



FINAL REPORT

SERDP and ESTCP Expert Panel Workshop on Research and Development Needs for the Environmental Remediation Application of Molecular Biological Tools

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Acronyms and Abbreviations

ASTM	American Society for Testing and Materials
CSIA	compound specific isotope analysis
DCE	dichloroethene
DDBJ	DNA Data Bank of Japan
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DoD	Department of Defense
Eh	redox potential
EMBL	European Molecular Biology Laboratory
EPA	Environmental Protection Agency
ES/MS	electrospray mass spectrometry
ESTCP	Environmental Security Technology Certification Program
FGPR	functional gene pipeline/repository
FISH	fluorescence in situ hybridization
ITRC	Interstate Technology & Regulatory Council
MALDI-TOF	matrix-assisted laser desorption/ionization – time of flight
MBT	molecular biological tool
MNA	monitored natural attenuation
MS	mass spectroscopy
MTBE	methyl-tert-butyl-ether
NDMA	N-Nitrosodimethylamine
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PLFA	phospholipid fatty acid analysis
QA/QC	quality assurance/quality control
qPCR	quantitative real-time polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RDase	reductive dehalogenase
RDP	Ribosomal Database Project
RDT&E	research, development, test, and evaluation
rRNA	ribosomal ribonucleic acid

Acronyms and Abbreviations (continued)

SERDP	Strategic Environmental Research and Development Program
SIP	stable isotope probing
TEAP	terminal electron accepting processes
T-RFLP	terminal restriction fragment length polymorphism
VC	vinyl chloride

Acknowledgements

This report summarizes the results of a workshop sponsored by the Department of Defense's (DoD) Strategic Environmental Research and Development Program (SERDP) and Environmental Security Technology Certification Program (ESTCP) that sought to determine the research, development, test, and evaluation (RDT&E) needs for molecular biological tools (MBTs) that have the potential to improve the design, implementation, field performance, and monitoring of remediation technologies at DoD field sites.

A steering committee composed of Dr. Todd Anderson, Ms. Erica Becvar, Dr. Linda Chrisey, Dr. John Cullinane, Dr. Terry Hazen, Dr. Rob Hinchee, Dr. Frank Löffler, Dr. Gary Sayler, Dr. Rob Steffan, Dr. Hans Stroo, Dr. James Tiedje, and Dr. Herb Ward assisted SERDP and ESTCP in determining the scope and structure of the workshop.

To communicate the state of the science and engineering associated with current molecular biological tools and techniques, potential future applications of tools, and field use and considerations, background papers were authored and presented at the workshop by Dr. Frank Löffler, Dr. Darrell Chandler, Dr. Rolf Halden, Dr. Derek Lovley, Dr. Syed Hashsham, Dr. Wen-Tso Liu, Dr. Suresh Pillai, Dr. James Cole, and Mr. Patrick Haas. These background papers established a foundation for discussions at the workshop.

Overview presentations of investments in MBTs by SERDP, ESTCP, the Services, the Department of Energy, and the U.S. Environmental Protection Agency were provided by Dr. Andrea Leeson, Dr. John Cullinane, Dr. Ed Perkins, Dr. Linda Chrisey, Mr. Cliff Casey, Dr. Todd Anderson, and Dr. Mitch Lasat as a basis for identifying and prioritizing needs.

Breakout group discussions to identify key issues, barriers, and RDT&E needs were led by Dr. Terry Hazen, Dr. James Tiedje, Dr. Paul Johnson, Dr. Bruce Alleman, Dr. Herb Ward, and Dr. Perry McCarty. Discussions were documented by rapporteurs, including Dr. Elizabeth Edwards, Dr. Wen-Tso Liu, Dr. Kent Sorenson, Dr. Matthew Fields, Dr. Hans Stroo, and Dr. Rob Steffan. These rapporteurs then integrated their notes and graciously authored significant sections of this final report.

Within SERDP and ESTCP, Dr. Jeffrey Marqusee, Mr. Bradley Smith, and Dr. Andrea Leeson provided leadership in the conception and implementation of this workshop. Ms. Alicia Shepard, Mr. Scott Dockum, Ms. Deanne Rider, Ms. Jenny Rusk, and Ms. Veronica Rice from HGL, Inc. facilitated all developmental activities for the workshop.

Most importantly, we acknowledge the input of all workshop participants, which has resulted in a strategic plan to guide investments in the area of molecular biological tools over the next 5 to 10 years by SERDP and ESTCP. Attributions appear in Appendix B.

1. INTRODUCTION

The Strategic Environmental Research and Development Program (SERDP) and Environmental Security Technology Certification Program (ESTCP) are Department of Defense (DoD) programs designed to support research, development, demonstration, and transition of environmental technologies required by DoD to perform its mission. Remediation of hazardous waste in the environment is an area of emphasis for both programs.

1.1 Department of Defense Liabilities

For nearly a century, DoD manufactured, operated, maintained, and repaired thousands of vehicles and hundreds of weapons systems at its 1,700 installations. Following standard industrial practices, millions of pounds of powerful chemicals and solvents were used annually. These weapons systems also consumed billions of gallons of gasoline, diesel, and jet fuel. In addition, the need for realistic training resulted in the annual expenditure of millions of rounds of ammunition, missiles, and pyrotechnics on training ranges. The result of many decades of military operations was the inadvertent contamination of soil, sediments, and groundwater.

These contaminants can affect human and ecological health in complex ways, and the technologies used for cleanup can impact the environment. Assessing the human and ecological risks and monitoring remediation performance can be difficult and costly given the currently available tools, particularly when mixtures of contaminants are present or when residual contamination remains after treatment.

Much of the contamination at DoD sites is susceptible to multiple natural and enhanced degradation processes. Biodegradation plays a prominent role in the fate and transport of these contaminants and represents a promising remediation method. Although the potential for biodegradation has been well documented in the scientific literature, there is a significant burden of proof and lag time associated with achieving the acceptance of natural and/or enhanced bioremediation by regulatory and public stakeholders – especially with respect to chlorinated solvents. The burden of proof that bioremediation is occurring requires the proponent to provide compelling evidence of ongoing treatment efficacy. Converging lines of evidence include contaminant flux and concentrations, degradation activity (geochemical and microbial), and hydrogeological complexities. Furthermore, the field practitioner must have the knowledge and tools necessary to determine if natural or enhanced bioremediation will meet specified remedial action objectives.

1.2 Molecular Biological Tools

Rapid advances in molecular biology impact practices in many fields, including bioremediation. Molecular biology, by definition, is the study of the structure, function, and activity of macromolecules (e.g., nucleic acids, protein, and lipids) essential to life. For the purposes of this workshop, we have defined molecular biological tools (MBTs) as tools that target biomarkers (e.g., specific nucleic acid sequences, peptides, proteins, or lipids) to provide information about organisms and processes relevant to the assessment and/or remediation of contaminants in the

environment or other engineered systems. In the context of bioremediation, MBTs also include any other modern technology that measures microbial activity *in situ*.

