Bioremediation and monitored natural attenuation are among the most cost-effective approaches to manage soil and groundwater contamination by hazardous organic pollutants. However, these remediation alternatives are not universally applicable and may be marginally effective for recalcitrant pollutants if the necessary microbial catabolic capacity is not present or expressed. Thus, regulatory and public approval of bioremediation and natural attenuation requires documentation of the efficacy of microbial degradation of the target pollutants. Performance assessment generally consists of three components: documented contaminant mass loss, geochemical fingerprints associated with biodegradation, and microcosm studies that show direct evidence of biodegradation. More recently, new molecular and isotope fractionation techniques have emerged to complement existing technologies for the forensic analysis and the demonstration of bioremediation and natural attenuation. This critical review examines the current state-of-art in performance assessment methods and discusses future research directions.

KEY WORDS: bioremediation, monitored natural attenuation, stable isotope analysis, chemical fingerprinting, molecular biological tools
INTRODUCTION

Bioremediation and natural attenuation are remediation technologies that rely on natural or stimulated microbial degradation processes and hold great promise for the cost-effective removal of a wide variety of environmental pollutants. In particular, successful applications of bioremediation have been well documented for many sites contaminated with three major classes of hazardous wastes: petroleum hydrocarbons, creosotes, and chlorinated solvents (NRC, 1993). Bioremediation offers several advantages and limitations compared to traditional site remediation approaches, such as pump-and-treat or soil excavation followed by incineration. The principal advantages generally include lower cost and the ability to eliminate pollutants in situ, often transforming them into innocuous byproducts such as CO₂ and water. This eliminates potential liability costs associated with hazardous waste transportation and storage. However, bioremediation is not universally applicable, and it may be marginally effective for recalcitrant pollutants if the necessary microbial catabolic capacity is not present or expressed. For example, adverse environmental conditions such as extreme pH, temperature, or the presence of heavy metals at toxic concentrations may hinder specific microbial activities (Alvarez & Illman, 2006).

Monitoring efforts and a complex site characterization may be necessary to demonstrate the efficacy of bioremediation and natural attenuation. In the United States, the Environmental Protection Agency (EPA) directive (as well as the ASTM standard developed for the remediation industry) requires essentially three lines of evidence to demonstrate bioremediation and natural attenuation: 1) documented mass loss of contaminant; 2) geochemical fingerprints showing operation of biodegradation; and 3) microcosm studies that show direct evidence of biodegradation (EPA, 1999). The quantity and type of information that needs to be collected at a given site depends on factors such as the size and the nature of the contamination, the potential risk to receptors, the type of contaminants involved, and complexity in hydrogeologic conditions.

There are many methods to assess the performance of bioremediation and natural attenuation (see Table 1). More recently, new techniques have emerged that can complement existing technologies for the performance assessment and demonstration of bioremediation and natural attenuation. This critical review examines the state of the art in performance assessment methods of bioremediation and natural attenuation and suggests future research directions.

DIRECT EVIDENCE OF CONTAMINANT MASS LOSS IN THE FIELD

To demonstrate a clear and meaningful trend of decreasing contaminant mass and/or concentration over time at the field scale, data from several
**TABLE 1.** Comparison of established and emerging techniques to assess bioremediation and natural attenuation (Alvarez & Illman, 2006)

<table>
<thead>
<tr>
<th>Method of investigation</th>
<th>Applicability</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graphical and statistical analyses, analytical and numerical models</td>
<td>Established</td>
<td>Determine whether plume is stabilizing, increasing or decreasing in size.</td>
</tr>
<tr>
<td>Geochemical parameters</td>
<td>Established</td>
<td>Observe contaminant trends; daughter product formation; presence of geochemical footprints to indicate biogeochemical process responsible for biodegradation.</td>
</tr>
<tr>
<td>Push-pull tests</td>
<td>Established</td>
<td>Single-well tracer tests used to evaluate in situ degradation rates and metabolite formation.</td>
</tr>
<tr>
<td>Microcosm studies</td>
<td>Established</td>
<td>Observe presence of microorganisms, protozoan predators, cellular biomarkers, nucleic acids, and phospholipids; determination of cellular contents of ribosomes, intracellular energy reserves, nutritional status; measure cell growth and uptake of physiological substrates, respiratory activity from the reduction of dyes; determination of novel organisms from DNA sequencing; compute relative abundances of each phylogenetic group; observe unique intermediary metabolites indicative of biodegradation.</td>
</tr>
<tr>
<td>Stable isotope analysis</td>
<td>Emerging but in greater use</td>
<td>Demonstrate biodegradation of organic compounds; quantify microbial activities in situ; quantify relative extent of biodegradation between zones of contaminant plume.</td>
</tr>
<tr>
<td>Chemical fingerprinting</td>
<td>Emerging but in greater use</td>
<td>Identification of fuel types, determination of contaminant sources that will assist in the mapping of subsurface contamination and backward in time contaminant transport simulations.</td>
</tr>
<tr>
<td>Molecular techniques</td>
<td>Emerging</td>
<td>Used to increase understanding of biochemical reactions and their mechanisms; techniques allow one to obtain answers to questions such as, “What microorganisms are there?”, “What are microorganisms doing?”, and “When are the microorganisms active?” It also will likely provide a comprehensive mechanistic understanding of processes that regulate the genes that encode the proteins that actually degrade the contaminants.</td>
</tr>
</tbody>
</table>
monitoring or sampling locations must be collected over several time periods. However, the decrease could be due to plume migration, dispersion, and dilution. Therefore, a data set showing a stabilizing or shrinking plume may not necessarily prove that the contaminants are being destroyed. This suggests that alternative methods are needed to prove that bioremediation and natural attenuation are operating at a given site. We discuss later performance assessment techniques that can be used to help prove that bioremediation and natural attenuation are taking place at a site.

The first step in documenting the direct evidence of contaminant mass loss in the field is to develop an initial site conceptual model. At this stage, at which minimal data is available, the focus is on formulating a basic conceptual model from site hydrogeological conditions, including the direction of groundwater flow, background concentrations, and the location of the contaminant plume. Once an initial conceptual model is formulated for the site, groundwater monitoring strategies may be developed based on the preliminary information. It is important to remember that the formulation of a conceptual model is an iterative process with increased availability of information allowing for the updating of the model. Briefly, a conceptual model is an evolving hypothesis identifying the important features, processes, and events controlling fluid flow and contaminant transport of consequence at a specific field site in the context of a recognized problem (National Research Council, 2001). A conceptual model may also be a mental construct or hypothesis accompanied by verbal, pictorial, diagrammatic, and/or tabular interpretations and representations of site conditions as well as corresponding flow and transport dynamics (Neuman & Wierenga, 2003). The main difference between a site conceptual model and analytical or numerical model is that the latter is a quantitative representation of the former.

Once a preliminary site conceptual model is formulated, decisions need to be made to allocate resources for additional characterization efforts. The goal here is to document contaminant mass loss; hence, detection of changes in contaminant concentration both spatially and temporally will depend heavily on the proper location and construction of monitoring wells. The location of monitoring wells is important in all remediation efforts, but in the case of natural attenuation, this takes a greater importance because contaminant migration is controlled mainly by advective transport provided by ambient groundwater flow. The installation of monitoring wells to adequately quantify contamination concentrations is very important in determining the physical, chemical, and biological contributions to mass destruction. The location, number, and other pertinent data on monitoring wells to evaluate natural attenuation is a site-specific problem, as the network design will be determined by plume size, site complexity, source strength, groundwater/surface water interactions, distance to receptors, and the confidence limits that involved parties wish to place in the data (Azadpur-Keeley et al., 2001). One suggested monitoring network design is of McAllister and Chiang (1994), which is shown in Figure 1. It requires the monitoring of at least two
Assessment of Bioremediation and Natural Attenuation

FIGURE 1. Recommended groundwater monitoring network (modified after McAllister and Chiang, 1994).

Well transects throughout the plume, one in the direction of groundwater flow and another in the transverse direction to flow. At least one well should be placed upgradient of the source to obtain background concentrations, as well as beyond the downgradient edge of the plume to bracket the region of influence of the contamination.

Using a monitoring network such as the one shown in Figure 1, different groundwater monitoring strategies can be employed at various stages of remediation:

- **Site characterization** to describe the disposition of contamination and forecast its future behavior. It is also used to set site-specific cleanup levels in accordance with the risk-based corrective action (RBCA) paradigm.
- **Validation monitoring** to determine whether predictions of site characterization are accurate (demonstrate performance according to expectations, ensure that there is no impact to downgradient receptors, detect any new releases that could impact effectiveness, detect changes in environmental conditions, etc.).
- **Long-term or performance monitoring** to ensure that plume behavior does not change. Required for as long as a contamination remains above cleanup goals and beyond.

It is important to recognize that the network suggested in Figure 1 is for a generic plume, as the subsurface is generally heterogeneous. This is the rule rather than the exception, so plumes will take different shapes. Hence, the monitoring network will be needed to be customized for a given site. There are several issues that one must also consider in placing wells. For example, the longitudinal dispersivity of plumes is typically 10 times larger than the transverse dispersivity. This causes the plume to be elongated like a cigar; therefore, a sparse network of wells could potentially cause the plume...
to bypass a monitoring well network. Also, buried channels and backfill for infrastructure such as sewers, water mains, and cables may offer higher hydraulic conductivity and serve as preferential channels for contaminants to travel, causing the plume to miss the monitoring well network completely.

Documentation of Contaminant Mass Loss

To analyze and document contaminant mass loss at a given site, we advocate the hierarchical approach proposed by the NRC (2000). There are four levels of data analysis that one can perform at a given site, with the complexity of analysis increasing as we go down the list:

1) graphical and statistical analyses of contaminant concentration trends;
2) mass budgeting and mass flux analysis to track contaminant mass;
3) analytical modeling of solute transport; and
4) numerical modeling of flow and solute transport.

There are a number of factors that determines the suitability of one approach over another at a given site. Factors such as the characteristics of the contaminants, site hydrogeology, and the anticipated risk associated with the spreading of the contamination are all considered to be important in determining the approach. Table 2 provides guidance in choosing the level of data analysis appropriate for a site under investigation. In general, simple analyses such as graphical and statistical analyses of data are sufficient for sites with uniform and simple hydrogeological conditions and when the contaminant concentrations are low. If the contaminants readily attenuate, then a simple analysis is considered in most cases to be adequate. On the other hand, more detailed and complex analyses are required when the contaminants concentrations are high, when they are not readily biodegradable, and/or when the site hydrogeology is considered to be complex. The simple methods of analyses should also be performed when a suite of complex analyses are required at a given site. In such cases, a simple analysis should be conducted at the initial stages of the project, and as more data become available, more complex analyses should be performed.

Graphical and Statistical Analyses of Contaminant Concentration Trends

A plume stability analysis can be conducted to determine whether a particular plume is shrinking, stable, or expanding. One approach examines concentration trends of contaminants obtained from monitoring wells over several time periods. A decrease in contaminant concentrations with time may be circumstantial evidence that contaminant mass loss is occurring. The
<table>
<thead>
<tr>
<th>Hydrogeology</th>
<th>Contaminant characteristics</th>
<th>Analysis Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple flow, uniform geochemistry, and low concentrations</td>
<td>Biodegrades under most conditions (e.g., BTEX)</td>
<td>Graphical and statistical analyses</td>
</tr>
<tr>
<td>Simple flow, small-scale physical or chemical heterogeneity, and medium-high concentrations</td>
<td>Immobile under most conditions (e.g., Pb)</td>
<td>Graphical and statistical analyses</td>
</tr>
<tr>
<td>Strongly transient flow, large-scale physical or chemical heterogeneity or high concentrations</td>
<td>Biodegrades under limited conditions (e.g., chlorinated ethenes)</td>
<td>Mass budgeting and mass flux analysis</td>
</tr>
<tr>
<td></td>
<td>Immobile under some conditions (e.g., Cr)</td>
<td>Analytical solute transport model</td>
</tr>
<tr>
<td></td>
<td>Mobile and degrades or decays slowly (e.g., tritium, MTBE)</td>
<td>Mass budgeting and mass flux analysis with numerical solute transport model</td>
</tr>
</tbody>
</table>

Notes. The table lists the recommended levels of natural attenuation data analysis for different contaminants and site conditions. In the site descriptions given along the left-hand side, the recommended data analysis strategy applies when all of the conditions are satisfied unless the term “or” is used. Data completeness and consistency are to be evaluated in all cases. All techniques listed in higher rows of the same column are to be applied, along with the methods in the applicable row. Where mixed concentrations are present, the most thorough analysis recommended for any single contaminant should be applied to the entire site. Abbreviations: BTEX = benzene, toluene, ethyl benzene, and xylene; MTBE = methyl tert-butyl-ether.
detection and estimation of trends can be confounded by changes in sample collection procedures or analytical techniques, seasonality, and variations in groundwater flow and dilution processes (Gilbert, 1987). Contaminant concentration can also be obtained and plotted in the direction of contaminant transport. For example, concentrations of contaminants can be obtained in the source zone and at a number of locations downgradient to the source. Other graphical methods include the preparation of contour maps of contaminant concentrations obtained through synoptic sampling. Contour plots can also be produced over multiple dates to obtain the spatial-temporal coverage of the plume and analyze plume dynamics to determine whether the plume is decreasing, stable, or increasing in size. As natural attenuation progresses, a decline in contaminant concentration should be observed, while an increase in daughter product concentration occurs.

