combination with other EMDs, like polymerase chain reaction (PCR). For example, this method can reveal whether key organism(s) needed for biodegradation are present in the sample material and allow estimation of their abundance, similar to other EMDs. However, this method can also allow investigators to explore their structure, form, and spatial distribution and association with other microorganisms. FISH signals can provide some information about activity of the target organisms although no precise rate information can be obtained.

### Example Environmental Remediation Questions FISH Can Help Answer

- **Site Characterization**
  - Are the right microorganisms and/or genes present that are capable of degrading the contaminant? If so, how many and where?
  - What other microorganisms are present in the environment, and what impact do they have on the microorganisms or processes of interest?
- **Monitoring**
  - Does the microbial community change in response to an amendment?

## How does it work?

Short sequences of single-stranded nucleic acids (such as DNA), called "gene probes," are designed to match a portion of a gene or metabolic product of the organism or population of interest. A fluorescent dye is attached to the probe so that when the probe binds to target sequences within a cell, it emits fluorescent light that can be observed through a microscope (i.e., using an epifluorescent microscope) or sorted with flow cytometry. Cells emitting a fluorescent light are called "hybridized cells." In flow cytometry, labeled cells are diluted or concentrated (depending on the initial cell concentration in the sample) so that individual cells pass through a laser beam that detects and counts fluorescently labeled cells. Flow cytometry can be significantly more efficient than counting cells using a microscope. Cells in environmental samples are handled in such a way that the cell structure remains intact while still allowing the comparatively large gene probe to enter the cell and bind to the target gene within the microorganism of interest (Figure 1). Under ideal conditions, only cells that contain the target gene are recognized by the probe and become fluorescently labeled. Various cell staining procedures are sometimes combined with FISH probes to allow quantification of various parameters such as the total number of microorganisms or the presence of specific compounds. Table 1 presents selected FISH probes and cellular stains.

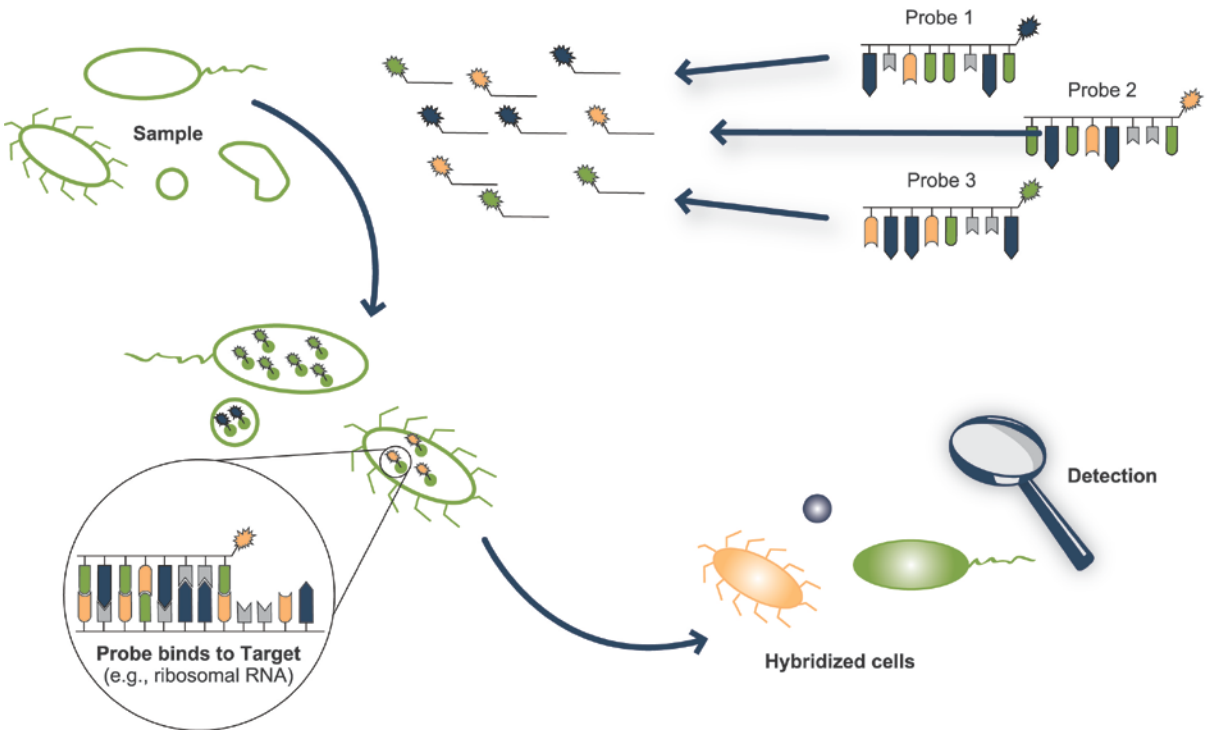


**Figure 1. Fluorescence in situ hybridization method.**

## How are the data reported?

Depending on the method, FISH results can be presented in two different ways:

- If FISH is evaluated using a microscope and manual counting of labeled cells, the results are presented as cells per unit (liter of liquid or gram of solid) analyzed.
- If FISH is evaluated with advanced microscopy techniques and digital image processing, the results are usually presented on a relative volume or area basis, which can be converted by the laboratory to cells per unit of liquid or solid.

**Table 1. Selected FISH probes or cellular stains**

| FISH probes or cellular stains | Contaminants | Target microorganism(s) | Reference |
|---|---|---|---|
| DAPI | NA | DNA of all microorganisms (live and dead) | This is a very common laboratory cellular stain. Not unique to environmental contaminants. |
| Acridine orange | NA | DNA of all microorganisms (live and dead) | This is a very common laboratory cellular stain. Not unique to environmental contaminants. |
| Dhe1259t | Chlorinated solvents | Some *Dehalococcoides* spp. 16S rRNA | Yang and Zeyer 2003 |
| Dhe1259c | Chlorinated solvents | Some *Dehalococcoides* spp. 16S rRNA | |
| KT1phe | Trichloroethene | *Ralstonia eutropha* KT1 16S rRNA | Tani et al. 2002 |
| Ac627BR | Naphthalene | Naphthalene dioxygenase (*nahAc*) mRNA | Bakermans and Madsen 2002 |
| RhLu | s-Triazine herbicides | *Rhodococcus wratislaviensis* 16S rRNA | Grenni et al. 2009 |

## Advantages

- FISH does not require cultivation of the organisms or any technology-based gene amplification (see PCR Fact Sheet), which can lead to false negatives and positives.
- In contrast to some other EMDs, FISH allows visualization of whole cells that are important to environmental remediation activities. FISH can thus provide complementary information to other EMDs, such as morphology of the cells or association of groups of microorganisms with relationship to one another.
- FISH can target several different genes simultaneously, for example, genes associated with specific degrading species of interest (e.g., *Dehalococcoides*) and broader microbial groups, such as methane-producing organisms.
- Depending on the species, and in combination with other appropriately validated activity-targeted approaches, FISH can provide general information about the activity of the organisms or populations of interest.
- FISH enables single-cell microbial studies and allows for subsequent studies, such as gene sequencing (see the Microbial Fingerprinting Methods Fact Sheet).

## Limitations

- The detection limit of FISH is high (~$10^6$ cells/mL). However, in some cases high detection limits can be corrected by sample concentration or cell extraction methods which lower the detection limits to a few hundred cells per concentrated sample.
- Validated probes and FISH procedures are not available for a wide range of organisms within the bioremediation field. Additionally, standard protocols for sample collection and storage prior to FISH analysis have not yet been developed.
- FISH can also be used to target not only ribosomal genes (which indicate the type of organism) but also functional genes (via mRNA) relevant in bioremediation. These other genes indicate what the microorganisms can do with regards to contaminant biodegradation, for example, naphthalene dioxygenase or reductive dehalogenase. However, laboratory protocols are often time-consuming and complicated and not yet validated for field applications.
- The FISH method is not widely commercially available. Currently, mainly specialized research laboratories are performing these analyses to explore and optimize the potential of FISH for validated and cost-effective applied studies.
- The FISH method is currently expensive because of the expertise and labor needed for development of validated FISH protocols and direct microscopic counting. Once validated protocols have been

developed, FISH can be automated to some extent by using flow cytometry to count target cells more efficiently, reducing the analytical costs. However, when using flow cytometry for cell counting, all information regarding spatial relationships (among and between the cells) is lost.