While advances in molecular biology have had a profound effect on the understanding of biological remedial processes and are used extensively in the research community, their use in the operational cleanup community is limited at present. There is, however, tremendous potential for use of these technologies to improve the design, implementation, field performance, and monitoring of remediation technologies.

The rapid progress in sequencing capabilities, database development, bioinformatics, environmental genomics, transcriptomics, metabolomics, and proteomics promises that relevant processes can be studied and manipulated even if the key organisms involved have not been cultivated. Critical issues related to MBT utility include specificity, sensitivity, quantitation, calibration, and consistent sampling methods. Further, practical knowledge and guidance are lacking regarding how to alter the design, implementation, operation, and monitoring of enhanced bioremediation systems based on the results of these analyses.

1.3 Workshop Objectives

SERDP and ESTCP must determine how their limited research, development, and demonstration funds can best be invested to improve DoD's ability to effectively address its requirements to remediate contaminated sites. The objectives of this workshop were to (1) examine the current state of the science and technology of molecular biological tools that are applicable to the cleanup of hazardous waste in the field, (2) assess the current operational usage of such tools and identify technical and other barriers to their use, (3) identify promising areas of research and development that have the potential to lead to improved cost-effective tools to support remedial design and decisions, and (4) identify the most promising areas that are ready for and could benefit from rigorous field-scale demonstrations. This report, which documents the findings and recommendations of workshop participants, will serve as a strategic plan to guide future investments in molecular biological tools that can ultimately improve the design, implementation, monitoring, and/or performance assessment of remedial technologies.

2. METHOD

The *SERDP and ESTCP Expert Panel Workshop on Research and Development Needs for the Environmental Remediation Application of Molecular Biological Tools* was held August 9-10, 2005, in Charlottesville, Virginia. Fifty experts, including researchers, engineers, and practitioners, from within the DoD, other federal and state agencies, academia, and the private sector accepted the invitation to participate in the workshop (Appendix B: Attendees). Further, a steering committee composed of representatives from the various sectors aided SERDP and ESTCP in defining the scope of the workshop and determining the format.

Background papers were prepared and distributed in advance of the workshop to communicate the state of the science and engineering associated with current molecular biological tools and techniques; the potential application of microarrays, proteomics, systems biology, next-generation real-time polymerase chain reaction, nanoparticles, tools from other fields, and bioinformatics; and the current use and limitations of molecular biological tools. Titles and authors are provided below:

- *MBTs to Support Hazardous Waste Site Remediation*
Dr. Frank Löffler, Georgia Institute of Technology
- *Transforming Microarray Technology from a Research Tool to a Diagnostic Environmental Test*
Dr. Darrell Chandler, Argonne National Laboratory
- *The Role of Proteomics in Applied Environmental Microbiology*
Dr. Rolf Halden, Johns Hopkins University
- *Systems Biology Approach to Bioremediation*
Dr. Derek Lovley, University of Massachusetts
- *Next Generation Real Time Polymerase Chain Reaction*
Dr. Syed Hashsham, Michigan State University
- *Nanoparticles and Their Biological and Environmental Applications*
Dr. Wen-Tso Liu, National University of Singapore
- *Molecular Methods for Microbial Detection and Characterization*
Dr. Suresh Pillai, Texas A&M University
- *Bioinformatics Resources for Bioremediation*
Dr. James Cole, Michigan State University
- *Field Perspective: Current Use of Molecular Biology Tools and Limitations*
Mr. Patrick Haas, P. E. Haas & Associates, LLC

At the workshop, presentations on the content of the background papers as well as overviews of investments in MBTs by SERDP, ESTCP, the Services, the Department of Energy, and the U.S. Environmental Protection Agency (EPA) set the stage for follow-on breakout group discussions by participants (Appendix C: Agenda). Leveraging the background paper topics, participants identified key issues and prioritized gaps in knowledge and technology during breakout sessions.

The first breakout session focused on the following topics:

- Current field considerations for use of MBTs;
- MBTs in use in the field or those MBTs nearing field implementation (referred to as near-term MBTs); and
- MBTs that have potential for practical field implementation in 5 to 10 (or more) years (referred to as long-term MBTs).

Participants were assigned to groups that focused on one of the three topics. During the second breakout session on research, development, test, and evaluation (RDT&E) needs to impact environmental remediation, participants from the initial breakout groups were intermixed. Breakout sessions were led by a chair, with discussions documented by a rapporteur, who was tasked with compiling relevant sections of this summary document. Following each breakout session, the entire group reconvened to review and discuss findings.

Breakout Session I: Key Issues

The first breakout session addressed key issues related to the level of MBT development and field considerations. Topics addressed by group were as follows:

Field Considerations

- What questions are we trying to answer with MBTs for field remediation system design, monitoring, and/or performance assessment?
- Where can the application of MBTs have the greatest impact on understanding environmental processes?
- What are realistic endpoints and benefits in applying MBTs?
- What information do people in the field need? At what cost?
- What successes have we really had in applying MBTs, and what can be learned?
- How can MBTs contribute to understanding issues of environmental heterogeneity?
- How can MBTs be used to understand microbially catalyzed reactions occurring at different scales in the environment?
- What are the regulatory perspectives on the use of MBT information?
- What sampling techniques and quality assurance/quality control (QA/QC) measures are needed for molecular analyses?

Near-Term MBTs

- What is the state of the science for existing MBTs?
- What techniques are available for evaluating microbial communities in environmental samples? What improvements are needed?
- What techniques are available for assessing and quantifying microbial activity? How should the results be interpreted and modeled?
- How can contaminant degradation/transformation activity be measured using MBTs in the field to provide substantiation of *in situ* bioremediation?
- What techniques are available to link MBT detection of activity with contaminant degradation/transformation in the environment?
- What other MBTs may be useful and how would you envision their application?

Long-Term MBTs

- What do we want MBTs to ultimately tell us about microbial communities?
- What additional information is needed and/or can be obtained from MBTs?
- Can genomics, proteomics, and metabolomics data enable new technology development for the field?
- What is the role of eco- or metagenomics in developing new MBTs?
- What opportunities exist to adapt MBT-related technologies from other fields?
- What means exist to apply MBTs in real time or near real time?
- What tools are needed from an academic point of view?

Breakout Session II: MBT RDT&E to Impact Environmental Remediation

The second breakout session integrated the key issues identified from Breakout Session I into discussions of MBT RDT&E to impact environmental remediation. All groups addressed the following topics:

- Identify and prioritize the major barriers preventing field implementation.
- Identify and prioritize critical research paths to achieve practical field implementation.
- Identify and prioritize critical demonstrations that could be conducted in the near term to achieve design, implementation, monitoring, or performance assessment goals.

Research paths and demonstrations were prioritized as either critical or high, largely based on the sequence of events required to impact environmental remediation (see Table 1).