Mass Budgeting and Mass Flux Analysis to Track Contaminant Mass

Mass budgeting is another approach that is used to assess natural attenuation. It involves evaluating whether the rate at which footprint compounds are produced is commensurate with the rate at which the contaminant is destroyed or sequestered (NRC, 2000). One is able to determine the importance of natural attenuation processes by comparing the relative rates. More specifically, one defines a domain which encompasses the plume and assumes 1-D advective flux and a steady-state plume. Observations of electron acceptors up- and down-gradient of this domain are then used in conjunction with stoichiometric relationships in order to assess whether biodegradation is responsible for contaminant destruction or immobilization and to estimate its depletion rate. Further details to the approach are provided in NRC (2000).

Another method to evaluate whether a plume is shrinking, stable, or expanding is to estimate the mass fluxes of contaminants at specified transects. The total mass flux—that is, the amount of contaminant mass migrating through cross-sections of the aquifer perpendicular to groundwater flow (see Figure 2)—decreasing over time is one indication that natural attenuation is taking place.

We summarize below the methodology developed by Einarson and Mackay (2001a, 2001b; see also American Petroleum Institute [2003]) to estimate contaminant mass flux using well transects. Contaminant mass flux is estimated using sampling transects consisting of either traditional monitoring wells or multi-level samplers. An array of monitoring wells or multi-level samplers is positioned perpendicular to the flow path of the contaminant plume with the transect extending both horizontally and vertically beyond the extent of the contaminant plume. Groundwater samples are collected from each monitoring point and analyzed for the contaminant under scrutiny. One also calculates the specific discharge using the local hydraulic conductivity and
Figure 2. Investigation of a contaminant plume using well transects to determine whether a plume is shrinking, stable, or expanding where total mass flux is defined according to Eq. (1) (modified after American Petroleum Institute, 2003).

Hydraulic gradient. A cross-section of the subsurface is made at the location of the transect, and a contour plot of concentrations is made. Polygons are defined based on the cross-section and contour plot. The total contaminant flux is then calculated using the following equation:

\[ M_d = \sum_{i=1}^{n} M_{di} = \sum_{i=1}^{n} C_i A_i q_i \]  

(1)

where \( M_d \) is the total mass flux from the source zone [MT\(^{-1}\)], \( M_{di} \) is the contaminant mass flux through polygon \( i \) [MT\(^{-1}\)], \( C_i \) is the contaminant concentration within the flow area of polygon \( i \) [ML\(^{-3}\)], \( A_i \) is the cross-sectional area of polygon \( i \) [L\(^2\)], and \( q_i \) is the specific discharge through polygon \( i \) [LT\(^{-1}\)]. Here, the dimensions of each variable are provided in mass [M], length [L], and time [T].

Einarson and MacKay (2001b) present an example in which they applied this technique to estimate contaminant mass flux of cis 1,2-dichloroethene (DCE) flowing in a dissolved contaminant plume at Site 1, Alameda Point, California, USA. Figure 3a shows contours of cis 1,2-DCE concentrations measured along the transect. The concentrations were obtained from groundwater samples taken from a seven-zone multi-level sampler installed at the site. Each concentration value obtained from the sampling point is assigned to polygons (in this case, rectangular cells) with the sampling point located at the center of the polygon (see Figure 3b). The contaminant mass flux in each polygon is then calculated by multiplying the averaged contaminant concentration, with the area of the polygon and the specific discharge. The total contaminant mass flux is obtained by summing the individual \( M_{di} \) values. The average hydraulic conductivity value determined at the site was 6.6 \( \times \) 10\(^{-3}\) cm/sec. Together with the measured horizontal hydraulic gradient of 0.0029, the calculated cis 1,2-DCE \( M_d \) is approximately 31 g/day (Einarson & MacKay, 2001b).
FIGURE 3. Estimates of contaminant $M_d$ can be obtained by sampling a transect of multi-level monitoring points installed perpendicular to the axis of the dissolved plume: (a) contours of cis 1,2-DCE concentrations measured along the transect; (b) discrete concentration measurements are assigned to rectangular cells centered around each monitoring point. The mass discharge within each cell ($M_{di}$) is calculated by multiplying the concentration value by the flux of groundwater through the cell. Total contaminant $M_d$ is obtained by summing the individual $M_{di}$ values (modified after Einarson & MacKay, 2001b).

Conventional water samples are representative of small subsurface volumes relative to the scale of the plume. As such, these point measurements are subject to high uncertainties with regard to contaminant concentrations between sampling points. To minimize this uncertainty, an Integral pumping test (IPT) has been suggested by Teutsch et al. (2000) and Ptak and Teutsch (2000). Bayer-Raich et al. (2004) provide an analytical framework...
to determine average contaminant concentration and mass flux in aquifers during an IPT. The method complements conventional approaches determining contaminant concentrations and mass fluxes, especially when sampling density is low, which can lead to misinterpretations.

Analytical Modeling of Solute Transport

Analytical models are useful in estimating how far a contaminant plume will migrate and how long it will take for a plume to reach steady state. The latter is useful in predicting when the plume will become stable, which could mean that natural attenuation is working. Analytical models can also be used to assess the age and stability of contaminant releases. One common approach is to use the available data on the spatial distribution of the target contaminant(s) with appropriate hydrogeologic parameters to calibrate the model. Using standard data fitting techniques, such as non-linear regression, one can estimate site-specific migration rates and assess the release age. Another use of an analytical model is the estimation of a biodegradation rate. Analytical models can be fit to plume concentrations by trial-and-error or nonlinear regression by adjusting the degradation rate.

The key assumptions that go into analytical solute transport models have been described in various publications (e.g., Baetlé, 1969; Bear, 1979; Domenico, 1987; Domenico & Robbins, 1985b). Deviations from model assumptions are commonly due to heterogeneity of the aquifer material, which causes spatial variability in model parameters such as hydraulic conductivity, porosity, sorption capacity, and biodegradation kinetics (due to changes in availability of nutrients or electron acceptors needed for microbial activity). Other deviations from ideal behavior include seasonal changes in hydraulic gradient and associated variability in groundwater flow velocity and direction, artificial hydraulic gradients caused by pumping wells, and variable source flux with undefined shape. Such complexities cannot be incorporated in analytical models, which feature their relative simplicity as both their main advantage and disadvantage. Therefore, analytical model results should be interpreted with caution. These analytical solutions are often used in screening studies (Tiers 1 and 2 RBCA) and are generally limited to steady, uniform flow and should not be used for groundwater flow or solute transport problems in strongly anisotropic or heterogeneous aquifers. These models additionally should not be applied under non-uniform flow conditions. It should be kept in mind that analytical models are best utilized for order-of-magnitude estimations, as a number of potentially important processes are treated in the models in an approximate manner or sometimes ignored totally. Analytical models are also useful in exploring a variety of worst case scenarios for plume migration, if site conditions are relatively simple and the assessments are preliminary (NRC, 2000).
When dealing with uncertainty and variability of model parameters, it is good practice to conduct sensitivity analyses using a reasonable range of all parameters that are not well defined for the site of interest. This type of analysis allows the modeler to demonstrate worst case and best case scenarios, and to provide valuable input for risk assessment and control decisions. The use of analytical fate-and-transport models is likely to increase with the adoption of risk-based corrective action (RBCA) in the environmental protection rules of numerous countries.

Numerical Modeling of Flow and Solute Transport

Numerical flow and solute transport models are needed when the site is complicated and the complexity has to be captured in the model. The main advantage of numerical approaches over analytical solutions is that it is flexible and can handle complex geometries. This flexibility allows one to incorporate complex geological features and spatial variability in flow and transport parameters. It can also incorporate spatial variation in the initial condition and both space and time variation in boundary conditions (as well as biogeochemical processes in both space and time). Time and space variations in source/sink terms are readily incorporated in numerical models. If site heterogeneities are large but well known, it will probably be more appropriate to use a numerical model that discretizes the site into small elements or blocks and assigns appropriate parameters to different domains. Conditions under which a numerical solute transport models should be used include (NRC, 2000):

- when chemical reactions take place that need to be incorporated to accurately track the contaminant transport;
- when multiple reactions take place (i.e., the products of key reactions participate in other reactions, such as precipitation or complexation, that affect aqueous-phase concentrations);
- when the partitioning of contaminant materials to other phases occur;
- when loss reactions occur in multiple steps that produce and consume intermediates; and
- when the site hydrogeology is complex and highly transient in nature, both of which have to be incorporated.

Table 3 is a list of numerical models that can be used to assess bioremediation and natural attenuation.

GEOCHEMICAL INDICATORS OF INTRINSIC BIOREMEDIATION

The second line of evidence relies on hydrogeochemical data that can be used to demonstrate indirectly the type(s) of natural attenuation processes
<table>
<thead>
<tr>
<th>Name of code</th>
<th>Description</th>
<th>Distributor/Contact</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIO1D®</td>
<td>One-dimensional, finite difference, numerical simulation code for simulation of biodegradation and sorption of hydrocarbons. BIO1D can be applied to solute transport problems involving a reactive substrate. The reactions included are aerobic and anaerobic degradation and/or adsorption described by a linear, Freundlich or Langmuir isotherm. It has a preprocessor and display graphics.</td>
<td>Scientific Software Group (<a href="http://www.scisoftware.com">http://www.scisoftware.com</a>)</td>
<td>Srinivasan and Mercer (1988)</td>
</tr>
<tr>
<td>BIOPLUME III</td>
<td>Two-dimensional, finite difference numerical simulation code for reactive transport of multiple hydrocarbons under the influence of advection, dispersion, sorption, first-order decay, and reactant-limited biodegradation. Development commissioned by the Airforce Center for Environmental Excellence (AFCEE). The code has interactive, graphical pre- and postprocessing capabilities. Based on the United States Geological Survey (USGS) two-dimensional Method of Characteristics (MOC) model (including a finite-difference flow model) by Konikow and Bredehoft (1978). Oxygen-limited biodegradation is a reactive transport process. It is a public domain code with a menu-driven preprocessor and limited postprocessing capabilities.</td>
<td>EPA</td>
<td>Rifai et al. (1998)</td>
</tr>
<tr>
<td>CompFlow Bio</td>
<td>CompFlow Bio (Yu et al., 2009) is a multi-phase multi-component compositional thermal model that considers three mobile phases, namely the aqueous, non-aqueous, and gas phases, following from the work of Forsyth (1993) and Unger et al. (1995). The components can be summarized as water, air, any number of organic components, plus oxygen, methane and carbon dioxide. It also includes enhancements for dual-Monod biodegradation due to aerobic and anaerobic biomass, as well as branched first-order chain-decay.</td>
<td>Andre Unger (<a href="mailto:aunger@uwaterloo.ca">aunger@uwaterloo.ca</a>)</td>
<td>Forsyth (1993), Unger et al. (1995), Yu et al. (2009)</td>
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<th>Name of code</th>
<th>Description</th>
<th>Distributor/Contact</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEHM</td>
<td>Three-dimensional, finite element or finite volume numerical simulation code for time-dependent, multiphase, multicomponent, nonisothermal, reactive flow/transport through porous and fractured media. It can accurately represent complex 3-D geologic media and structures and their effects on subsurface flow and transport.</td>
<td>Los Alamos National Laboratory (<a href="http://www.ees5.lanl.gov/fehm/">http://www.ees5.lanl.gov/fehm/</a>)</td>
<td>Zyvoloski et al. (1997)</td>
</tr>
<tr>
<td>FEFLOW</td>
<td>Three-dimensional, finite element, numerical simulation flow and transport code for saturated and unsaturated zones with capabilities for heat transport as well. Sorption is modeled through Henry, Freundlich, or Langmuir isotherms. It also considers variable fluid density due to temperature or (salt) concentration effects; and additional capabilities include 1D/2D finite elements for flow and transport in fractures, channels, or tubes.</td>
<td>Waterloo Hydrogeologic, Inc. (<a href="http://www.waterloohydrogeologic.com">www.waterloohydrogeologic.com</a>)</td>
<td>Diersch (2005)</td>
</tr>
<tr>
<td>FEMWATER,</td>
<td>Finite element flow (FEMWATER) and transport (FEMWASTE) models. FEMWATER can simulate variably saturated conditions in two and three dimensions. FEMWASTE can simulate transport in one, two, and three dimensions. The system may be heterogeneous and anisotropic, and the code can account for advection, dispersion, first-order decay, and three types of sorption. Public domain codes developed by researchers at Oak Ridge National Laboratories. Some proprietary versions of FEMWATER are available; they are included in the Department of Defense' Groundwater Modeling System (GMS) modeling and data management package.</td>
<td>Oak Ridge National Laboratories; Environmental Modeling Systems, Inc. (<a href="http://www.ems-i.com">www.ems-i.com</a>)</td>
<td>Yeh et al. (1992)</td>
</tr>
<tr>
<td>FEMWASTE</td>
<td></td>
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<tr>
<td>FLONET®,</td>
<td>Two-dimensional steady-state groundwater flow (FLONET) and transient solute transport (FLOTANS) models for cross-sectional problems. FLOTANS is an extension of FLONET that can simulate transport under the influence of advection, dispersion, linear sorption, and first-order decay. A proprietary program with an interactive graphical user interface and extensive pre- and postprocessing capabilities.</td>
<td>Waterloo Hydrogeologic, Inc. (<a href="http://www.waterloohydrogeologic.com">www.waterloohydrogeologic.com</a>)</td>
<td>Guiguer et al. (1994); Waterloo Hydrogeologic Inc. (1997)</td>
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<tr>
<td>FLOTANS®</td>
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</table>
FRAC3DVS is a three-dimensional, finite element model for simulating steady-state or transient, variably-saturated groundwater flow and advective-dispersive solute transport in porous or discretely-fractured porous media.

