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.

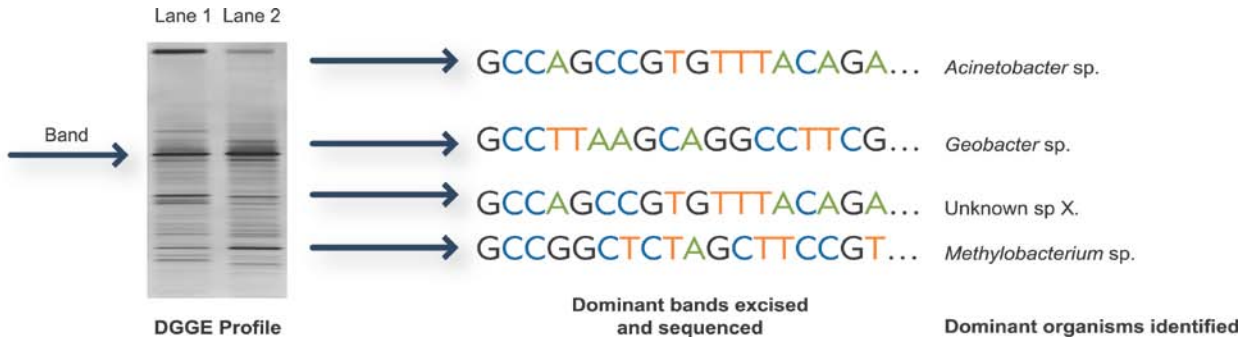

Figure 1. DGGE bands, with diagnostic sequences and identifications of the microorganisms responsible for a given band. The figure illustrates the separation and visualization (step 3) and sequence identification (step 4) aspects of the DGGE fingerprinting method.
Source: Microbial Insights, Inc., 2010,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

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