Table 1. Definition of Research Need Prioritization

	Critical	High
Research	Research that potentially could have a significant impact on the use and understanding of MBTs in the design, implementation, monitoring, and/or performance assessment of remedial technologies	Research that is of high priority but may not be able to be initiated until critical research needs are addressed or may be more clearly defined after critical research needs are addressed
Demonstration	Field demonstrations or assessments that can impact our near-term ability to implement MBTs in the field to improve the design, implementation, monitoring, and/or performance assessment of remedial technologies	Field demonstrations or assessments that are of high priority but may not be able to be implemented until critical demonstrations or assessments are completed

3. FIELD CONSIDERATIONS

The field considerations session was convened to develop an understanding of current field experience with MBTs, to identify opportunities for MBTs to improve the understanding and optimization of field processes, and to determine the barriers to more widespread use of MBTs. This section presents the output of the session in the context of the current state of MBT application in the field with perceived potential advances, followed by a discussion of the key issues identified with respect to applying MBTs in the field.

3.1 Current State of Field Application of MBTs

In order to discuss the state of current field applications and the potential for creating new applications, it is important to identify the questions that could be answered using MBTs. These tools potentially can contribute to site characterization and to performance assessment for remediation technologies. For site characterization, three primary questions were identified:

- What is the potential for degradation based on the presence/ absence of genes or microorganisms of interest?
- What is the link between the presence of target genes or microorganisms and the activity of interest?
- Is the spatial and temporal distribution of organisms appropriate to meet goals?

For performance assessment, seven questions were identified, some of which overlap the site characterization questions, but they are included here for completeness:

- Is the desired microbial process active?
- Are we achieving appropriate spatial and temporal distribution of organisms to meet goals?
- Is the desired process adequate (in terms of rates, degradation products, etc.)?
- Is the process limited by an environmental constraint?
- What can be done operationally to improve/maintain the environment?
- Can MBTs be used for continuous monitoring to improve process control and management?
- Can we predict how to achieve optimal performance under possibly variable site conditions?

These questions are useful for summarizing the current field experience with MBTs and for discussing potential future developments and their impact on the field. Table 2 lists MBTs used in the field to varying degrees to date and provides a qualitative assessment of the relative frequency of use, the perceived advantages and disadvantages, and current and possible future

Table 2. Summary of the State of Current Applications for Various MBTs

Tools	Current Relative Frequency of Use	Perceived Advantages	Perceived Disadvantages	Current Applications	Comments
Direct PCR	Moderate	Easy to perform	False negatives	Screening tool for presence/absence	Replaced by qPCR
Nested PCR	Moderate	Unsurpassed sensitivity	Requires two PCR steps	Screening tool for presence/absence	Replaced by qPCR
qPCR (16S rRNA gene)	High	Provides information on presence/absence/abundance of organisms of interest; nearly reaches the sensitivity of nested PCR; commercially available for a few key organisms (e.g., <i>Dehalococcoides</i> spp.); estimates of total bacterial numbers possible	Does not provide confirmation of activity; sampling, handling, and analysis not standardized	Screening tool for presence/absence of desired or indicator organisms; monitoring of growth and distribution of individual organisms	Expansion to wider range of organisms; standardized procedures; availability of standards
qPCR mRNA	Low	Provides information on gene expression (i.e., activity); quantitative approaches under development	Relative instability of RNA presents sampling and preservation challenges; not commercially available to a significant extent; sampling, handling, and analysis not standardized	A few experimental applications for confirming expression of functional genes	Needs wider range of genes of interest; standardization of approach; clarification of how mRNA abundance relates to activity
qPCR (functional gene)	Low	Provides information on presence/absence/abundance of functional genes of interest; commercially available for a few key genes (e.g., reductase dehalogenase genes)	For DNA, does not provide confirmation of activity; sampling, handling, and analysis not standardized	Screening tool for presence/absence of target functional genes; monitoring of distribution and proliferation of specific genes	Needs wider range of functional genes; extension to mRNA; standardized procedures; availability of standards

Tools	Current Relative Frequency of Use	Perceived Advantages	Perceived Disadvantages	Current Applications	Comments
DGGE	Low	Provides information regarding presence/absence of 16S rRNA and/or functional genes of interest; can provide an indication of target gene diversity; increased resolution with specific primers	inconclusive results with unspecific primers; short amplicon length with insufficient information; not quantitative; no standardized procedures; cumbersome	Screening tool for presence/absence of indicator genes; sequencing of amplicons for positive identification	Use is quite specialized; will likely be replaced by qPCR methods; standardized procedures lacking
T-RFLP	Low	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	Limited resolution; does not provide sequence information; not quantitative; biased towards dominant community members	Screening tool for community diversity; analysis of community structure; tracking of microbial groups within a community over time during and after active remediation	Standardized sample preparation procedures; guidance document for data interpretation
Clone Libraries (16S rRNA genes and functional genes)	Low	Indication of gene diversity; individual clones can be sequenced	Labor intensive and expensive; not widely available commercially	Community structure analysis; identification of new genes	Will remain a research tool; limited applications for bioremediation monitoring

Tools	Current Relative Frequency of Use	Perceived Advantages	Perceived Disadvantages	Current Applications	Comments
PLFA	High	Community screening tool; monitoring individual groups of organisms; total biomass determination, etc.; commercially available; can be quantitative	Other methods provide more specific information for similar cost and effort	Biomass measurements; specialized application for screening of exposure to vegetable oil; screening of high-level community structure and microbial ecosystem health	May be useful for identifying specific organisms; may have potential for measuring respiratory activity
Enzyme Probes	Low	Provides most direct measurement of the activity of interest (i.e., measures presence/absence of the actual enzyme)	Very few enzyme probes have been developed; not widely available	Direct measurement of soluble methane monooxygenase	Needs wider range of enzymes; experimental and practical validation
FISH	Low	Provides measurement of activity of organisms of interest; can be quantitative; visual information on spatial distribution	Not widely available; probes not available for a wide range of organisms; method development for each target organism required	A few experimental applications	Needs wider range of target organisms; more commercial availability; standardized protocols
CSIA	Moderate	This method distinguishes transformation from dispersion, dilution or volatilization; estimates of in situ activity are theoretically possible	Fractionation factors not always characterized; need more labs with capability to analyze samples; cost perceived as high	Used to delineate or confirm presence of multiple contaminant sources, to confirm transformation or biodegradation and estimate degradation rates	Needs fractionation factors for key contaminants and relevant degrading organisms with variability of those factors; field demonstrations; more commercial availability; integration of data with other MBTs such as qPCR

applications. These assessments were recast as information needs that may be addressed by MBTs in Table 3, which also presents the perceived ability for MBTs to address these needs.