FRACTRAN is a 2-D (cross-section and areal) finite-element model for simulating steady-state groundwater flow and transient contaminant transport in a discretely fractured, porous media. The porous media is represented by block elements while fractures (optional) are represented by line elements. Unlike other fracture flow models, FRACTRAN accounts for flow and transport mechanisms through both the discrete fractures and the matrix block. FRACTRAN also has the option of solving for flow and transport in non-fractured porous media.

Graphical user interface (GUI)-based software that combines applications in 3-D groundwater flow and contaminant transport modeling. It supports both finite-difference and finite-element models in 2D and 3D including MODFLOW 2000, MODPATH, MT3DMS/RT3D, SEAM3D, ART3D, UTECHM, FEMWATER, PEST, UCODE, MODAEM, and SEEP2D. Model calibration can be done with a parameter estimation package (PEST) implemented in the code. Pre-processing of input data and post-processing of results can be done within the software.

Numerical simulation code for modeling groundwater flow and associated heat and solute transport in three dimensions. Solute transport is for a single solute with advection, dispersion, linear sorption, and first-order decay. A public domain code available at the USGS with no pre- and postprocessors.
<table>
<thead>
<tr>
<th>Name of code</th>
<th>Description</th>
<th>Distributor/Contact</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>HydroGeoSphere</td>
<td>A three-dimensional numerical model for fully-integrated subsurface and surface flow and solute transport. Surface domain is represented as 2-D areal flow for the entire surface or as 2-D runoffs into 1-D channels. The subsurface domain consists of 3-D unsaturated and/or saturated flow. Both domains naturally interact through physically based fluid exchange terms. The code also handles density-dependent flow and transport. As for transport, the code handles simulation of non-reactive and reactive chemical species transport, and heat transport in the associated surface and subsurface flow fields.</td>
<td>University of Waterloo and Université Laval</td>
<td>Therrien et al. (2005)</td>
</tr>
<tr>
<td>MOC, USGS2D-MOC</td>
<td>Two-dimensional model for simulation of groundwater flow and non-conservative solute transport. Derived from the original model developed by Konikow and Bredehoeft (1978). The latest version (March 1995) simulates transport under the influence of advection, dispersion, first-order decay, reversible equilibrium-controlled sorption, and reversible equilibrium-controlled ion exchange. The flow model is a finite-difference model, while transport is simulated using MOC methods. A public domain code with an interactive preprocessor.</td>
<td>USGS, IGWMC</td>
<td>Konikow and Bredehoeft (1978)</td>
</tr>
<tr>
<td>MODFLOW, MODFLOW1996, &amp; MODFLOW2000</td>
<td>Block centered finite difference code for steady-state and transient simulation of groundwater flow in two and three dimensions. Consists of main program and a large number of subroutines (modules) that are used to simulate a wide variety of boundaries and stresses on the hydrogeologic system. Originally coded by McDonald and Harbaugh (1988) of the USGS. Possibly the most widely used flow model in the United States and Canada, MODFLOW can be used to generate flow fields that may be coupled with a wide variety of transport models (e.g., MT3D, BioTrans®, or RAND3D). A public domain code available at the USGS.</td>
<td>USGS, IGWMC; in addition, many companies have developed pre- and post-processing programs with a wide variety of capabilities and features</td>
<td>McDonald and Harbaugh (1988), Harbaugh and McDonald (1996), and Harbaugh et al. (2000)</td>
</tr>
<tr>
<td><strong>MODFLOWP</strong></td>
<td>Extension of MODFLOW that includes a package that uses nonlinear regression techniques to estimate model parameters under constraints given by the modeler. Model input includes statistics for analyzing the parameter estimates and the model to quantify the reliability of the resulting model, to suggest changes in model construction, as well as to compare results of models constructed in different ways. A public domain code available at the USGS.</td>
<td>USGS, IGWMC</td>
<td>Hill (1998)</td>
</tr>
<tr>
<td><strong>MODFLOW-SURFACT</strong></td>
<td>The code is based on the USGS MODFLOW code with flow and transport modeling capabilities for both the saturated and unsaturated zones. The code has an axisymmetric option for quick analyses of slug tests or tracer tests. It can accommodate up to five contaminant species in a single simulation, including first-order chain reactions, which may occur due to radioactive decay (species dependent) or biochemical transformation (species and soil/location dependent). Other highlights include options for linear or nonlinear (Freundlich) equilibrium adsorption and two, three, and four component dispersivity options for various anisotropic conditions.</td>
<td>Scientific Software Group; Waterloo Hydrogeologic, Inc. (<a href="http://www.waterloohydrogeologic.com">www.waterloohydrogeologic.com</a>)</td>
<td>Hydrogeologic Inc. (1996)</td>
</tr>
<tr>
<td><strong>MODPATH</strong></td>
<td>A particle tracking code that is used in conjunction with MODFLOW. Particles are tracked through time assuming that advection is the only transport mechanism. Users can define location of particles. The code is useful for defining capture zones, well head protection zones, and potential locations for contaminant source zones.</td>
<td>USGS</td>
<td>Pollock (1994)</td>
</tr>
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<table>
<thead>
<tr>
<th>Name of code</th>
<th>Description</th>
<th>Distributor/Contact</th>
<th>Reference</th>
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<tbody>
<tr>
<td>MT3D</td>
<td>Three-dimensional transport model for simulation of advection, dispersion, linear or nonlinear sorption, and first-order decay of single species. Uses a modular structure similar to that of MODFLOW. Intended for use with any block-centered finite difference model, such as MODFLOW, on the assumption that concentration changes will not affect the flow field. MT3D uses one of three methods (all based on MOC) for solution of the transport equation. MT3D is available in the public domain and proprietary versions. Proprietary versions are typically the most advanced in terms of pre- and post-processing capabilities.</td>
<td>S.S. Papadopulos &amp; Associates, Inc.; IGWMC; many versions available from many companies with pre- and post-processing programs with a wide variety of capabilities and features. Often coupled with MODFLOW in such codes. Public domain version may be acquired from USEPA.</td>
<td>Zheng (1990)</td>
</tr>
<tr>
<td>MT3DMS</td>
<td>A new version of MT3D that has a comprehensive set of options and capabilities for simulating advection, dispersion/diffusion, and chemical reactions of contaminants in groundwater flow systems under general hydrogeological conditions. The key features of MT3DMS are summarized below. It includes the standard finite difference method; the particle-tracking-based Eulerian-Lagrangian methods; and the higher-order finite-volume TVD method. The code also supports an optional, dual-domain formulation for modeling mass transport. It can be used to simulate changes in concentrations of miscible contaminants in groundwater considering advection, dispersion, diffusion, and some basic chemical reactions, with various types of boundary conditions and external sources or sinks. The chemical reactions included in the model are equilibrium-controlled or rate-limited linear or non-linear sorption, and first-order irreversible or reversible kinetic reactions.</td>
<td>C. Zheng, University of Alabama</td>
<td>Zheng and Wang (1999)</td>
</tr>
<tr>
<td>Code</td>
<td>Description</td>
<td>Institution</td>
<td>Reference</td>
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<tr>
<td>RT3D</td>
<td>Modification of MT3DMS, developed by Battelle Pacific Northwest National Laboratory. RT3D is designed to describe multispecies transport and reactions, including attenuation of chlorinated compounds and their daughter products, and fate of solid-phase species. Also included are reaction packages for aerobic, instantaneous BTEX reactions (similar to Bioplume II) and multiple-electron-acceptor, kinetically limited BTEX reactions (similar to Bioplume III). The code is well suited for simulating natural attenuation and bioremediation.</td>
<td>Battelle Pacific Northwest National Laboratory</td>
<td>Clement (1997)</td>
</tr>
<tr>
<td>SEAM3D</td>
<td>A reactive transport model used to simulate complex biodegradation problems involving multiple substrates and multiple electron acceptors. The code is based on MT3DMS. In addition to the regular MT3DMS modules, SEAM 3D includes a biodegradation package and NAPL dissolution package.</td>
<td>M. Widdowson at Virginia Tech University</td>
<td>Widdowson et al. (2002)</td>
</tr>
<tr>
<td>SWMS2D</td>
<td>Two-dimensional model for simulating water and solute movement in variably saturated media. Includes dispersion, linear sorption, zero-order production, and first-order decay. A public-domain code prepared by researchers at the U.S. Salinity Lab.</td>
<td>U.S. Salinity Laboratory, IGWMC</td>
<td>Simunek et al. (1994)</td>
</tr>
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<table>
<thead>
<tr>
<th>Name of code</th>
<th>Description</th>
<th>Distributor/Contact</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMVOC</td>
<td>A numerical simulator for three-phase non-isothermal flow of water, soil gas, and a multicomponent mixture of volatile organic chemicals (VOCs) in multidimensional heterogeneous porous media. It is an extension of the TOUGH2 general-purpose simulation program developed at the Lawrence Berkeley National Laboratory. TMVOC is designed for applications to contamination problems that involve hydrocarbon fuel or organic solvent spills in saturated and unsaturated zones. It can model contaminant behavior under “natural” environmental conditions, as well as for engineered systems, such as soil vapor extraction, groundwater pumping, or steam-assisted source remediation. TMVOC is upward compatible with T2VOC and can be initialized from T2VOC-style initial conditions.</td>
<td>Lawrence Berkeley National Laboratory</td>
<td>Pruess and Battistelli (2002)</td>
</tr>
<tr>
<td>UTCHEM</td>
<td>A program for modeling transient and steady-state three-dimensional flow and mass transport in the groundwater (saturated) and vadose (unsaturated) zones of aquifers. Physical, chemical, and biological process models important in describing the fate and transport of NAPLs in contaminated aquifers are incorporated into the simulator. These include multiple organic NAPL phases; the dissolution and/or mobilization of NAPLs by non-dilute remedial fluids; chemical and microbiological transformations; and changes in fluid properties as a site is remediated. The model allows for non-equilibrium interphase mass transfer; sorption; geochemical reactions; and the temperature dependence of pertinent chemical and physical properties. It can simulate the flow and transport of remedial fluids for which the density, temperature, and viscosity are variable, including surfactants, co-solvents, and other enhancement agents. The biodegradation model includes inhibition, sequential use of electron acceptors, and cometabolism, and can be used to model a very general class of bioremediation processes.</td>
<td>G. Pope at University of Texas at Austin</td>
<td>Reservoir Engineering Research Program (2000)</td>
</tr>
<tr>
<td>Software</td>
<td>Description</td>
<td>Website</td>
<td>Author/Reference</td>
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</tr>
<tr>
<td>Visual Modflow Pro</td>
<td>GUI-based software that combines applications in 3D groundwater flow and contaminant transport modeling. Flow and transport codes implemented include MODFLOW2000, MODPATH, MT3DMS, RT3D. Model calibration can be done with a parameter estimation package PEST implemented in the code. Pre-processing of input data and post-processing of results can be done within the software.</td>
<td>Waterloo Hydrogeologic, Inc. (<a href="http://www.waterloohydrogeologic.com">www.waterloohydrogeologic.com</a>)</td>
<td>Waterloo Hydrogeologic Inc. (2006)</td>
</tr>
<tr>
<td>VSAFT2</td>
<td>A finite element program for solving flow and reactive solute transport in variably saturated porous media (2-D horizontal plane, vertical plane, or axisymmetrical plane). A GUI has been developed for easy generation of irregular solution domain with homogeneous properties, zonal properties, or randomly distributed hydraulic properties, and easy definition of boundary/initial conditions, and observation wells. In addition, a geostatistics package (GSLIB) is being implemented in the program to allow variogram analysis and estimation using kriging and cokriging for parameter estimations. The code also includes a nonlinear least squares approach to estimate homogeneous hydraulic properties of aquifers. More importantly, it has the ability to analyze hydraulic tomography data to obtain estimates of spatial distributions of hydraulic properties including storage terms. These are unique features of VSAFT2 that are not available in any other codes at this moment.</td>
<td>T.-C. Jim Yeh, University of Arizona</td>
<td>Yeh et al. (1993)</td>
</tr>
</tbody>
</table>
that are active at the site, as well as the rate at which such processes will reduce contaminant concentrations to required levels. Some changes in geochemical parameters are known as “footprints” of natural attenuation (NRC, 2000). Such footprints or indicators can be used as secondary evidence to demonstrate whether natural attenuation is occurring at a site or not. Table 4 lists NRC case studies from sites across the country with a variety of contaminants, whether the contaminants were degraded or not, and their corresponding footprints. For example, at Dover Air Force Base in Delaware, USA, the contamination of the aquifer with trichloroethylene (TCE) and trichloroethane (TCA) is of great concern. According to Table, 4 the contaminants are degraded, and the geochemical footprints at the site suggest that reductive dechlorination is most likely taking place. The presence or absence of these compounds alone is not proof of natural attenuation, but their presence and the changes in concentration over time can be indicative of intrinsic remediation.