## Sampling Protocols

Sample matrices that can be analyzed by FISH include most kinds of environmental samples, such as wastewater, groundwater, filters, soil, and sediments. However, depending on the sample type, different types of sample preparations and FISH protocols may have to be employed. Basic sampling for microbiological samples can be easily incorporated into routine environmental monitoring programs. The following items are typical requirements for microbiological sampling: (a) use of aseptic sample collection techniques and sterile containers, (b) shipment of the samples to the laboratory within 24 hours of sample collection, and (c) maintenance of the samples at an appropriate temperature (e.g., 4°C during handling and transport to the laboratory). Sample collection techniques and containers may vary depending on the matrix sampled and the laboratory analyzing the samples. Users should work with the analytical laboratory to ensure sampling protocols for collecting, handling, and transporting the samples are in place and understood.

## Quality Assurance/Quality Control

To date, most EMDs do not have standardized protocols accepted by the U.S. Environmental Protection Agency or other government agencies. However, most laboratories work under standard operating procedures (SOPs) and good laboratory practices, which can be provided to the user (e.g., consultant, state regulator) on request.

Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific quality assurance project plan (QAPP). This plan should include identification of the EMDs being used; the field sampling procedures, including preservation requirements; the SOPs of the laboratory performing the analysis; and any internal quality assurance/quality control information available (such as results for positive and negative controls). Sample collection, preservation, and laboratory protocols for FISH have been standardized for only certain types of organisms and ecosystems.

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## Glossary

**biodegradation**—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

***Dehalococcoides*—**A specific group (genus) of bacteria. *Dehalococcoides* species are the only microorganisms that have been isolated to date which are capable of complete reductive dechlorination of PCE and TCE to ethene. Some *Dehalococcoides* species are also capable of reductive dechlorination of chlorinated benzenes, chlorinated phenols, and polychlorinated biphenyls.

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**epifluorescent microscope**—A type of microscope that uses a high-energy light source (e.g., ultraviolet light) and specialized filters to visualize fluorescently stained specimens. Epifluorescent microscopy procedures can be used to determine both the total number of cells and total number of viable or active cells in a sample.

**FISH probes**—Short sequences of single-stranded DNA carrying a fluorescent label. When the probe binds to the target DNA/RNA sequence of the microorganism(s) of interest in an environmental sample, the target cell fluoresces and can be visualized and counted using a specialized microscope or a flow cytometer.

**flow cytometry**—A method whereby cells or particles move in a liquid stream past a laser or light beam and a sensor detects the relative light scattering and fluorescence of the particles.

**functional gene**—A segment of DNA that encodes an enzyme or other protein that performs a known biochemical reaction. For example, the functional gene *tceA* encodes the reductive dehalogenase enzyme that initiates reductive dechlorination of TCE. Other genes can code for RNA entities which can regulate the activity of other DNA target sequences.

**gene**—A segment of DNA containing the code for a protein, transfer RNA, or ribosomal RNA molecule (based on Madigan et al. 2010).

**labeled cell**—A microorganism in which a gene probe has bound to a matching sequencing within the microorganism and released a fluorescent dye, resulting in a cell that is emitting fluorescent light.

**metabolic product**—Products generated by a microorganism whose structure and function are defined by DNA sequences also called genes. Example metabolic products include RNA and proteins or enzymes.

**probes**—(1) short DNA strands (see *microarray probes*, Microarray Fact Sheet; *FISH probes*; *qPCR probes*, qPCR Fact Sheet); (2) surrogate compounds (see *enzyme activity probes*, EAP Fact Sheet).

**RNA (ribonucleic acid)**—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

**whole cell**—The entirety of a microbial cell, without extraction of DNA, RNA, etc. A whole-cell preparation does not modify the cell but evaluates it as unit.

# EMD Sampling Methods

## EMD Team Fact Sheet—November 2011

*This fact sheet, developed by the ITRC Environmental Molecular Diagnostics (EMD) Team, is one of 10 designed to provide introductory information about and promote awareness of EMDs. Please review the Introduction to EMDs Fact Sheet along with this one. A glossary is included at the end of this fact sheet.*

## What EMD sampling methods are used?

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

## How are the data used?

Microbial sampling devices are versatile platforms that can be used in conjunction with a broad spectrum of EMDs, including the following, each of which is described in more detail in other fact sheets:

- quantitative polymerase chain reaction (qPCR and RT-qPCR)
- microbial fingerprinting methods, such as phospholipid fatty acids (PLFA) analysis, denaturing gradient gel electrophoresis (DGGE)
- microarrays
- compound specific isotope analysis (CSIA)
- stable isotope probing (SIP)

Selecting microbial sampling methods and subsequent EMD analyses depends on the site-specific questions that need to be addressed. For example, an appropriate microbial sampling method can be paired with qPCR to quantify known key microorganisms capable of biodegradation of a contaminant of interest to assess MNA.