As indicated in Table 2, quantitative real-time polymerase chain reaction (qPCR) for nucleic acid analysis has been recognized as a useful tool in the field, perhaps more than any other MBT to date. Along with phospholipid fatty acid analysis (PLFA), qPCR for the 16S rRNA gene used for bacterial identification is currently the most widely used MBT in the field. This is because qPCR is offered as a commercial service by multiple laboratories that have the ability to detect and roughly quantify key genes, and thus bacterial cells, of interest, especially *Dehalococcoides spp.*, for which this tool is currently predominantly used. It also has been shown that the technique can be applied to functional genes, such as reductive dehalogenase (RDase) genes, an application that is increasing at field sites. Of particular interest have been RDase genes associated with enzymes involved in dechlorination of the various chloroethenes. While this work has been limited mostly to deoxyribonucleic acid (DNA) thus far, the potential exists for detecting mRNA and expressed proteins from environmental samples, which would bridge the gap from detecting a potential capability to detecting an actual activity. The primary research needs identified in Table 2 for qPCR are extensions of the technique to a wider range of organisms and functional genes, increased development of RNA-based and protein-based applications, and validation by testing its relationship to other measurements and analyses.

As shown in Table 3, qPCR is used to address four of the seven information needs listed. It is noted that MBTs currently in use assess an organism's activity only indirectly. This is accomplished by qualitatively evaluating trends of target genes over time. A statistically significant increasing trend in target gene numbers, for example, would be indicative of an increase in the number of target organisms. It should also be noted that the extent to which current DNA-based techniques address the need for operational improvements is limited primarily to delineating the adequacy of distribution of an organism or functional gene of interest. On the other hand, mRNA-based qPCR may have the potential to address the remaining information needs. With regard to process adequacy, the extent of gene expression may be useful for inferring general metabolic activity and contaminant degradation rates. Transcription of certain functional genes also could be a strong indicator of specific and active degradation pathways. Trends in gene expression may be quite useful for identifying opportunities for process optimization, potentially even in a near-continuous monitoring mode.

Denaturing gradient gel electrophoresis (DGGE) has been applied at field sites for identification of specific organisms, but with the development and commercialization of qPCR, the rate of application has been on the decline. DGGE also has been applied to evaluate microbial diversity because it can distinguish closely related species, or even strains within a species, and it could potentially be applied to RNA to detect metabolic activity.

Terminal restriction fragment length polymorphism (T-RFLP) is useful for screening microbial diversity, as it facilitates differentiation of a large number of community members. Some indication of the relative abundance of the species can be inferred, although the technique should be considered qualitative at best for this purpose because of complicating factors such as potentially large differences in the number of target gene copies in different organisms. While T-RFLP has been used in conjunction with *in silico* (computer model) predictions of fragment

Table 3. Applications of MBTs for Providing Critical Information for Remediation in the Field

Tools	Degradation Potential	Specific Organism Detection	Organism Activity	Process Adequacy (rates, completeness)	Environmental Limitations	Operational Improvements	Continuous Monitoring/ Process Control
qPCR 16S rRNA gene	Current	Current	Current (indirect)	No ¹	No	Current	Future potential
RT-qPCR (rRNA)	Future potential	Future potential	Current	Future potential	Future potential	Future potential	Future potential
RT-qPCR (mRNA)	Current	Current	Current	Future potential	Future potential	Current	Future potential
DGGE	Current	Current	Future potential	No	Future potential	No	Future potential
T-RFLP	Current ²	Current ²	Future potential ²	No	No	No	No
Clone Libraries	Current	Current	Future potential	No	No	No	No
PLFA	Future potential	Future potential	No	No	Future potential	Future potential	Future potential
Enzyme Probes	Future potential	No	Current	Future potential	No	No	Future potential
FISH	Future potential	Current	Current	Future potential	Future potential	Future potential	Future potential
Proteomics	Future potential	Future potential	Future potential	Future potential	Future potential	Future potential	Future potential
CSIA	Current	No	Current	Current	Current	Future potential	Future potential

Notes:

Predictive design will require an understanding of the information provided by a suite of MBTs as well as conventional site characterization information. The fact that something has a current use does not imply that it's particularly well-suited for that use.

¹ In this column, "No" indicates the technique is not currently used for a given purpose, and the panel considered it unlikely that the technique has significant potential to be useful for such a purpose in the foreseeable future.

² Coupled with clone libraries

length for identification of specific organisms, this is a tenuous practice in general. Only when coupled with 16S rRNA gene clone libraries, can organisms producing a certain terminal restriction fragment be identified by 16S rRNA gene sequencing. Another useful application is to evaluate T-RFLP profiles for a given location in a time series to assess community changes and dynamics. Like DGGE, T-RFLP can potentially be applied to 16S rRNA to detect activity of various species, but again, the application of interest would most likely be community-level analysis. Ultimately, research on community dynamics may lead to significant improvements in the application of remediation strategies. Thus, while these tools are useful for understanding microbial communities and interactions among different populations, it appears that T-RFLP and clone libraries are unlikely MBTs for widespread field use although further advances may decrease current cost constraints.

PLFA has been widely applied at field sites, in large part because it was probably the first MBT to be commercialized for environmental applications. Applications to date have provided high-level, qualitative information about overall community structure and “health,” and quantitative information about biomass (see Table 2). With the increasing availability of qPCR, T-RFLP, and clone libraries, the utility of the tool for community structure and biomass assessment is probably minimal. PLFA may still have applications for identifying environmental limitations such as nutrient deficiency or other stresses (see Table 3), though additional development would probably be required to achieve specificity in this application. Given this potential, PLFA also might be useful for identifying operational improvements and could perhaps be applied in a near-continuous monitoring mode. It also should be noted that specialized PLFA analysis shows some promise for identifying a specific organism, although it remains to be seen whether the tool will be superior to qPCR for this application. There is a need to develop and integrate extraction methods that allow PLFA analyses in parallel with other MBT analyses on single samples.

Enzyme probes potentially can provide the most direct measure of the activity of a degradation pathway of interest, but to date they have only been applied for various oxygenase enzymes (see Table 2). The greatest research need for this MBT if it is to be widely used in the field is to develop and validate probes for a wider range of enzymes. Given that the enzyme is directly detected, this is one of the few MBTs with the potential to provide information regarding degradation rates and the activity of specific degradation pathways of interest (see Table 3).

Fluorescence in situ hybridization (FISH) is an MBT that has not been applied in many field settings (see Table 2). It is of interest, however, because of its ability to detect and quantify an organism of interest. To be useful for widespread field use, FISH probes for a wider range of organisms or functional genes would be needed. The quantitative potential of FISH makes it attractive for assessing general metabolic activity as well (see Table 3). It may be possible to use FISH to identify environmental limitations on activity, depending on the genes targeted, thereby making this tool useful for making operational improvements and supporting process control. Further development would be required to achieve this objective. Although FISH probes for key organisms relevant in bioremediation are desirable for addressing ecological questions, the application is cumbersome and may not be practical for bioremediation monitoring, particularly for very small organisms (less than 0.4 μm) that can be common in nutrient-poor environments. The panel also noted the desirability of integrating FISH with other analyses, such as qPCR and enzyme probes, to produce multiple lines of evidence.

Compound-specific isotope analysis (CSIA) is not strictly a “molecular biology” tool. However, it has proven to be a very promising approach to measure in-situ transformation processes of pollutants in contaminated aquifers as well as to help determine the sources of groundwater contaminants. Several academic laboratory studies have demonstrated the potential of this tool (Ahad et al., 2000). To date, the use of CSIA in field studies is, however, confined to locations near source zones of groundwater contamination with high pollutant concentrations because the detection limits are relatively high (Hunkeler and Aravena, 2000).

