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.

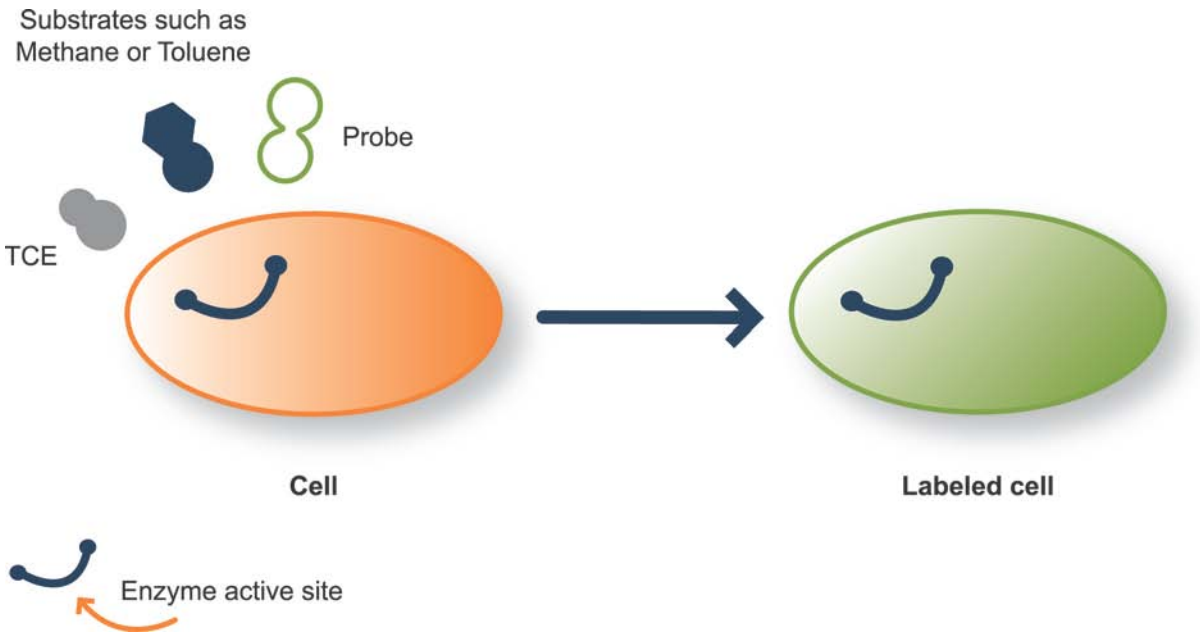

Figure 1. In this example of one class of EAPs, the target enzyme is an oxygenase. These enzymes are found in bacteria that can grow on substrates such as methane, toluene, propane, or ammonia. These enzymes can also often attack and degrade contaminants such as chlorinated solvents (e.g., TCE). The 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.

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