**Active** microbial sampling methods are widely used when collecting grab samples for EMD analysis. These sampling methods are similar to traditional soil and groundwater sample collection for volatile organic compound (VOC) analyses (e.g., low-flow groundwater sampling with peristaltic pumps). Since samples are collected from single points in time, the data are representative “snapshots” of the microbial community. Thus, multiple sampling events are typically used to describe how microbial conditions vary over time. The same is also true of sampling for chemical and geochemical parameters. Typically, samples are collected quarterly or annually from selected groundwater monitoring wells as they are for chemical or geochemical analyses. For example, *Dehalococcoides* analyses are quantified as cells per milliliter before, during, and after bioremediation treatment to evaluate system performance.

**Passive** microbial sampling devices are incubated within the sampled environment for several weeks (typically 30–90 days) and depend on the formation and collection of biofilms that grow on or within a solid matrix. Thus, the passive microbial samplers provide a more time-integrated sample of microorganisms from the sampled environment. Passive microbial sampling devices can be amended with potential remediation amendments (e.g., electron donors, electron acceptors, etc.) and/or microbial cultures of known degraders. These amended passive microbial sampling devices, combined with EMD analysis, have been used to evaluate biostimulation and bioaugmentation as remediation strategies. If the passive microbial sampler contains an adsorptive surface, such as activated carbon, the sampler can be amended with a specially synthesized form of the contaminant (e.g., VOC) containing “heavy” stable carbon ($^{13}C$) isotope as a label. Since $^{13}C$ is relatively rare, carbon originating from labeled contaminant can be readily distinguished from carbon (predominantly $^{12}C$) from other sources (see the SIP Fact Sheet for additional information). During in-well deployment, the $^{13}C$-labeled contaminant is subject to the same

physical, chemical, and microbiological processes as the unlabeled contaminant present at the site. For many contaminants (e.g., benzene, methyl *tert*-butyl ether), biodegradation is a process whereby microorganisms use the contaminant as a carbon and energy source producing new cells (biomass) and carbon dioxide. Thus, if biodegradation is occurring during field deployment, the $^{13}C$ label from the synthesized contaminant in the passive microbial sampling device will be incorporated into the end products of biodegradation: microbial biomass and dissolved inorganic carbon ($HCO_3^-$ and $CO_2$). Upon recovery of the passive microbial sampling device and subsequent EMD analysis, incorporation of the $^{13}C$ label into biomolecules (DNA or PLFA) and dissolved inorganic carbon provides evidence of in situ biodegradation. Figure 1, an example of SIP, illustrates the process. Here the passive microbial sampling device is a Bio-Trap™ in which the solid matrix is Bio-Sep®. This matrix contains powdered activated carbon to which $^{13}C$-labeled compounds can be tightly adsorbed prior to incubation in groundwater.

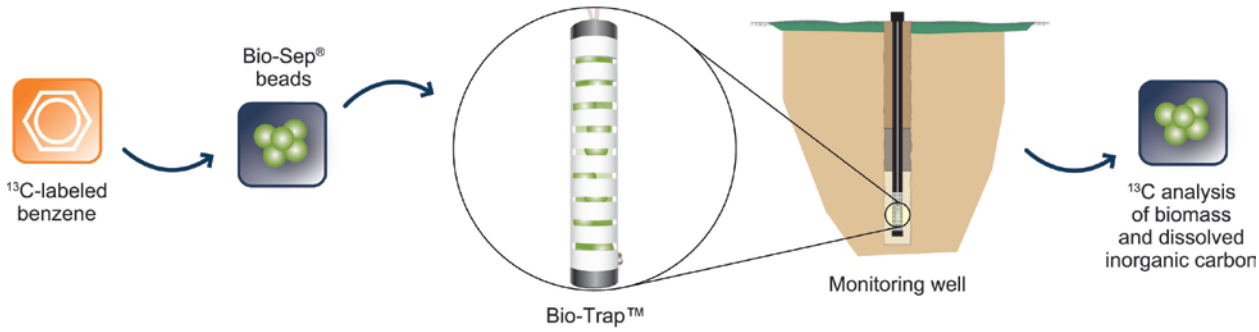


**Figure 1. Illustration of stable isotope probing with a Bio-Trap™.**
*Source:* Microbial Insights, Inc., 2010, used with permission.