Methods based on proteomics (i.e., the study or analysis of all the proteins in a cell or system) were not included in Table 2 because of the limited number of environmental field studies to which they have been applied. However, they were included in Table 3 for their potential to address a number of the information needs. Due to the many different types of proteins expressed by cells, protein analysis has the potential to provide information regarding organism distribution and activity, degradation pathways, degradation rates, environmental stresses, operational improvements, and through all of those, process control. However, research into protein expression and detection will have to advance significantly to realize this potential.

3.2 Key Issues

3.2.1 Areas of Potential Greatest Impact

One of the key issues regarding field application of MBTs is determining at what point within the remedial process, or for which specific remedial approaches, these tools can have the greatest impact. Several field scenarios and the manner in which MBTs could influence decisions at a site are presented below with a brief description of the significance of each.

3.2.1.1 Field Rate Constants for Monitored Natural Attenuation (MNA) Sites

One of the more significant challenges in evaluating the applicability of natural attenuation as a remediation strategy and in predicting its long-term effects is the estimation of field degradation rates. This challenge arises from the fact that it is typically quite difficult to distinguish degradation from dispersion within contaminated groundwater. If MBTs can be developed that contribute significantly either to estimating degradation rates directly or to documenting the extent of microbial degradation processes, they would substantially decrease the uncertainty involved in selecting MNA as a remedy.

3.2.1.2 Rate Information for Active Bioremediation

As suggested above, direct measurement of contaminant degradation rates at a field site might not be possible using MBTs; however, the tools have the potential to provide important data related to the activities of microorganisms (i.e., rate information). These data could significantly reduce uncertainty in biodegradation rate estimates. Further, MBTs have the potential to provide information regarding factors such as nutritional status and stress responses that may limit degradation rates and that could be modified to improve degradation performance.

3.2.1.3 Process Optimization

MBTs have the potential to provide the causal link between operational parameters or environmental conditions and degradation performance. If high frequency monitoring could be performed cost-effectively, such a tool may be used to maintain optimal degradation

performance near continuously. MBTs used in this application could monitor degradation activity, microbial response to environmental stresses, or other related parameters.

3.2.1.4 Characterization of Poorly Understood Pathways

The use of MBTs in environmental remediation applications is still in its infancy. As such, important functions of many relevant microorganisms are not well documented. In particular, only a few contaminant degradation pathways are characterized at all with respect to the enzymes involved and the genes that code for them. Once more pathways are better characterized, MBTs should be able to document the potential for and activity of pathways of interest. This can be a significant benefit for monitoring and/or optimizing natural attenuation and engineered bioremediation techniques.

3.2.2 Regulatory Perspective

A second key issue is the regulatory perspective regarding the use of MBTs for field applications. One significant limiting factor is that the regulatory community is largely uninformed about the potential advantages and limitations of MBTs. MBT development should incorporate the regulatory community into the process to maximize appropriate use of the tools. One approach to this issue would be to work with the Interstate Technology & Regulatory Council (ITRC) either to form a new team to develop guidance in this area or to incorporate MBTs into the scope of an existing ITRC team. Such a team may develop a “technology overview” document outlining the “state of the science” for these methods, what they could be useful for now, what is on the horizon, and of which QA/QC concerns regulators and practitioners should be aware. In many cases, ITRC develops nationwide, web-based training programs for all interested parties on such topics.

3.2.3 Standardized Analytical Methods and QA/QC Protocols

A third key issue in the use of MBTs for field applications is the lack of standardized analytical methods and QA/QC protocols among MBT service providers and laboratories. For example, information is often limited regarding false positive and false negative results for many of the methods in current use. In some cases this is an analytical issue, in other cases it is a sampling issue. For the former, standardization of methods may be useful, similar to American Society for Testing and Materials (ASTM) or EPA standard methods. Analytical methods are constantly being optimized, but it is likely that significant elements of the procedures could be generalized to help mitigate concern that analyses between laboratories are not comparable and might in fact be completely different, even though MBT service providers call the analyses by the same name.

In the case of sampling methods, it is well known that the method or the medium (i.e., soil versus groundwater) can have a dramatic impact on the results obtained from MBTs. Nevertheless, the most representative sampling method and medium for various MBTs is not known. Further research in this area is required to reduce the uncertainty in data interpretation for MBTs. Ideally, this research would lead to generalized sampling protocols for specific MBTs. Overall, improved understanding of the effectiveness of the analytical and sampling methods and improved QA/QC protocols will increase confidence in MBTs for practitioners and for regulators, helping MBTs achieve their maximum benefit in the field.

3.3 Summary

MBTs have the potential to answer important questions pertaining to remediation processes in the field. While some MBTs are already being used at several sites, this technology is in its infancy for environmental applications, especially in the field. It appears that the areas of remediation practice where MBTs could have the greatest impact are:

- Field rate constants for MNA sites;
- Rate information for active bioremediation;
- Process optimization; and
- Characterization of poorly understood pathways.

The method that appears most likely to contribute to field applications in these areas in the near term is qPCR, especially as the range of genes analyzed broadens and the technique extends to RNA. Other MBTs that show promise for field applications but may require more development to impact the above areas significantly include PLFA, enzyme probes, and FISH. Proteomics also appears to have great potential but probably will require the most development prior to widespread use in the field. The other MBTs discussed—DGGE, T-RFLP, and clone libraries—appear to be useful primarily as research tools that may provide important advances in the understanding of biodegradation processes that will ultimately improve remediation applications, but they seem unlikely to be used as routine monitoring tools in the field.

Two significant issues that need to be addressed for MBTs to reach their full potential to beneficially impact the remediation practice are engaging the regulatory community and improving and/or standardizing QA/QC protocols. The first issue can be addressed largely through dissemination of current and developing information through existing organizations such as the ITRC. The second issue will require significant further research as alluded to above and discussed in more detail later in this document. It also will require significant cooperation among method developers, practitioners, and stakeholders to establish appropriate guidelines for protocols development.

4. NEAR-TERM APPLICATIONS OF MOLECULAR BIOLOGICAL TOOLS TO SUPPORT ENVIRONMENTAL REMEDIATION

The Near-Term session was convened to discuss the current state of the science for existing MBTs and to review various techniques available for evaluating microbial communities and for assessing and quantifying microbial activity. Specifically, the group was to examine how data or results from MBTs should be interpreted and modeled so that they can provide substantiation of *in situ* bioremediation.

Discussions focused mainly on limitations to the current use of MBTs in the field. It became clear that field practitioners and scientists valued these techniques very differently; therefore, there was considerable discussion about what these tools actually can or cannot tell us, what they may in the near future be able to tell us, and how we can improve communication between these two groups (academic and industry).

Overall, the breakout group felt that it was important not to focus on a particular MBT and ask what it can tell you but rather to start from an important question or common problem a field practitioner may encounter during site assessment and remediation, and ask how MBTs can help address and solve this real problem.