### Attenuation of Petroleum Hydrocarbons

Intrinsic biodegradation is currently best documented and the processes are best understood for petroleum hydrocarbon sites. BTEX (benzene, toluene, ethylbenzene, and xylene) compounds biodegrade through microbial activity and ultimately produce non-toxic end products such as water and carbon dioxide. At sites where the source is controlled and where the microbial activity is fast relative to groundwater flow, the dissolved BTEX plume may stabilize. When the source is removed, the dissolved BTEX plume may shrink over time. Geochemical footprints that are indicative of natural attenuation of petroleum hydrocarbons taking place at a site (NRC, 2000) include:

- loss of electron acceptors (mainly O$_2$, NO$_3^-$, Fe(III), and SO$_4^{2-}$); in particular, one should look for O$_2$, NO$_3^-$, and SO$_4^{2-}$ levels below background in the core of the plume;
- generation of the products of acceptor reduction (such as Fe(II) and CH$_4$); again, Fe(II) and CH$_4$ levels should be highest in the core of the plume;
- the presence of organic acids that are known intermediate products of petroleum hydrocarbon degradation;
- an increased concentration of dissolved inorganic carbon (CO$_2$); and
- a characteristic increase in the alkalinity.

The above are general guidelines that apply to most petroleum spill sites. However, for large sources, a short-term criterion may not be justified. That is, in some cases, electron acceptors may be completely depleted when the hydrogeochemical conditions are such that the sustainability of electron acceptors is in question. If this is the case, the biodegradation rate may
### TABLE 4. Natural attenuation footprints from National Research Council case studies (NRC, 2000)

<table>
<thead>
<tr>
<th>Case study</th>
<th>Contaminant(s)</th>
<th>Contaminants controlled?</th>
<th>Footprints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Traverse City</td>
<td>BTEX</td>
<td>Yes</td>
<td>Depletion of O₂; formation of CH₄ and Fe²⁺</td>
</tr>
<tr>
<td>Vandenberg Air Force Base</td>
<td>MTBE</td>
<td>No</td>
<td>Insignificant decrease in O₂ and SO₄²⁻ concentration; extension of MTBE plume far beyond BTEX plume</td>
</tr>
<tr>
<td>Borden Air Force Base</td>
<td>Five chlorinated solvents</td>
<td>Partially</td>
<td>Detection of metabolites of solvent degradation</td>
</tr>
<tr>
<td>St. Joseph</td>
<td>TCE</td>
<td>Partially</td>
<td>Formation of CH₄; detection of degradation byproducts (vinyl chloride and ethene)</td>
</tr>
<tr>
<td>Edwards Air Force Base</td>
<td>TCE</td>
<td>No</td>
<td>Documentation of high NO₃⁻ and SO₄²⁻ concentration; demonstration that TCE moves with water</td>
</tr>
<tr>
<td>Dover Air Force Base</td>
<td>TCE, TCA</td>
<td>Yes</td>
<td>Formation of degradation byproducts (cis-1,2-DCE, 1,1-DCA, vinyl chloride, and ethene); CH₄ and H₂S formation; increase in Cl⁻ concentration</td>
</tr>
<tr>
<td>Hudson River</td>
<td>PCBs</td>
<td>Partially</td>
<td>Detection of breakdown products; detection of unique transient metabolites; observation of microbial metabolic adaptation</td>
</tr>
<tr>
<td>South Glens Falls</td>
<td>PAHs</td>
<td>Yes</td>
<td>Depletion of O₂; detection of unique metabolic byproducts; detection of genes for degrading PAHs in site microorganisms; rapid PAH degradation in soils taken from site</td>
</tr>
<tr>
<td>Pinal Creek Basin</td>
<td>Metals, acid</td>
<td>Yes, but may not be sustainable</td>
<td>Observation of carbonate dissolution, leading to pH increase coincident with metal precipitation; observation of Mn oxide precipitates in stream sediments</td>
</tr>
<tr>
<td>Hanford 216-B-5</td>
<td>Radionuclides</td>
<td>Yes</td>
<td>Sorbed radionuclides observed in site samples</td>
</tr>
<tr>
<td>Anonymous field site</td>
<td>BTEX</td>
<td>Yes</td>
<td>Loss of O₂, NO₃⁻, and SO₄²⁻; formation of Fe²⁺ and CH₄; increase in inorganic carbon concentration; increase in alkalinity</td>
</tr>
<tr>
<td>Bemidji</td>
<td>Petroleum hydrocarbons</td>
<td>Partially</td>
<td>Loss of O₂; formation of Fe²⁺, Mn²⁺, and CH₄; formation of intermediate metabolites; observation of selective degradation of petroleum hydrocarbons relative to more stable chemicals</td>
</tr>
</tbody>
</table>

Abbreviations: BTEX = benzene, toluene, ethylbenzene, and xylene; MTBE = methyl tert-butyl ether; TCE = trichloroethylene; TCA = trichloroethane; PCBs = polychlorinated biphenyls; PAHs = polycyclic aromatic hydrocarbons; DCE = dichloroethene; DCA = dichloroethane.
change over time, causing the natural attenuation of contaminants to fail. In such a case, the slowest sustainable degradation mechanism (which may be methanogenesis) and its corresponding degradation rate have to be compared to the minimum travel time to humans or sensitive ecosystems (NRC, 2000).

Another factor to consider is the kinetics of the reactions that are taking place. If microbial kinetics are limiting the biodegradation rate, anaerobic electron acceptors (e.g., nitrate and sulfate) would be constantly decreasing in concentration as one moved downgradient from the source zone, and anaerobic byproducts such as Fe(II) and methane would be increasing in concentration. In contrast, if microbial kinetics are considered to be relatively fast, electron acceptors through anaerobic degradation (nitrate and sulfate) would be mostly or totally consumed in the source zone, while the anaerobic by-products Fe(II) and methane would be found in the highest concentration in the source zone (Newell et al., 1996).

A good example that shows a number of geochemical footprints that indicates intrinsic bioremediation taking place is a spill site in New York. Figure 4 shows the distribution of BTEX (Figure 4a), electron acceptors (Figures 4b–4d), and geochemical footprints of electron acceptor utilization (Figures 4e and 4f) at a former fire training area at Plattsburgh Air Force Base, New York. Groundwater flow at the site is to the southeast, the dissolved BTEX plume extends about 2000 feet downgradient from the source zone, and the plume has a maximum width of approximately 500 feet. Within the source zone, a total BTEX concentration as high as 17,000 µg/L have been observed. It is evident from Figures 4a–4d that the BTEX plume and its electron acceptors are migrating in the direction of groundwater flow. We also see the strong correlation between elevated BTEX concentrations with depleted electron acceptors. The absence of electron acceptors suggests that intrinsic bioremediation is taking place through aerobic respiration as well as anaerobic processes of nitrate and sulfate reduction. Figures 4e and 4f reveal that Fe(II) and methane concentrations are highest in the core of the BTEX plume, suggesting that BTEX degradation near the source zone is coupled to Fe(III) reduction and methanogenesis.

An alternative yet complementary geochemical indicator for the natural attenuation of petroleum hydrocarbons is H2 concentrations. Lovley and Goodwin (1988) proposed the use of H2 concentration as a microbially based, non-equilibrium alternative to pe analysis for determining which oxidation-reduction reactions are taking place in anoxic sedimentary environments. Molecular hydrogen (H2) is produced through microorganisms metabolizing natural or anthropogenic organic matter under anaerobic conditions. The produced H2 is then utilized by microorganisms that most commonly utilize Fe(III), sulfate, or carbon dioxide as terminal electron acceptors (Chapelle et al., 1997). The technique then is based on the fact that each of these Terminal Electron Accepting Processes (TEAP) has a different affinity
for $\text{H}_2$ uptake, and organisms using thermodynamically weaker TEAP need higher $\text{H}_2$ concentrations to yield a given amount of energy. Table 5 summarizes the dominant redox processes associated with $\text{H}_2$ concentrations.

The technique is highly useful because in some cases, these redox processes are hard to determine from geochemical footprints alone. Large differences in geochemical conditions may obscure the TEAP operating.
TABLE 5. Dominant redox processes associated with H₂ concentrations (modified after Chapelle et al., 2001)

<table>
<thead>
<tr>
<th>Characteristic H₂ concentration (nM)</th>
<th>Dominant TEAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01–0.05</td>
<td>Denitrification</td>
</tr>
<tr>
<td>0.1–0.3</td>
<td>Mn(IV) reduction</td>
</tr>
<tr>
<td>0.2–0.8</td>
<td>Fe(III) reduction</td>
</tr>
<tr>
<td>1.0–4.0</td>
<td>Sulfate reduction</td>
</tr>
<tr>
<td>&gt;5.0–15.0</td>
<td>Methanogenesis</td>
</tr>
</tbody>
</table>

Attenuation of Chlorinated Solvents

Chlorinated solvents such as TCE, TCA, and tetrachloroethylene (PCE) represent another class of contaminants that are commonly found in groundwater. Research over the last decade has identified several mechanisms that can intrinsically biodegrade these compounds. Research also suggests that the intrinsic bioremediation of these compounds to the point that is compliant under regulatory standards only takes place under hydrogeologically and biogeochemically favorable conditions that are anticipated to occur at a limited number of sites. For these compounds, reductive dechlorination is thought to be the most widely applicable mechanism.

For reductive dechlorination, many indicators for attenuation are same as those for the attenuation of petroleum hydrocarbons. The presence of electron donors is the most important screening criterion used to determine the potential for reductive dechlorination of chlorinated solvents (NRC, 2000). That is, one should look for low redox potential (<−100 mV) above-background Cl⁻ and the presence of volatile fatty acids that tend to accumulate in anaerobic environments. For example, degradation of chlorinated solvents under anaerobic conditions involves total depletion of oxygen, nitrate, and sulfate in the source area and downgradient, as well as the appearance of ferrous iron, methane, acetate, and high levels of organic carbon. For the same chlorinated solvents, under aerobic conditions, the depletion of oxygen and nitrate and the appearance of methane, ferrous iron, and acetate in the source area, as well as the reappearance of oxygen and disappearance of methane downgradient of the plume, illustrate that natural attenuation is occurring. Another indication of natural attenuation can be illustrated by comparing dissolved oxygen (DO) concentrations with redox potentials. As DO decreases, redox potential also decreases. Low DO values typically have negative redox potentials, which are indicative of anaerobic conditions that can lead to reductive dechlorination. Other footprints that one should look for in the reductive dechlorination of chlorinated solvents include:

- an increase in degradation rates or daughter products appearance. For example, the reductive dechlorination of PCE will yield TCE, which in turn
degrades to \textit{cis}-DCE and finally VC in the core of the plume. Because of the non-sorptive, sequential nature of the reaction kinetics, the plume center for each degradation component will be further downgradient compared to the parent components.