Both active methods and passive devices are easy to use and are useful tools for microbial sampling and supporting remedial investigation and design efforts.

## How does it work?

Descriptions for how both active sampling methods and passive sampling devices work in conjunction with EMDs are presented separately below.

**Active Microbial Sampling Methods**—For practical reasons, active sampling for EMDs at remediation sites generally focuses on groundwater. The focus on groundwater is justified for the analysis of targets like *Dehalococcoides* that are found in the aqueous phase (e.g., planktonic microbial cells which grow in a suspended state in an aqueous environment as opposed to attached to a surface). Various active microbial sampling approaches are available for collection of biomass from environmental media, ranging from commonly used peristaltic pumps for groundwater sampling to direct-push coring or split-spoon sampling for soils that incorporate aseptic techniques for collecting microbial samples. Until recently, groundwater samples were typically collected and sent to a laboratory for biomass extraction. However, based on field trials conducted as part of the Environmental Security Technology Certification Program (ESTCP) Project ER-0518 and guidance from commercial vendors, field filtration is recommended for collection of biomass from groundwater (Lebrón et al. 2011, Ritalahti et al. 2010). Field filtration increases the likelihood of collecting suspended particles, decreases shipping costs, and significantly reduces the costs associated with laboratory extraction procedures. Whether sending samples to a laboratory for biomass extraction or using the field filtration approach, the active sampling methods enable analysis of virtually all of the biomass (alive, dead, and dormant) within the sample.

**Passive Microbial Sampling Devices**—When sampling groundwater, passive microbial sampling devices typically consist of a solid matrix as a surrogate for aquifer material within a slotted or otherwise permeable housing. Although a number of solid matrix materials have been used (e.g., sterilized sand, glass or ceramic beads, glass wool, granular activated carbon), Bio-Trap samplers are commonly used

and commercially available passive microbial sampling devices. Bio-Traps contain Bio-Sep beads, a composite of Nomex® and powdered activated carbon (PAC), as the solid matrix. Nomex allows beads to be heat sterilized prior to in-well deployment, while the PAC provides adsorptive properties and a large surface for microbial growth. When sampling groundwater, passive microbial sampling devices are typically deployed in an existing monitoring well for 30–90 days. During in situ deployment, active microorganisms grow on and/or within the solid matrix similar to biofilm formation on native aquifer materials. Once recovered from the well, DNA, RNA, or phospholipids can be readily extracted from the solid matrix for analysis by the EMD methods to characterize the subsurface microbial community. If the solid matrix contains activated carbon, organic aquifer contaminants will adsorb to the matrix during incubation and may also be extracted for VOC/semivolatile organic compound analyses or CSIA.

The solid matrix in passive microbial sampling devices is not a perfect surrogate for the aquifer material; thus, the microbial community colonizing the surface or interior of this solid matrix may not perfectly reflect the community composition of the aquifer.

## Advantages of Active Microbial Sampling Methods

- Active microbial sampling methods can be easily integrated into existing site monitoring programs since the sample collection techniques are comparable (e.g., low-flow groundwater sampling from monitoring wells).
- Since actual environmental media (e.g., soil and groundwater) are collected and biomass extraction/filtration methods have become highly efficient, the resulting EMD data are considered to represent in situ conditions at the time of sampling relatively well.
- Field filtration increases the likelihood of collecting suspended particles, decreases shipping costs, and significantly reduces costly laboratory extraction procedures.

## Limitations of Active Microbial Sampling Methods

- Active microbial sampling devices give a “snapshot” of the microbial community; therefore, periodic sampling is required to evaluate variations over time.
- Active microbial sampling is targeted at collection of site media samples only and does not allow for in situ assessments (e.g., in-well SIP or treatability studies).
- Filters can clog during sampling, which would limit the sample size and potentially reduce the representativeness of the sample.
- Active sampling methods may use sterilized materials and aseptic techniques, requiring additional training for field personnel.

## Advantages of Passive Microbial Sampling Devices

- Passive microbial sampling devices are relatively easy to deploy and recover.
- Passive sample collection over an extended period of time may be more representative of actual subsurface conditions compared to single, “snapshot” grab-sample collection of a microbial community.
- EMD results based on passive microbial sampling devices can reflect temporal changes in aquifer microbial community composition that cannot always be discerned from analysis of groundwater samples.
- Passive microbial sampling devices can be amended with potential remediation amendments (e.g., electron donors or electron acceptors) or microbial cultures to evaluate treatment alternatives.
- Passive microbial sampling devices that contain activated carbon have been used for SIP studies to provide evidence of in situ biodegradation potential of a contaminant by indigenous microorganisms under actual aquifer conditions.
- Passive microbial sampling devices that contain activated carbon can concentrate contaminants for CSIA.