A summary of key issues and research needs that emerged from this breakout session is provided in Table 4. The discussions leading to the development of this table are summarized in the following sections.

4.1 State of the Science for Existing MBTs

There are two distinct uses of MBTs in the context of bioremediation. The primary use is in scientific research, where these constantly evolving tools are used to gain new knowledge. The second use is as a commercially available measurement or diagnostic tool used by remediation practitioners to obtain information about *in situ* biological processes. These need to be considered separately.

Many molecular techniques and combinations of these techniques are used in scientific research, but most are not used in the field. To date, PLFA analysis, PCR, qPCR, and CSIA are the only MBTs practitioners use on a somewhat regular basis (see Table 2). Certainly there have been field applications of other techniques (clone libraries, DGGE, stable isotope probing [SIP], enzyme assays, and others), but mostly in the context of field research, not as a routinely adopted monitoring or assessment tool. While use of these tools is quite limited, it is anticipated that their use will grow, especially as compelling evidence for the value added becomes more available and as protocols and methodology become more standardized and automated.

4.1.1 Field-User Perspective

Concerning the current use of MBTs in the field, practitioners see two types of needs for new tools: (1) validating the reliability of MNA (perhaps the most pressing need) and (2) monitoring the performance of engineered bioremediation systems. These two needs were considered

different, because in the former, biomass concentrations are usually very low and relatively constant over time, while in the latter, biomass levels are higher and should increase during a successful operation. The perception from practitioners was that MBTs could be very useful for making a case for MNA, with some additional understanding of the data and how they can be used for this purpose, e.g., demonstrating that the appropriate biodegrading organisms are present and active. For now, the value added is not always clear, especially to those who have limited time to invest in understanding the technology. The bottom line is always cost and compliance. Regulators and stakeholders need to understand the MBTs, and they must be easy to implement and easy to explain. The consensus is that there is currently too little understanding of how these tools work and how they can be used. Better technology transfer is needed, and in particular, examples of successful uses of MBTs should be highlighted.

4.1.2 Activity Measurement

The current state of the practice of bioremediation is quite empirical, and degradation rates are inferred from chemical data and the experience gained during prior projects. Thus, projections and predictions can be highly unreliable. Can MBTs help reduce this uncertainty and provide insight regarding how to sustain the desired microbial activities? There was considerable discussion and some skepticism about what MBTs can tell us about rates of degradation, indicating a clear need to demonstrate to practitioners how this kind of science and technology can help them in this regard. The most pressing question from practitioners is: Can MBT data tell us if current rates will be sustained? The answer is most likely yes, but two types of efforts are required: 1) an improved understanding of subsurface processes (basic research) and 2) development of tools and methodologies to monitor the subsurface processes affecting contaminant fate. Without a fundamental understanding of subsurface microbiology, it is difficult or impossible to predict the sustainability of microbially mediated processes.

4.1.3 Key Organism Identification

The general relevance of and need for identifying specific microorganisms involved in contaminant degradation or transformation was discussed. Certainly there is value in working with isolates and defined cultures but many isolates do not accurately represent dominant organisms within the subsurface. It was noted that *Dehalococcoides* is somewhat of an exception in bioremediation, where there is a strong link between the organism type (identification) and the activity (i.e., reductive dechlorination). This is not the case for most other contaminants. For example, knowledge of the specific organisms present is not necessary for successful operation of an activated sludge plant; only the activity must be monitored. Because of the diversity of microbial types, it actually is desirable to have tools that can measure or predict activity where knowledge of the organisms involved is not needed. However, having the ability to define the functional groups of microbes that are present could indicate the sustainability of desired processes and important nutrient requirements, as well as any competing terminal electron accepting processes that may cause stalls or lead to the formation of undesirable products.

4.1.4 Sampling

It was unanimously agreed that a major barrier to further implementation of those MBT tools currently in use (mostly qPCR) is the lack of uniformity and standardization in the protocols for obtaining this kind of data. There is a need to better understand the effects of sample matrix, sample collection, preservation, extraction, and analysis, and to provide quality assurance and

standardization of the results. This was seen as a critical need to improve the utility of these methods, to provide a basis for comparison of data, and to increase regulatory acceptance.

4.2 Techniques for Evaluating Microbial Communities in Environmental Samples

A discussion of which techniques are being used, and which are not, led to the consensus that each technique has its application in specific instances and that we should not be ruling one out over another. Some tools that appear to be outdated are still evolving and may actually be very useful for answering a particular question. New tools or novel combinations of tools are constantly being proposed so it is important to consider suggestions for a particular application.

4.2.1 Database of Existing Information

There is clearly a need to summarize what has already been done at contaminated sites and how MBT data were employed (perhaps in the form of a web site). This repository of information should include sampling protocols and data interpretation methods that were used to develop and/or defend a particular remedy. For example, a relatively wide range of MBTs have been used at chlorinated solvent contaminated sites. A synthesis of this existing data, including site-specific hydrogeologic, physical, and chemical information, may indicate which organisms are frequently found in a given environment or may reveal insights into environmental factors that affect biological activity. A compilation of clone libraries or data from phylochips (or other analyses) from sites where dechlorination was stimulated relative to sites where no activity was stimulated, could provide some insight into reasons for failure such as the absence of appropriate organisms and/or genetic potential.

The group also felt that improvements were needed in the use of these tools to reduce uncertainty in the rates of contaminant degradation. One example cited was that bioremediation processes sometimes fail due to a lack of information about the site. For example, co-contaminants such as chloroform will inhibit at least some *Dehalococcoides* organisms. The use of MBTs could possibly prevent such failures. It was noted that such field-scale experiments could be very useful in identifying new biomarkers for tracking bioremediation processes. An experiment suggested was to compare several sites where dechlorination progressed to ethene with other sites where this did not occur in order to identify differences in gene content and expression. Such an experiment would require a sufficiently detailed site assessment to clearly define whether a particular site is exhibiting complete dechlorination or not. Because geographic separation can lead to different microbial communities, the testing would need to establish the range of changes that occur at different sites.

4.2.2 Improvements Needed

A better understanding of key microbes, community structure, co-contaminant effects, inhibitors, genetic systems, and regulation *in situ* is required. To date, this research has had comparatively little funding. Given the importance of *Dehalococcoides*, it was considered surprising that there has not been more investment in genomics and systems biology of this organism. Scientifically, the community is on the verge of an explosion in information and technology. For example, the genome sequence determination of the “average” microbe can now be conducted in a few hours; however, the closing and annotation of that sequence may take weeks or months. In the next

months to years, numerous potential new targets will be sequenced and demonstrated, but the availability of appropriate biomarkers is currently limiting.