- generation of products such as \textit{CO}_2, ethane, and chloride should be highest downgradient of the parent plume.

**Attenuation of Other Contaminants**

The footprints of contaminants other than petroleum hydrocarbons and chlorinated solvents such as inorganic contaminants are less well established. According to the EPA (1999), a number of mechanisms for natural attenuation of inorganic compounds have been postulated, but the scientific basis for the postulation is weak. This will likely change over time, but as of 1999, the EPA advocated the strategy for evaluating natural attenuation of inorganic contaminants by postulating several geochemical footprints. Natural attenuation processes besides intrinsic bioremediation, such as sorption, decay, and redox reactions, can effectively mitigate the concentrations of these contaminants or transform them into non-toxic forms.

It is of interest to note that many of the inorganic contaminants are persistent in the subsurface as they are not degraded readily by natural attenuation, perhaps with the exception of radionuclides, which undergo radioactive decay. Therefore, natural attenuation is applicable to inorganic compounds that are relatively immobile and undergo radioactive decay (EPA, 1999). The key issue is that these processes (particularly sorption) need to be irreversible, such that remobilization of these contaminants does not take place with changes in geochemical conditions at a later time.

**MICROCOSM STUDIES**

Microcosm studies can demonstrate the occurrence of a particular natural attenuation process at the site and its ability to degrade the contaminants of concern. Currently, microbiological investigations related to bioremediation are conducted through in situ microcosms (Gillham et al., 1990a, 1990b; Higgo et al., 1996; Nielsen et al., 1996) or by obtaining samples from the field, which are incubated, and rates of contaminant degradation or immobilization are quantified. Such studies provide estimates of metabolic activity of the microbial community but little insight into the actual mechanisms for bioremediation or how some amendments may or may not stimulate microbial activity (Lovley, 2003).

The habitat in which the microorganisms live is very intricate. It involves the continuous availability (e.g., cycling) of nutrients, water, and gases, as
well as chemical constituents. Within such a habitat, there are complex interactions among various species of microorganisms that are very difficult to decipher and quantify. We also know that the subsurface is highly heterogeneous at a multiplicity of scales (i.e., from micrometers to kilometers), and that the habitat of microorganisms depends on the pore structure. Very little is known about these microhabitats because the act of studying it involves destroying the habit. Unlike other organisms that are commensurate in scale with humans (e.g., plants in landscapes), detailed knowledge of where microorganisms live is very difficult to obtain because of scale- and sampling-related physical characteristics of microhabitats and microorganisms therein (Madsen, 1998).

Therefore, field sampling will undoubtedly disturb the habitat of microorganisms. It is also well known that the conditions in the field are very difficult to duplicate in the laboratory; thus, biodegradation rates determined in the laboratory may not be entirely representative of field conditions.

However, biodegradation can also be assessed using more innovative laboratory analyses on environmental samples. These techniques include documentation of enhanced numbers of protozoan that feed on higher concentrations of bacteria inside but not outside the contaminant plume; extracting total RNA to compute relative abundances of each phylogenetic group at different depths; extracting nucleic acids from sediment to identify unique transient intermediary metabolites, indicative of biodegradation; assessment of growth using flow cytometry; and cloning, sequencing, and analyzing 16S rRNA genes to identify novel organisms (Madsen, 1998). The quest for the improved understanding of biodegradation mechanisms continues. A comprehensive mechanistic understanding of such processes will have to involve the comprehension of processes that regulate the genes that encode the proteins that actually degrade the compounds.

STABLE ISOTOPE ANALYSIS

Stable isotope analyses of site data have been increasingly used to assess bioremediation. The approach is based on the premise that many biodegradation processes are accompanied by isotope fractionation. The weaker bond strength of light isotopes results in the preferential enrichment of the lighter isotopes in the product of a degradation reaction, while the heavy isotopes accumulate in the non-reacted material. Therefore, microbial or chemical degradation of contaminants increases the number of heavier isotopes as biodegradation proceeds; thus, the ratio between heavy and light isotope is expected to increase.

Isotopic ratios are reported as the difference between the investigated sample ratio and a standard ratio, divided by the standard ratio and expressed
as $\delta$ values in per mil ($\permil$) according to:

$$\delta = \left[ \frac{R_{\text{sample}} - R_{\text{reference}}}{R_{\text{reference}}} \right] \times 10^3 \permil \quad (2)$$

where $R_{\text{sample}}$ is the ratio of the heavy isotope to the light isotope for some compound (e.g., $^{13}\text{C}/^{12}\text{C}$), and $R_{\text{reference}}$ is the corresponding ratio for the international Vienna Pee Dee Belemnite (VPDB) standard. The reference $^{13}\text{C}/^{12}\text{C}$ ratio for VPDP is 0.011180 (Schmidt et al., 2004).

A significant carbon isotope fractionation accompanies in situ microbial degradation of a variety of common pollutants, including BTEX, chlorinated solvents, methyl tert-butyl ether (MTBE), and tert-butyl alcohol (TBA) (Beneteau et al., 1999; Holt et al., 1997; Jendrzejewski et al., 1997, 2001; Morasch et al., 2002; Poulson et al., 1997; Sherwood Lollar et al., 2001; van Warmerdam et al., 1995). In addition, isotopic signatures can be used to quantify the relative extent of biodegradation between different zones of the contaminant plume (Lollar et al., 2001). Figure 5 presents an example of isotope fractionation by sulfate-reducing bacteria degrading toluene. In this example, toluene concentrations decrease with time, while sulfide concentrations and $\delta^{13}\text{C}$ increase over time, indicating a kinetic isotope effect. Other examples relying on the stable isotope analysis of carbon, chlorine, and/or hydrogen to verify the biodegradation of various common priority pollutants include those by Migaud et al. (1996), Richnow et al. (1998), Hanson et al. (1999), Meckenstock et al. (1999), Pelz et al. (1999), Sherwood-Lollar et al. (1999), and Illman (2006).

**FIGURE 5.** Results from a growth experiment with the sulfate-reducing strain TRM1. Toluene and sulfide concentrations are measured over time together with the isotope value $\delta^{13}\text{C}$ in the residual toluene fraction. Data are from Meckenstock et al. (1999), and the figure is modified after Alvarez and Illman (2006).
et al. (1999), Ahad et al. (2000), Bloom et al. (2000), Wilks et al. (2000), Rodgers et al. (2000), Hunkeler et al. (1999, 2001, 2004), Pelz et al. (2001), Slater et al. (2001), Morasch et al. (2001), Barth et al. (2002), Gray et al. (2002), Kolhatkar et al. (2002), Meckenstock & Richnow (2002), Mancini et al. (2002), Meckenstock et al. (2002), Brungard et al. (2003), Griebler et al. (2003), Mancini et al. (2003), Richnow et al. (2003), Shouakar-Stash et al. (2003, 2006), Kuder et al. (2005), Morrill et al. (2005), Zwank et al. (2005), Chartrand et al. (2005a, 2005b), Elsner et al. (2007), Abe et al. (2009), and Yanik et al. (2003). Many of studies conducted at the batch-, column-, and field-scales reported a substantial isotope fractionation during microbial degradation of investigated compounds. This implies that the technique is promising; however, there are a few studies that revealed the lack of stable isotope fractionation despite biodegradation taking place (see Drenzek et al., 2001; Mazeas et al., 2002; Morasch et al., 2002), implying that further research is needed to understand such departures from the norm.

CHEMICAL FINGERPRINTING

Similar to human fingerprints, chemical fingerprints can provide unique signatures of different contaminants and demonstrate changes resulting from natural weathering processes, including biodegradation (Stout et al., 1998). The measurement of stable isotopic ratios discussed in the previous section is a form of chemical fingerprinting. However, the most common approach for chemical fingerprinting involves analysis of contaminant mixtures (usually petroleum hydrocarbons) by gas chromatography/mass spectrometry (GC/MS).

Numerous fingerprinting techniques have been used (and misused) in the environmental profession to assess the age and liability of petroleum product releases. Such techniques often involve chemical analysis of groundwater or free product sampled from the subsurface in conjunction with precise knowledge of historical usage and chemical compositions. For example, high resolution gas chromatography might be useful in liability assessment to distinguish among different types of fuel types that might be present in a single location. The presence of gasoline additives can be particularly revealing, yielding valuable insight for forensic age dating. For example, tetraethyl lead was added to gasoline prior to 1985 (at 400 to 800 mg/L) to suppress pre-ignition and enhance the octane rating (Watts, 1997). Therefore, the presence of organic lead in free product is indicative of a relatively old (pre-1985) release. Similarly, methyl-tert-butyl ether (MTBE) has been used as a gasoline oxygenate since 1979 (Steffan et al., 1997), and its national use increased rapidly in the early 1980s at 40% per year (Suflita & Mormile, 1993). MTBE is not used in all current gasoline formulations, but this additive has been used in amounts up to 15% by volume by many marketers. Thus,
high MTBE concentrations in contaminated groundwater suggest a relatively recent (post-1980) gasoline release to the subsurface. It should be kept in mind, however, that atmospheric deposition of MTBE that had been previously volatilized can be a non-point source in urban areas (Pankow et al., 1997). Thus, traces of MTBE in groundwater (<10 ppb) do not necessarily indicate recent gasoline contamination. Forensic analysis based on trace constituents is further confounded by the existence of additional unknown sources of contaminant release, which is common in urban areas.

Fingerprinting analysis and examination of spatial and temporal concentration trends of groundwater contaminants are bona fide components of liability assessment, but caution should be exercised to avoid stretching forensic inferences beyond scientific constraints. For example, there is a strain of folklore asserting that the age of a petroleum product release can be established in any situation by comparing aqueous concentration ratios such as benzene to xylenes (B/X) or benzene-plus-toluene to ethylbenzene-plus-xylenes (B+T)/(E+X). This provocative idea is based on the fact that benzene and toluene are more soluble than ethylbenzene and xylenes and thus are preferentially solubilized from the gasoline. Thus, when solubilization is the sole fate and transport mechanism considered, B/X and (B+T)/(E+X) ratios tend to decrease near the source as the time since the spill occurred increases. Such changes in BTEX ratios can be described empirically with an exponential decay function that permits solving for the time required to achieve a given ratio and estimate the age of a release. However, inferring the age of a release based on BTEX ratios alone is a valid approach only in rare, well-constrained situations (Alvarez et al., 1998). Different attenuation mechanisms can affect the relative proportion of BTEX compounds at different rates, and sometimes in opposing directions. For example, biodegradation could increase B/X and (B+T)/(E+X) ratios in groundwater near the source, especially when anaerobic conditions prevail. This is because benzene degrades very slowly if at all under anaerobic conditions. Downgradient from the source, BTEX ratios are predominantly influenced by adsorption-induced “chromatographic” separation (i.e., retardation) as BTEX compounds migrate at different velocities through the aquifer, as well as by differential biodegradation. Retardation tends to increase downgradient B/X and (B+T)/(B+X) ratios due to the higher solubility of benzene, whereas biodegradation can either increase or decrease such ratios, depending on the substrate preference of the prevailing phenotypes and the available electron acceptor pools. The aquifer properties that control the rate and extent of different attenuation mechanisms are site-specific. Therefore, one should not use the relative concentrations of individual BTEX compounds in groundwater to determine the age of a petroleum product release reliably. Such attempts bear a burden of proof that is often beyond the limits of scientific constraints and require far more data than can be obtained given common technical and economic constraints.
The above discussion illustrates that liability assessment via fingerprinting techniques requires consideration of how the composition may have weathered since its release because of physical, chemical, and biological processes. Changes in fingerprints are inevitable but occur to different degrees and at different rates in nearly every situation (Stout et al., 1998).

Another caveat for liability allocation is the presumption that the presence of trace levels of polynuclear aromatic hydrocarbons (PAH) is due to contamination from anthropogenic sources. PAHs are also byproducts of incomplete combustion of organic matter. Thus, natural sources such as forest fires and volcanic activity can contribute to background PAH contamination. However, such (more recent) naturally occurring PAHs are typically alkylated (Mantseva et al., 2002). The anthropogenic contribution of these contaminants (e.g., from coal gasification and petroleum refining operations), which far exceeds natural sources (Blumer, 1976), can thus be discerned by the lack of alkyl substituents.

Forensic analysis is further confounded by the existence of additional unknown sources of contaminant release. This is common in urban areas.

**MICROBIAL ANALYSIS AND MOLECULAR TECHNIQUES**

It has become broadly accepted that communities of microorganisms rather than single strains are most important in bioremediation. Microbial communities usually contain significant metabolic diversity as well as some metabolic redundancy that contributes to process robustness, and their characterization can yield valuable information to assess the efficacy of bioremediation. In particular, establishing that specific microorganisms that can degrade the target pollutants are present, and that their concentrations are higher in the treatment zone compared to background samples, is an important line of evidence to demonstrate that bioremediation is working. Microbial analysis is also important in identifying the main organisms responsible for the reactions of interest and to evaluate the efficacy of biostimulation or bioaugmentation approaches.