- Certain passive sampling media, such as Bio-Sep, collect only organisms that are actively reproducing under local aquifer conditions.

## Limitations of Passive Microbial Sampling Devices

- Passive microbial sampling devices typically require 30–90 days of incubation in the sampled environment and require two mobilizations to the site to install and then retrieve the sampling devices.
- The solid matrix of most passive microbial sampling devices is a surrogate; thus, differences may exist between organisms colonizing the sampling device and native aquifer material.
- Regulatory approval may be required to deploy amended sampling devices, depending on the amendment and the applicable regulations.
- Data cannot be normalized to a unit volume of groundwater.

## Sampling Protocols

**Active** microbial sampling involves biomass extraction/filtration from environmental media samples. Based on field trials conducted as part of ESTCP Project ER-0518 and guidance from commercial vendors, field filtration is recommended for collecting biomass from groundwater. A field filtration approach involves low-flow groundwater purging and sampling from monitoring wells, using the same methods that are generally recommended when sampling for VOCs. Representative groundwater is passed through a filter (e.g., Sterivex™), which isolates biomass from the sample. The filter is then shipped overnight on ice to a laboratory for analysis. A guidance protocol is available under ESTCP Project ER-0518 (Lebrón et al. 2011; Petrovskis, Amber, and Walker, in press), which provides a step-by-step approach to groundwater sampling using field filtration methods.

**Passive** microbial sampling devices are typically deployed in purged groundwater monitoring wells located within and upgradient of the dissolved contaminant plume to compare results of analyses between impacted and background conditions. Comparing the impacted area to a background control clearly illuminates the effect of a contaminant on the groundwater community. A typical in-well incubation period is 30–90 days. Following in well deployment, samplers are recovered and shipped overnight on ice for analysis. If recovered passive microbial sampling devices have been frozen, it is important that they not thaw in route to the laboratory for analysis.

Users of all types of microbial sampling devices should work with the analytical laboratory to ensure that sampling protocols for collecting, handling, and transporting the samples are in place and understood.

## Quality Assurance/Quality Control

Commercial filters for active sampling and passive microbial sampling devices are assembled under sterile conditions and shipped in sterile bags. Following deployment both types of samplers should be shipped cold by overnight delivery to their respective locations for analysis. Currently, users can best ensure data quality by detailing the laboratory requirements in a site-specific quality assurance project plan (QAPP). This plan should include identification of the sampling devices and procedures being used; the field locations and procedures, including preservation requirements; the EMDs being used; the standard operating procedures of the laboratory performing the analyses; and any internal quality assurance/quality control information available (such as results for positive and negative controls).

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## Glossary

**bioaugmentation**—The introduction of cultured microorganisms into the subsurface environment for the purpose of enhancing bioremediation of organic contaminants (EPA 2011).

**biodegradation**—A process by which microorganisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment (EPA 2011).

**biostimulation**—A remedial technique which provides the electron donor, electron acceptor, and/or nutrients to an existing subsurface microbial community to promote degradation.

***Dehalococcoides***—A specific group (genus) of bacteria. *Dehalococcoides* species are the only microorganisms that have been isolated to date which are capable of complete reductive dechlorination of PCE and TCE to ethene. Some *Dehalococcoides* species are also capable of reductive dechlorination of chlorinated benzenes, chlorinated phenols, and polychlorinated biphenyls (PCBs).

**DNA (deoxyribonucleic acid)**—A nucleic acid that carries the genetic information of an organism. DNA is capable of self-replication and is used as a template for the synthesis of RNA. DNA consists of two long chains of nucleotides twisted into a double helix (EPA 2004).

**electron acceptor**—A chemical compound that accepts electrons transferred to it from another compound (based on EPA 2011).

**electron donor**—A chemical compound that donates electrons to another compound (based on EPA 2011).

**phospholipid**—A type of biomolecule that is a primary structural component of the membranes of almost all cells.

**PLFA—**Phospholipid fatty acids derived from the two hydrocarbon tails of phospholipids.

**RNA (ribonucleic acid)**—Single-stranded nucleic acid that is transcribed from DNA and thus contains the complementary genetic information.

# Appendix A

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