4.3 Techniques for Assessing and Quantifying Microbial Activity

A discussion of techniques that could be used in the near term to provide information for quantifying microbial activity revealed shortages of available tools for measuring activity in the field. SIP in combination with PLFA/nucleic acid/protein analysis can identify which populations are active in a complex environment. SIP/PLFA has been used with ^{13}C carbon substrates in the field. These kinds of techniques will lead to new biomarker identification. However, once biomarkers are identified via such labor-intensive and costly screens, the detection method must be adapted for a tool such as qPCR, which is more applicable for routine, cost-effective use. Information on which populations are being stimulated can also be used to understand the physiology and nutritional requirements of the key players involved in contaminant degradation.

Microbial growth can be demonstrated by an increase in cell numbers (or other biomass indicators) starting from a low concentration state. Similarly, a system can be initially starved such that any increases in specific DNA, RNA, or other biomarker may represent an indicator of activity. PLFA has been used for such community and/or activity measurements and is also an excellent technique for quantifying biomass. With the development of electrospray mass spectrometry (ES/MS), it is now possible to analyze larger lipids, which should yield more specific functional information. The recent addition of respiratory quinone analysis should also greatly improve our use of signature biomarkers. Until 2005, commercial PLFA analyses exceeded commercial PCR analyses, but PCR analyses are now more frequently requested³.

There was some discussion about the potential for mRNA analysis to yield useful information on degradation rates. In other words, is there a correlation between the abundance of a certain gene transcripts as measured by reverse transcriptase qPCR (mRNA analysis) with degradation rates? This is still at the conceptual level now, but by using a better combination of target genes, improving RNA recovery from environmental samples, and developing appropriate standards, this may be possible.

Protein and enzyme assays also could be useful; there have been several documented uses of enzyme probes to assess activity. Metaproteome analysis, i.e., direct analysis of all the proteins in a sample, is probably not a near-term tool. However, a recent report in *Science* demonstrated metaproteome analysis at an acid mine drainage site, building on previous genomic analyses (albeit the site had low diversity) (Ram et al., 2005). It is also possible to analyze a groundwater sample using matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectroscopy (MS) for specific proteins; however, the need to identify a specific target protein and the lack of protein fragment databases is currently limiting this approach.

Current databases (GenBank, etc.) have gaps relevant to bioremediation and are difficult to search from a remediation perspective. For example, it is impossible to find a single source that identifies all genes that have been isolated from chlorinated solvent contaminated sites. A

³ Information provided by A. Peacock of Microbial Insights, Inc.

SERDP database of sequences found at contaminated sites could be a valuable resource. There is also a need for consistent naming of genes and proteins and where they came from. A database of information about remediation MBTs, genes, and proteins could be very useful to practitioners, stakeholders, and regulators. SERDP/ESTCP will need to identify data that should be in a database, determine how this would be entered in a consistent and validated way, and consider mechanisms for long-term maintenance and updating of information.

Key questions and needs identified by the group included:

- Identify more of the key organisms and/or genes responsible for degradation and additional targets (biomarkers) that can be monitored or measured using an MBT.
- What genes are associated with a successful process?
- How do we model pathways?
- What controls the metabolic state of the key organisms in a given environment?
- Use genome/transcriptome/proteome/lipome-based studies of the physiology of important model organisms to reveal key physiologic needs.
- Need to integrate all data on donors, acceptors, rates, and MBTs with fate and transport models to assess the big picture.

A team approach should be used to develop this integrated model. Rates depend on many parameters in the field such as donor and acceptor concentrations, biomass, redox, and the presence of potential inhibitors. We need to link all pertinent models with all relevant data. This kind of integration will provide guidance to the practitioner to identify all the possible mechanisms that could be operative at a particular site. We need tools to support or refute hypotheses for the site conceptual model.

4.4 Linking MBTs to Contaminant Degradation/Transformation

How can MBTs be used to obtain transformation rates? It was agreed that CSIA is probably the technique closest to being ready for commercial use for this purpose, although it is still a technique performed primarily by academic and government laboratories.

Contaminant degradation also can be partially inferred currently from analysis of specific organisms or groups of organisms such as methanogens, methanotrophs, sulfate reducers, iron reducers, etc., and knowing the physiology of these groups of organisms. MBTs cannot be used alone and should be combined with fundamental biogeochemical measurements such as terminal electron accepting processes (TEAP), limiting nutrients, or redox potential (Eh).

There are RNA-based (as opposed to DNA-based) tools being used to infer metabolic rates in marine systems. For example, there is evidence from both pure cultures and environmental samples that *rbcL* (ribulose-1,5-bisphosphate carboxylase/oxygenase [or RUBISCO] gene) mRNA levels correlate with carbon dioxide fixation rates (Corredor et al., 2004). Research is needed to establish similar links for processes of interest at contaminated sites. In particular, data on the mRNA half-life and transcriptional regulation for various biogeochemical and degradative target genes from environmental samples will be necessary for reasonable interpretation of field measurements.

Little discussion was offered on the use of flow cytometry for cell enumeration although this technique could be readily used on groundwater or other field samples. In this technique, cells in a sample are stained or labeled with general or specific markers, and then analyzed in a flow cytometer. Cells of different kinds and size can be distinguished and each group can be quantified. Very high numbers of cells and samples can be processed easily and quickly, thus providing a high degree of statistical confidence in the result. This is an emerging technique that should be evaluated in combination with specific biomarker labels.

Linking MBTs to contaminant degradation/transformation was seen as a significant need. However, MBT data must be more reliable and quantitative, then integrated with all other available site information and basic knowledge to make predictions of rate or activity.

4.5 Key Issues

To summarize the discussion, the breakout group agreed on a list of key issues and research needs, then each participant was given three votes to prioritize the list. The number of votes reflects the group's attempt to prioritize the issues, not whether a particular issue was important; the group felt that all the issues were important. The results are provided in Table 4.

Table 4. Key Issues from Near-Term Breakout Session

Key Issue	Votes
Identification of key active players?	11
Sediment versus water: which medium is more reliable for sampling?	9
Community and activity comparisons at multiple sites undergoing different degrees of remediation	9
Systems analysis incorporating MBT data; using integrative approach combining all site data; coupling groundwater models with MBTs	8
Sampling and analysis protocol verification for existing MBTs using <i>Dehalococcoides</i> as a model organism	4
What are the key genes? Finding contaminant gene pathways	4
Linkage of SIP with MBTs	2
Database on genes involved in PCE degradation – bioinformatics	2
Others indicators of activity that are non nucleic acid-based (quinones, enzymes, fatty acids, other)	2

In addition to the above, this breakout session identified the need for:

- Better synthesis and compilation of existing data;
- Better communication to the user group of advantages and limitations of existing and new tools;
- Better integration of disparate types of field data and information into conceptual models; and
- Systematic evaluation of mRNA, cell numbers, protein levels and activity, to establish linkages and identify organism- and condition-specific limitations.