Historically, microbial analyses involved isolation and culture of specific microorganisms by virtue of their ability to grow on the pollutant of interest, followed by their identification based on morphological, physiological, and metabolic traits (e.g., Gram stain, enzyme activity, substrate utilization patterns). However, such traditional techniques are inadequate for microbial characterization of contaminated sites because they are selective and not quantitative (Vestal & White, 1989; White et al., 1997), provide little insight into microbial consortium interactions (White et al., 1997), and may introduce disturbance artifacts because these techniques involve subsampling and separation of microorganisms from the environmental matrix (Findlay et al., 1990). Furthermore, most microorganisms in the environment
are viable but not cultivable (McCarthy & Murray, 1996; Xu et al., 1982). Viable counts of bacteria in environmental samples determined with classical methods represent only a small fraction (0.1–10%) of the active microbial community (White et al., 1997). Furthermore, the most important “players” in the microbial community might not have been yet isolated and characterized. Such limitations have motivated the development of chemical and molecular characterization techniques to determine microbial biomass and community structure without prior isolation and cultivation of microorganisms.

Chemical Analyses

Current approaches used for chemical characterization of microbial populations in natural environments include two techniques that analyze the cell membrane phospholipids:

1. phospholipid ester-linked fatty acid (PLFA) analysis by gas chromatography/mass spectrometry (White et al., 1979); and
2. intact phospholipid profiling (IPP) using liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) analysis of bacterial membrane phospholipids (Fang et al., 2000a, 2000b).

Both techniques rely on the fact that phospholipids are found in the membranes of all living cells, but not in storage lipids, and are rapidly turned over in dead cells. Thus, their quantification provides an estimation of viable biomass (Balkwill et al., 1988).

The identification of microorganisms by either PLFA or IPP is possible, but this approach is subject to potential confounding effects of overlapping phospholipid profiles and potential changes in phospholipid composition due to differences in growth conditions (Haack et al., 1993; White et al., 1997). Nevertheless, both techniques can give valuable insight into microbial community structure, based on the premise that there are a great number of dissimilar fatty acids in bacterial phospholipids. Some bacteria also contain unique fatty acids that can be used as biomarkers for their identification.

Molecular Analyses

DNA is present in every independently living cell in order to translate the genetic information into working enzymes and other proteins. The advent of high-throughput methods of nucleic acid synthesis and the analysis of gene expression and function revolutionized the field of environmental microbiology and provided a boom to bioremediation (Lovley, 2003). These new molecular-based tools allow us to interrogate the genetic information of community members to learn who is there, what they are doing (including their metabolic state), and how they interact, as well as how the organisms
TABLE 6. Targets for microbial interrogation (after Alvarez and Illman, 2006)

<table>
<thead>
<tr>
<th>Target</th>
<th>Information gained</th>
<th>Question answered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal (r)RNA</td>
<td>Phylogenetic identity</td>
<td>Who is there?</td>
</tr>
<tr>
<td>Genes for rRNA on DNA</td>
<td>Phylogenetic identity</td>
<td>Who is there?</td>
</tr>
<tr>
<td>Catabolic genes on DNA</td>
<td>Phenotypic potential</td>
<td>What they can degrade?</td>
</tr>
<tr>
<td>Messenger (m)RNA</td>
<td>Phenotypic activity</td>
<td>What genes are being expressed?</td>
</tr>
<tr>
<td>Protein or other products</td>
<td>Phenotypic activity or</td>
<td>Who is active and what they</td>
</tr>
<tr>
<td></td>
<td>phylogenetic identity</td>
<td>are doing?</td>
</tr>
</tbody>
</table>

responsible for the reactions of interest respond to manipulations of environmental conditions. Common targets for microbial molecular analysis and the type of information gained are summarized in Table 6, while Table 7 summarizes tools for microbial interrogation by molecular methods.

The most basic molecular target for microbial identification is the ribosomal RNA (rRNA) (Lane et al., 1985). All living organisms contain ribosomal RNA, and the small sub-unit of prokaryotic rRNA (known as 16S rRNA) has approximately 1500 nucleotide bases that can be easily sequenced. Thus, sequences are already known for thousands of bacteria (Cole et al., 2003). The finding that 16S rRNA sequences are highly conserved allows the use 16S rRNA as an evolutionary clock that reveals phylogenetic relationships among members of complex microbial communities, as well as a measure of phylogenetic identity (Pace et al., 1986). Analysis of 16S rRNA is important to identify and quantify the presence of specific strains without having to rely on (biased) isolation and culturing techniques. Thus, depending on the primers used to amplify the 16S rRNA sequence (see PCR procedures, below), researchers can now track and quantify specific strains or groups of phylogenetically related microorganisms.

Microorganisms with a known 16S rRNA sequence can also be identified, quantified, and visualized without culturing by using fluorescent in situ hybridization analysis (FISH) (Thurnheer et al., 2004; Yang & Zeyer, 2003). Cells are hybridized with a molecular probe that is tagged with a fluorescent molecule, enabling microscopic visualization. Multiple different microorganisms can be visualized and counted by using probes tagged with different fluorescent molecules that vary in color.

Note that the same phylogenetic information inferred by 16S rRNA sequences can be obtained by analyzing the genes that code for 16S rRNA on the bacterial chromosome (i.e., 16S rDNA) (see Table 6). Phylogenetic probes commonly used to detect and quantify the presence of specific degraders are listed in Table 8. Interestingly, 16S rRNA analyses have revealed that the microorganisms that are responsible for bioremediation are often phylogenetically related to those that can be cultured (Watanabe & Baker, 2000). This has in many cases validated the relevance of studying isolates to learn about
### TABLE 7. Tools for microbial interrogation by molecular methods (after SERDP, 2005)

<table>
<thead>
<tr>
<th>Tools</th>
<th>Current relative to frequency of use</th>
<th>Perceived advantages</th>
<th>Perceived disadvantages</th>
<th>Current applications</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct PCR</td>
<td>Moderate</td>
<td>Easy to perform</td>
<td>False negatives</td>
<td>Screening tool for presence or absence of organisms</td>
<td>Replaced by qPCR</td>
</tr>
<tr>
<td>Nested PCR</td>
<td>Moderate</td>
<td>Unsurpassed sensitivity</td>
<td>Requires two PCR steps</td>
<td>Screening tool for presence or absence of organisms</td>
<td>Replaced by qPCR</td>
</tr>
<tr>
<td>qPCR (16S rRNA gene)</td>
<td>High</td>
<td>Provides information on the presence, absence, and/or abundance of organisms of interest; nearly reaches the sensitivity of nested PCR; commercially available for a few key organisms (e.g., <em>Dehalococciodes</em> sp.); estimates of total bacterial numbers possible</td>
<td>Does not provide confirmation of activity; sampling, handling, and analysis not standardized</td>
<td>Screening tool for presence or absence of desired or indicator organisms; monitoring of growth and distribution of individual organisms</td>
<td>Expansion to wider range of genes of interest; standardization of approach; clarification how mRNA abundance relates to activity</td>
</tr>
<tr>
<td>qPCR mRNA</td>
<td>Low</td>
<td>Provides information on gene expression (i.e., activity); quantitative approaches under development</td>
<td>Relative instability of RNA presents sampling and preservation challenges; not commercially available to a significant extent; sampling, handling, and analysis not standardized</td>
<td>A few experimental applications for confirming expression of functional genes</td>
<td>Needs wider range of genes of interest; standardization of approach; clarification how mRNA abundance relates to activity</td>
</tr>
</tbody>
</table>

(Continued on next page)
<table>
<thead>
<tr>
<th>Tools</th>
<th>Current relative to frequency of use</th>
<th>Perceived advantages</th>
<th>Perceived disadvantages</th>
<th>Current applications</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR (functional gene)</td>
<td>Low</td>
<td>Provides information on the presence, absence, and/or abundance of functional genes of interest; commercially available for a few key genes (e.g., reductase dehalogenase genes)</td>
<td>For DNA, does not provide confirmation of activity; sampling, handling, and analysis not standardized</td>
<td>Screening tool for the presence or absence of indicator genes; sequencing of amplicons for positive identification</td>
<td>Needs wider range of functional genes; extension to mRNA; standardized procedures; availability of standards</td>
</tr>
<tr>
<td>DGGE</td>
<td>Low</td>
<td>Provides information on the presence or absence of 16S rRNA and/or functional genes of interest; can provide an indication of target gene diversity; increased resolution with specific primers</td>
<td>Inconclusive results with unspecific primers; short amplicon length with insufficient information; not quantitative; no standardized procedures; cumbersome</td>
<td>Screening tool for the presence or absence of indicator genes; sequencing of amplicons for positive identification</td>
<td>Use is quite specialized; will likely be replaced by qPCR methods; standardized procedures lacking</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Low</td>
<td>Provides relatively inexpensive basic information on community diversity and changes in community structure over time; can provide means to track individual organisms over time or space within a community when combined with other methods</td>
<td>Limited resolution; does not provide sequence information; not quantitative; biased toward dominant community members</td>
<td>Screening tool for community diversity; analysis of community structure; tracking of microbial groups within a community over time during and after active remediation</td>
<td>Standardized sample preparation procedures; guidance document for data interpretation</td>
</tr>
<tr>
<td>Method</td>
<td>Complexity</td>
<td>Indication of gene diversity; individual clones can be sequenced</td>
<td>Labor-intensive and expensive; not widely available commercially</td>
<td>Community structure analysis; identification of new genes</td>
<td>Will remain a research tool; limited applications for bioremediation monitoring</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------</td>
<td>------------------------------------------------------------------</td>
<td>------------------------------------------------------------------</td>
<td>------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Clone Libraries (16S rRNA genes and functional genes)</td>
<td>Low</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLFA</td>
<td>High</td>
<td>Community screening tool; monitoring individual groups of organisms; total biomass determination, etc.; commercially available, can be quantitative</td>
<td>Other methods provide more specific information for similar cost and effort</td>
<td>Biomass measurements; specialized application for screening of exposure to vegetable oil; screening of high-level community structure and microbial ecosystem health</td>
<td>Might be useful for identification of specific organisms; might have potential for measuring respiratory activity</td>
</tr>
<tr>
<td>Enzyme Probes</td>
<td>Low</td>
<td>Provides most direct measurement of the activity of interest (i.e., measures presence/absence of the actual enzyme)</td>
<td>Very few enzyme probes have been developed; not widely available</td>
<td>Direct measurement of soluble methane monooxygenase</td>
<td>Needs wider range of enzymes; experimental and practical validation</td>
</tr>
</tbody>
</table>

*Continued on next page*
<table>
<thead>
<tr>
<th>Tools</th>
<th>Current relative to frequency of use</th>
<th>Perceived advantages</th>
<th>Perceived disadvantages</th>
<th>Current applications</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH</td>
<td>Low</td>
<td>Provides measurement of activity of organisms of interest; can be quantitative; visual information on spatial distribution</td>
<td>Not widely available; probes not available for a wide range of organisms; method development for each target organism required</td>
<td>A few experimental applications</td>
<td>Needs wider range of target organisms; more commercial availability; standardized protocols</td>
</tr>
<tr>
<td>CSIA</td>
<td>Moderate</td>
<td>This method distinguishes transformation from dispersion, dilution, or volatilization; estimates of in situ activity are theoretically possible</td>
<td>Fractionation factors not always characterized; need more labs with capability to analyze samples; cost perceived as high</td>
<td>Used to delineate or confirm presence of multiple contaminant sources, to confirm transformation or biodegradation and estimate degradation rates</td>
<td>Fractionation factors for key contaminants and relevant degrading organisms needed, with variability of those factors; need field demonstrations; need commercial availability; need to integrate data with other MBTs, such as qPCR</td>
</tr>
</tbody>
</table>
TABLE 8. Common phylogenetic probes used to detect and quantify specific degraders (after Alvarez and Illman, 2006)

<table>
<thead>
<tr>
<th>Target group</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria. Universal primers to determine total Bacteria concentration.</strong></td>
<td>Unibac 8F</td>
<td>5′-AGAGTTTGATCCCTGGCTCAG-3′</td>
<td>Löffler et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>Unibac 1541R</td>
<td>5′-AAGGAGGTAGTCAGCCGCA-3′</td>
<td></td>
</tr>
<tr>
<td><strong>Archaea. Universal primers to determine total Archaea concentration (including methanogens).</strong></td>
<td>ARCH1-1369F</td>
<td>5′-CGGTGAATACGTCCCTGC-3′</td>
<td>Suzuki et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>ARCH2-1369F</td>
<td>5′-CGGTGAATATGCCCCCTGC-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PROK1541R</td>
<td>5′-GGAGGAGGTAGTCCTGCCGCA-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM-5′-CITTGACACCCGCGGTGC-3′-BHQ-1</td>
<td></td>
</tr>
<tr>
<td><strong>Pseudomonas (16S rDNA), which is a catabolically versatile genus</strong></td>
<td>Forward</td>
<td>5′-ACTGCATCCAAAACCTGGCAA-3′</td>
<td>Duteau et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TCTCTGATGTCAGGGCCT-3′</td>
<td></td>
</tr>
<tr>
<td><strong>Dehalococcoides (16S rDNA), which dechlorinate PCE or TCE to ethane</strong></td>
<td>FpDHC1</td>
<td>5′-GATGACGTAGCGCGG-3′</td>
<td>Hendrickson et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>RpDHC1212</td>
<td>5′-GGATTAGCTGTTCACACT-3′</td>
<td></td>
</tr>
<tr>
<td><strong>Desulfitobacterium debalogenes (16S rDNA), which exhibits dechlorinating activity</strong></td>
<td>Dd1</td>
<td>5′-AATACCNGATAAGCTTACTCC-3′</td>
<td>El Fantroussi et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>Dd2</td>
<td>5′-CAAGTCTAGAGACATATC-3′</td>
<td>El Fantroussi et al. (1997)</td>
</tr>
<tr>
<td><strong>Desulfomonile (16S rDNA), which exhibits dechlorinating activity</strong></td>
<td>Dg-F</td>
<td>5′-GAAGAGATTGCTTTCCAGCA-3′</td>
<td>Sung (2004)</td>
</tr>
<tr>
<td></td>
<td>Dg-R</td>
<td>5′-ACCCCTACCTTTTATAG-3′</td>
<td></td>
</tr>
<tr>
<td><strong>Geobacter sp. SZ (16S rDNA), an iron-reducing bacterium with dechlorinating activity.</strong></td>
<td>Geo-F</td>
<td>5′-ACGCGCACGCGGATG-3′</td>
<td>Beller et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>Geo-R</td>
<td>5′-AGAGACAGAACCCGCTTC-3′</td>
<td></td>
</tr>
<tr>
<td><strong>Bacteriophage (λ) primers are used as internal standard to calculate DNA recovery (%) from environmental samples</strong></td>
<td>Forward</td>
<td>FAM-5′-CITTGAGGTCTCTGCAGCCTTCGACCC-3′-TMRA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td>5′-AGAAGGAGGTAGTCCTGCCGCA-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>probe</td>
<td>FAM-5′-CITTGACACCCGCGGTGC-3′-BHQ-1</td>
<td></td>
</tr>
</tbody>
</table>
their biodegradation reactions and aspects of their physiology that are likely to control their growth and performance in contaminated environments.

Another common molecular target is a catabolic or another functional gene on chromosomal or plasmid DNA, which code for enzymes in aerobic biodegradation pathways. Recently, several gene sequences associated with anaerobic biodegradation have been identified and targeted (see Table 9). These include bssA, which codes for benzyl succinate synthase (Beller et al., 2002), an enzyme that initiates anaerobic degradation of toluene and xylenes; and vcr, which codes for vinyl chloride reductase (Krajmalnik-Brown et al., 2004), an enzyme associated with dehalorespiration and conversion of vinyl chloride to ethene. In general, there is a positive correlation between the abundance of catabolic genes of interest and the potential for contaminant degradation (Lovley, 2003). Thus, determining which genes are present is important to establish what functions can be performed by the microbial community (i.e., phenotypic characteristics). However, because the presence of a gene does not guarantee its expression, this approach can only address the phenotypic potential, or “what the microorganisms could do.” As mentioned above, the gene that codes for the 16S rRNA (i.e., 16S rDNA) can also be targeted instead of the rRNA itself to address phylogenetic identity.

The messenger RNA (mRNA) can also be targeted by molecular probes to assess gene expression (see Table 6). Recall that mRNA is produced by transcription of the genes to form an RNA product that faithfully carries the genetic code from the DNA to the ribosome, where it is translated into an enzyme or another protein. Thus, detection of specific mRNA that is complementary to a gene of interest means that the microorganisms are expressing, or “turning on,” that specific gene. Thus, mRNA analysis provides genetic information on what the cells are doing. Higher biodegradation rates are often associated with higher concentrations of mRNA (Fleming et al., 1993). However, extracting and purifying intact mRNA from environmental samples can be a significant challenge due to the labile nature of mRNA (Sessitsch et al., 2002).

Because transcription of a gene does not guarantee translation, researchers can also target the final product of gene expression, often a catabolic enzyme. A common approach to assay enzyme activities is to measure the transformation rate of a specific substrate by whole cells or cell-free extracts and to normalize the rate to the protein concentration (see Table 10). The presence of some enzymes can also be detected using enzyme-linked immunoassays (ELISA) techniques with specific antibodies (Lynch et al., 1996), although this approach is subject to considerable interference by soil matrix constituents such as humic acids.

Chemical analyses of the outcome of the activity of proteins, such as fatty acid methyl esters (FAME), can also be conducted not only as an alternative to identify microorganisms, as described above, but also to evaluate microbial stress and nutritional status. For example, the ratio of trans/cis
### TABLE 9. Catabolic gene probes used to detect and quantify biodegradation potential (after Alvarez and Illman, 2006)

<table>
<thead>
<tr>
<th>Target group</th>
<th>Primer name 1</th>
<th>Primer name 2</th>
<th>Sequence 1</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aerobic processes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catechol 2,3-dioxygenase, catalyzes aerobic degradation of BTEX, phenol, naphthalene, and biphenyl.</td>
<td>23CAT-F 23CAT-R</td>
<td>5′-CGACCTGATCTCCATGACCGA-3′</td>
<td>Mesarch et al. (2000)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEG-F</td>
<td>5′-TCAGGTCAGACCGTCA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEG-R QUANT-F</td>
<td>5′-CGACCTGATC(AG)CATGACCGA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-TGACTCCATGACCGA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphtalene dioxygenase, which initiates the oxidation of naphthalene and co-oxidizes other PAHs.</td>
<td>NAH-F</td>
<td>5′-CAAAA(A/G)CACCTGATT(C/T)ATGG-3′</td>
<td>Baldwin et al. (2003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NAH-R</td>
<td>5′-A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTTCAA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene dioxygenase, which initiates aerobic degradation of BTEX, and chlorobenzene.</td>
<td>TOD-F</td>
<td>5′-ACCGATGA(A/G)GA(C/T)CTGTACC-3′</td>
<td>Baldwin et al. (2003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TOD-R</td>
<td>5′-CTTCGGTC(A/C)AGTAGCTTGAC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylene dioxygenase, which initiates aerobic xylene degradation.</td>
<td>TOL-F</td>
<td>5′-TGAGGCTGAAAATCTTACGTA-3′</td>
<td>Baldwin et al. (2003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TOL-R</td>
<td>5′-CTCACCTGAGTTGCTAC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl dioxygenase, which initiates the aerobic degradation of lightly chlorinated PCBs.</td>
<td>BPH1-F</td>
<td>5′-GGACGTGATGCTGAC(C/T)CGC-3′</td>
<td>Baldwin et al. (2003)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPH1-R</td>
<td>5′-TGTT(C/G)GG(C/T)ACGGCCAT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPH2-F</td>
<td>5′-GACGGCGCCCTATATGGA-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPH2-RBPH3-F</td>
<td>5′-AGCCGAGTTGCCAGGAAAAT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPH4-F</td>
<td>5′-CCGGAGAAGGACGGGATC-3′</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>BPH3-R</td>
<td>5′-AAGGCGCGCACTTCACTGAC-3′</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>5′-TGCTCCGCTGCGAATTCC-3′</td>
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</tr>
</tbody>
</table>

(Continued on next page)
TABLE 9. Catabolic gene probes used to detect and quantify biodegradation potential (after Alvarez and Illman, 2006) (Continued)

<table>
<thead>
<tr>
<th>Target group</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl monooxygenases in the large subunits of the toluene monooxygenase gene.</td>
<td>RMO-F</td>
<td>5′-TCTG(A/C/G)AGCAT(C/T)CAGAC(A/C/G)GACG-3′</td>
<td>Baldwin et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>RMO-R</td>
<td>5′-TT(G/T)TCGATGAT(C/G/T)AC(A/G)TCCCA-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RDEG-F</td>
<td>5′-TT(G/T)TCGATGAT(C/G/T)AC(A/G)AC(A/C/G)GA(C/T)GA-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RDEG-R</td>
<td>5′-TT(A/G/T)TCG(A/G)TG(TA/G)AT(C/G/T)AC(A/G)TCCCA-3′</td>
<td></td>
</tr>
<tr>
<td>Phenol hydroxylases, hydroxylate (halo)phenols, and other phenolics</td>
<td>PHE-F</td>
<td>5′-GTGCTGAC(C/G)AA(C/T)CTG(C/T)TGTTC-3′</td>
<td>Baldwin et al. (2003)</td>
</tr>
<tr>
<td>Anaerobic processes</td>
<td>PHE-R</td>
<td>5′-CGCCAGAACCA(C/T)TT(A/G)TC-3′</td>
<td></td>
</tr>
<tr>
<td>Benzylsuccinate synthase, which initiates anaerobic degradation of toluene and xylenes.</td>
<td>bssA-F</td>
<td>5′-ACGACGGYGGCATTTTCTC-3′</td>
<td>Beller et al. (2002)</td>
</tr>
<tr>
<td></td>
<td>bssA-R</td>
<td>5′-GCATGATSGGYACCGACA-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM-5′-CTTCTGGTTCTTGACACCTTGGACACC-3′-TAMRA</td>
<td></td>
</tr>
<tr>
<td>TCE reductive dehalogenase system, which mediates the dechlorination of TCE to ethane.</td>
<td>tceA-797F</td>
<td>5′-ACGCCAAAAGTGCGAAAAGC-3′</td>
<td>Magnuson et al. (2000)</td>
</tr>
<tr>
<td></td>
<td>tceA-2490R</td>
<td>5′-GAGAAAGGATGGAATAGATTA-3′</td>
<td></td>
</tr>
</tbody>
</table>
Vinyl chloride reductive dehalogenase, dechlorinates cis-DCE, and VC to ethane.

Oxygen insensitive, nitro- and chromate-reductase subfamily NsfA, which reduces oxidized nitrogen and metal groups.

NiFe hydrogenase gene, present in dissimilatory metal reducing bacteria.

**vcrA-F**

5’ – CTATGAAAGCCTCCAGATGC – 3’

Spormann et al. (2004)

**vcrA-R**

5’ – GTAAACGCCCCATATGCAAGTA – 3’

Kwak et al. (2003)

**NfsA-F**

5’-GTAGGATCCACGCCAACCATTGAAC-3’

Kwak et al. (2003)

**NfsA-R**

5’-ACTGAAATTCTAGCAGCGTCGCCAACC-3’

Kwak et al. (2003)

**NfsB-F**

5’-GTAGGATCCGATATCTATGTTCGTGCC-3’

Kwak et al. (2003)

**NfsB-R**

5’-ACTGAAATTCTATACCTGTTAGTGGTG-3’

Kwak et al. (2003)

**Hyd1F**

5’-CGCCCCGCCCGCCCGCCCGCCGT

C CCCGCC

Wawer et al. (1995)

**Hyd1R**

5’-GCAGGGGCTTCCAGGTAGTGGGCG

GTGCGATGAGGT-3’

Wawer et al. (1995)

Dissimilatory sulfite reductase (DSR) gene, present in all sulfate-reducing bacteria.

Nitrite reductases, present in denitrifying bacteria.

**dsr1F**

5’-ACSCACTGGAAGCACG-3’

Wagner et al. (1998)

**dsr4R**

5’-GTGTAATCGTACCGCA-3’

Braker et al. (1998)

**nirK1F**

GG(A/C)ATGGT(G/T)CC(C/G)TGGCA

Wagner et al. (1998)

**nirK2F**

GC(C/G)(C/A)T(C/G)ATGGT(C/G)CTGCC

Braker et al. (1998)

**nirK3R**

GAACCTGCGGT(A/C/G)(G/T)CCAGAC

Braker et al. (1998)

**nirK4R**

GG(A/G)AT(A/G)MA(A/G)CCAGGTTCC

Braker et al. (1998)

**nirK5R**

GCCTGATCAG(A/G)TAT(A/G)TGG

Braker et al. (1998)

**nirS1F**

CCT(A/T)GGGCGCC(A/G)CA(A/G)T

Braker et al. (1998)

**nirS2F**

TACCAACC(C/G)GA(A/G)CCGCGCGT

Braker et al. (1998)

**nirS3F**

TTCTC(C/G)TCA(C/T)GACGGCGGC

Braker et al. (1998)

**nirS4F**

TTCA(A/G)TCAAGAC(C/G)CA(C/T)CCGAA

Braker et al. (1998)

**nirS3R**

GCCGCCG(T/A/G)TG(A/C/G)AGAA

Braker et al. (1998)

**nirS5R**

CTTTGTG(A/T)ACTCG(C/G)CA(CTG(C/G)CTGCAC

Braker et al. (1998)

**nirS6R**

CGTGGAACTTR(A/G)CGG

Braker et al. (1998)
TABLE 10. Selected microbial enzyme assays (after Alvarez and Illman, 2006)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Function</th>
<th>Method reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arylphosphatase</td>
<td>Hydrolyzes pesticides with aryl phosphate esters at the O-P bond</td>
<td>Tabatabai and Bremmer (1970)</td>
</tr>
<tr>
<td>Catalase</td>
<td>Conversion of hydrogen peroxide, a powerful and potentially harmful oxidizing agent, to water and molecular oxygen.</td>
<td>Day et al. (2000)</td>
</tr>
<tr>
<td>Dehalogenase</td>
<td>Degradation of chlorinated compounds by hydrolytic cleavage of carbon-halogen bond, replacing halogen with hydroxyl group.</td>
<td>Holloway et al. (1998)</td>
</tr>
<tr>
<td>Fluorescein diacetate hydrolase</td>
<td>Simple, sensitive, and rapid method for determining microbial activity in soil</td>
<td>Adam and Duncan (2001)</td>
</tr>
<tr>
<td>Hydrogenase</td>
<td>Catalyzes the reversible reductive formation of hydrogen from protons and electrons. Used by hydrogen-oxidizing bacteria.</td>
<td>Watrous et al. (2003)</td>
</tr>
<tr>
<td>Nitrogenase</td>
<td>Nitrogen fixation (i.e., reduction of N₂ to NH₃, which is critical for the N cycle).</td>
<td>Staal et al. (2001)</td>
</tr>
<tr>
<td>Toluene dioxygenase</td>
<td>Aerobic BTEX degradation and cometabolism of chlorinated aliphatic compounds such as TCE.</td>
<td>Ensley and Gibson (1983)</td>
</tr>
<tr>
<td>Urease</td>
<td>Hydrolyzes urea to ammonia and CO₂</td>
<td>Kandeler and Gerber (1988)</td>
</tr>
<tr>
<td>Dechlorination activity (non-specific)</td>
<td>Colorimetric assay that measures dechlorination activity based on the release of chlorine ions.</td>
<td>Holloway et al. (2001)</td>
</tr>
</tbody>
</table>

C16:1Δ9 fatty acids in cell membrane phospholipids has been used as an indicator of stress due to toxicity, contamination, or oxygen tension on microbial communities (Guckert et al., 1985). Apparently, bacteria synthesize more trans-monounsaturated acids under stress conditions as a mechanism to maintain their membrane fluidity.

Because the DNA or RNA of interest is often present at very low, undetectable concentrations, assaying for it generally requires that the DNA be amplified first. This is often accomplished by the polymerase chain reaction (PCR). Nevertheless, only a small fraction of microorganisms in the environment have been isolated and studied. Thus, it is often necessary to track and identify strains for which we have no molecular information. In such cases, molecular fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) are important analytical tools that researchers often use to complement the study of microbial communities (see Table 7).
In addition to the detection of the target gene sequences, quantification is desirable in many cases to determine, for example, changes in the concentration of specific genotypes resulting from specific engineered manipulations (e.g., biostimulation). Real-time quantitative polymerase chain reaction (RTQ-PCR) was developed for this purpose. This recently developed technology offers several advantages over conventional methods to detect specific genes in environmental samples, including higher sensitivity, accuracy, and the ability to collect quantitative data rapidly in real-time during the amplification process (Bustin, 2000; Livak et al., 1995). RTQ-PCR is based on the real-time measurement of the PCR product throughout the amplification reaction using fluorescence labeling (i.e., fluorogenic probe or double-stranded DNA binding dye, such as SYBRgreen or TaqMan) (Heid et al., 1996).

**FINGERPRINTING TECHNIQUES**

Molecular fingerprinting is a useful approach to characterize microbial communities and track the emergence (or disappearance) of important strains or genes, even when the identity of the microorganisms of interest is unknown. Fingerprinting relies on the principle that similar base sequences exist for genes that code for similar products in different microorganisms. Thus, a generic primer can be designed for a given class of genes. When such a primer is used in PCR, the amplified (DNA or 16S rRNA) segments are from all members of the microbial community possessing similar genes. Different amplified products can be subsequently separated by electrophoresis to create a spectrum of bands known as the molecular fingerprint. Changes in the molecular fingerprint can be analyzed to track the evolution of the microbial community structure in space and in time.

Denaturing gradient gel electrophoresis (DGGE) is perhaps the most frequently used fingerprinting technique. DGGE involves separating the (selectively amplified) DNA sequences as they migrate under the influence of electrophoresis through a gel. The name of this technique is due to the fact that the gel contains a gradient of a denaturing chemical (usually formamide and urea) that denatures (or separates) the two DNA strands. When separation occurs, the mobility of DNA is retarded due to the branched structure of the single stranded molecule, and subsequent entangling of the DNA segment in the gel matrix prevents further movement. Different DNA segments are separated and trapped at different positions on the gel. This position depends on the size of the DNA segment and the content of guanine-cytosine (GC) nucleotide base pairs. Smaller DNA segments or those with higher GC content migrate farther through the gel. A high GC content is conducive to resistance to melting (and thus further migration) because GC pairs form triple hydrogen bonds. DNA regions that are rich in double-bonded adenine-thymine (AT) base pairs are easier to melt and do not migrate as far through the gel. The ability of DGGE to separate DNA from different strains is due
to the fact that similar DNA molecules that differ by only one nucleotide will have different melting properties that determine the migration distance. The trapped DNA can be visualized as bands in the gel that can be excised and sequenced to obtain phylogenetic information (based on 16S rDNA sequences) or knowledge of the function of the gene. The obtained sequences can also be used to design oligonucleotide probes to detect and quantify the presence of specific genes in other environmental samples.

DNA-microarray technology (also called a “DNA microchip”), which was developed in the medical field to study the genetic basis of many diseases, is also emerging as a valuable approach to expand the capabilities of molecular analysis of microbial communities. This is a powerful tool to simultaneously assess the presence or activity of up to thousands of different genes. Chips could be synthesized with arrays of hundreds to thousands of molecular probes, allowing us to easily track (simultaneously, with one assay) specific strains (by targeting 16S rRNA), catabolic or other functional genes (by targeting amplified DNA sequences), and gene expression (by tracking mRNA) (Bodorossy et al., 2003; Cho & Tiedje, 2002; Dennis et al., 2003; Wu et al., 2001; Zhou, 2002). However, microarrays can be difficult to use with environmental samples due to currently low detection sensitivity. To be detected by current microarray technology, a target gene sequence may need to comprise at least 5% of the total DNA in the sample (Cho & Tiedje, 2002).

FIGURE 7. Steps in biodiversity analysis of a microbial community using phylogenetic probes. (a) Total community DNA is used with PCR to amplify 16S rRNA genes using universally conserved primers for Bacteria or primers that will target only a particular phylum of bacteria. The PCR bands are excised and the different 16S genes separated by either cloning or by DGGE. Note how in the DGGE gel, samples 1, 2, and 4 share a common band (gene), whereas samples 2 and 3 each contain one unique band (Modified after Madigan et al., 2003).
Terminal Restriction Fragment Length Polymorphism (T-RFLP) is another common molecular fingerprinting technique used for microbial community analysis (see Figure 6). This technique measures the size polymorphism of terminal restriction fragments from a PCR amplified marker. Similar to DGGE, T-RFLP can be used for strain identification, comparative community analysis, and estimations of the diversity of a phylogenetic group within a community (Marsh, 1999). However, T-RFLP may offer greater detection sensitivity to successfully discriminate between common environmental microorganisms (Gu et al., 2004; Lendvay et al., 2003; Porteus et al., 2002). T-RFLP involves performing PCR with a fluorescently labeled primer to amplify 16S rRNA (see Figure 7). The PCR product is subsequently digested with several restriction endonucleases that cut the DNA into fragments. Different 16S rRNA fragment sizes are obtained from different microorganisms because each organism has a unique 16S rRNA sequence that is cut by endonucleases at different places. The enzyme digest is then separated in a sequencing gel. Capillary electrophoresis is then used to generate a graph of relative fluorescence versus fragment size in base pairs (bp). The number of labeled fragment peaks is proportional to the number of species in the community.

In summary, similar to stable isotope analysis, an increase in the concentration of catabolic genes could be considered to be very reliable and robust primary evidence of biodegradation, whereas phylogenetic probes, PFLA, and DGGE are useful secondary lines of evidence. Although the presence of a gene does not guarantee its expression, catabolic gene copy numbers tend to be temporally quite responsive to changing environmental conditions, and their expression is conducive to bacterial growth on the target pollutant with an associated significant increase in gene copy numbers relative to background levels as a result of bioremediation. Thus, mRNA analysis (which is a much more difficult task due to the instability of mRNA) may not always be required to demonstrate catabolic gene expression.

DISCUSSION AND CONCLUDING REMARKS

Bioremediation and monitored natural attenuation (MNA) are among the most cost-effective approaches to manage soil and groundwater contamination by hazardous organic pollutants (e.g., chlorinated solvents, energetic, and hydrocarbons). However, the regulatory approval of MNA requires extensive site characterization and documentation of contaminant removal and/or plume stability, which can be a labor-intensive and lengthy process that is subject to considerable uncertainty. Much of the uncertainty on the suitability and sustainability of MNA could be overcome by improving our ability to reliably determine in situ biodegradation rates, using simple and inexpensive approaches. The accurate estimation of biodegradation rates is of paramount importance to decrease the risk of selecting MNA as a remedy
because such rates influence plume length and longevity; thus, the likelihood and duration of exposure. Estimating biodegradation rates as part of the monitoring effort is also important for performance and compliance assessment, as well as to discern the role of microbial processes versus dilution and phase transfer (e.g., sorption) in contaminant “removal.”

Molecular biology tools (MBTs) have had a transformative impact in environmental biotechnology research, enabling us to advance our understanding of both microbial ecology and the genetic basis for biodegradation. MBTs can be used to establish that specific microorganisms that can degrade the target pollutants are present, and that their concentrations are higher in the treatment zone compared to background samples, which is an important line of evidence to demonstrate that bioremediation is working. However, the use of MBTs for the selection and performance assessment of bioremediation and MNA has been limited. This is partly due to a disconnection between the information typically provided by MBTs (e.g., who is there, what can they degrade, who is active, and what genes are being expressed) and the information most needed by the remediation professionals to decide on the appropriateness of MNA (e.g., how fast is a pollutant likely to be degraded and how long will it take to meet remedial goals?).

Further research is needed to determine whether biodegradation rates (averaged over large spatial and temporal scales that are relevant to MNA) are correlated to the concentration of specific degraders, which in turn could be determined by targeting selected biomarkers with MBTs. If so, MBT-targeted biomarkers might offer a relatively simple and inexpensive approach to reliably estimate biodegradation rates and their spatial variability to enhance the selection and performance assessment of MNA. In addition to the concentration of specific degraders, there are numerous site-specific variables that influence biodegradation rates, such as bioavailability and mass transfer limitations, nutrients and electron donor (or acceptor) availability, redox conditions, pH, temperature, and presence of inhibitory compounds. However, these confounding factors are also likely to influence the concentration of specific biomarkers, which would be responsive to changing environmental conditions and may follow similar trends as biodegradation activity over temporal and spatial scales that are relevant to MNA. Thus, exploring whether a correlation exists between degradation rates and biomarker concentrations is worthy of consideration. Such a correlation could significantly reduce the cost and enhance the reliability of biodegradation rate estimations.

Finally, to demonstrate a clear trend of decreasing contaminant mass and/or concentration over time at the field scale that is due to bioremediation and natural attenuation, a new paradigm is necessary. A stabilizing or decreasing contaminant concentration suggests that bioremediation and natural attenuation may be taking place. However, it is important to recognize that the decrease could be due to plume migration, dispersion, and dilution, or some other cause. Therefore, a data set showing a stabilizing or
shrinking plume may not necessarily prove that the contaminants are being destroyed. This suggests that the development of a comprehensive performance assessment tool that can handle disparate information from site data, converging results from analytical and reactive transport models, stable isotope analysis, chemical fingerprinting and molecular biological tools, may become necessary in the near future.

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