5. FUTURE APPLICATIONS OF MOLECULAR BIOLOGICAL TOOLS TO SUPPORT ENVIRONMENTAL REMEDIATION

MBTs have begun to increase our general understanding of certain bioremediation processes, but, because of the complexity of the systems, additional development is needed to realize their true potential value for environmental cleanup. This breakout group believed that the greatest value of MBT today has been in providing a general understanding of the microbial communities and specific key players involved in the degradation processes as opposed to the development of any particular tools that can be used in practice. In the near term, the group believes that some of the tools can be enhanced for specific remedial practices, but the robustness of the currently used MBTs for biomarker monitoring needs to be enhanced. In the long term, new biomarkers are needed to assess more processes. This section presents the output in the context of the long-term goal and state of the science and engineering of bioremediation, followed by a summary of the long-term needs identified for achieving the long-term goal of bioremediation.

5.1 Long-Term Goal and State of the Science and Engineering of MBTs

The long-term goal of the science and engineering of MBT development is defined for a period of 5 to 15 years. It is important to understand bioremediation processes so we can more reliably assess, predict, and manage bioremediation to a desired outcome (i.e., site closure). Microbial processes in the environment are complex; to predict function and/or activity, additional research is needed on the microbial communities, their physiology, and their interaction with the environment. Thus, the focus of this long-term goal should be on the bioremediation activity or potential activity, so the knowledge gained can enhance the capabilities of bioremediation. To facilitate the discussion, topics or questions to be addressed by this group included:

- Understanding how community composition can influence the process.
- Understanding the stability and resilience of community activity.
- How should the supply of electron donors and acceptors be managed in order to achieve and sustain desired functions?
- How can we increase the knowledge about the unknown organisms, genes, and enzymes important to bioremediation processes?

Based on the discussion regarding these questions, the general consensus on the long-term vision for the science and engineering of bioremediation is summarized in Figure 1. The first critical step after identifying a contaminated site is to have a well-planned sampling scheme. The samples could be used to gain knowledge or in diagnostics processes by employing different MBTs, for example, to discover novel biomarkers or to evaluate the potential activity of the known key biomarkers. The information could be channeled to the data management tool and protocols where the retrieved sequence information is shared and compared with those stored in the database in order to identify potential biomarkers involved in the bioremediation processes. This could further lead to the refinement of different MBTs used for exploratory and diagnostic purposes and the development of new bioinformatics tools to predict and discover new biomarkers and metabolic processes. In parallel, comprehensive physical and geochemical data should be obtained from the contaminated sites, and the responsible microorganisms involved in

the bioremediation processes can be enriched and, if possible, isolated. Eventually, the overall information obtained from various components can be combined and used in a systems biology approach to better understand the bioremediation process. To achieve this holistic approach for bioremediation, four long-term needs have been proposed—subsurface sampling, signature identification/biomarkers, database/modeling, and physiology—and are discussed in Section 5.2.

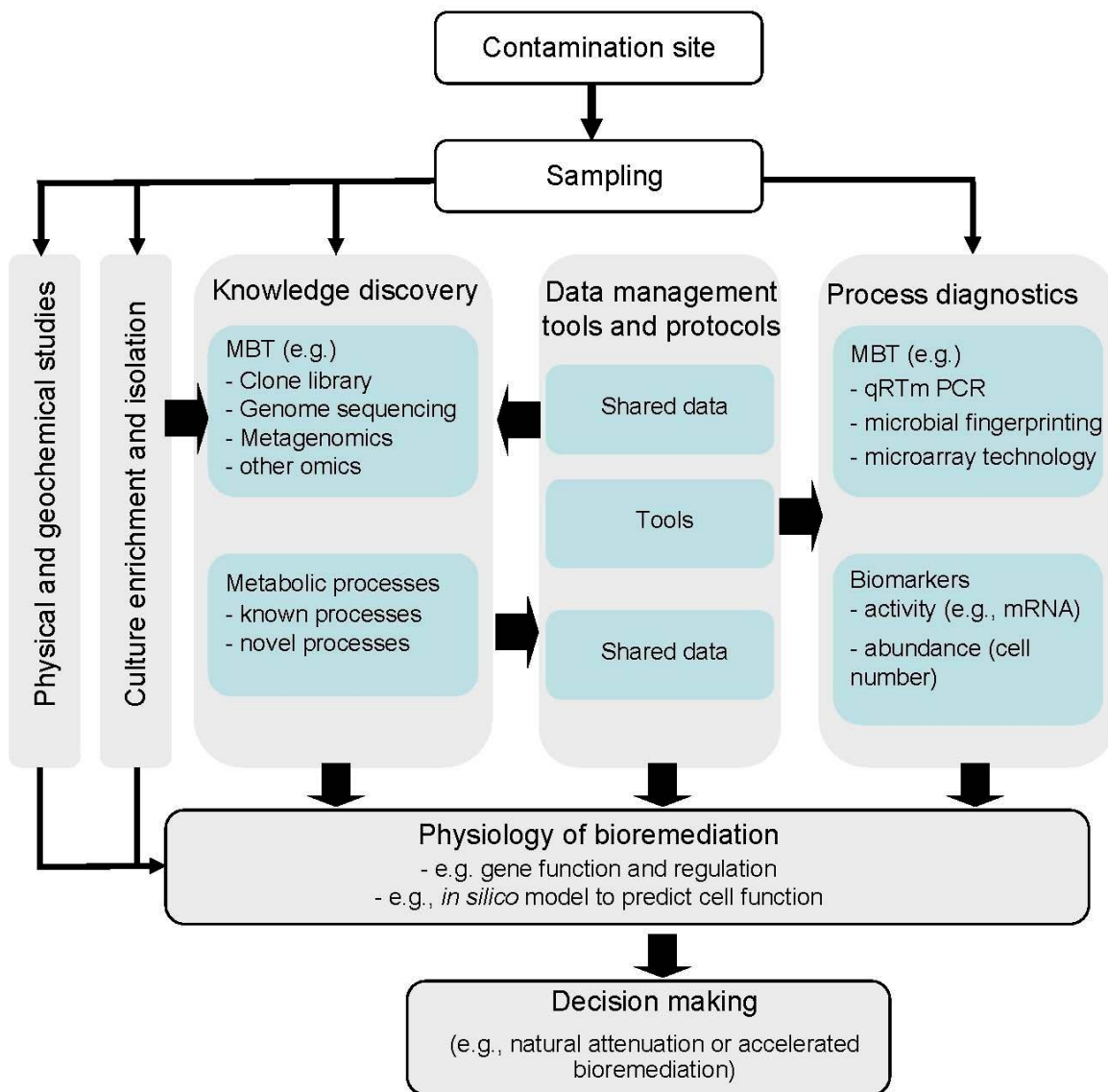


Figure 1. A Holistic Approach for Bioremediation

5.2 Long-Term Needs

5.2.1 Subsurface Sampling

Optimizing subsurface sampling procedures for MBTs in a cost-effective manner is suggested as the first priority of the long-term needs, as most of the contaminants of concern are within subsurface environments (i.e., saturated zone). A typical sampling procedure involves a series of steps: selecting the sampling locations; drilling wells at different spatial distributions into the subsurface; obtaining samples of aquifer materials (i.e., soil/sediments) and groundwater at different temporal intervals; sample handling, shipping, and storage; and sample processing. At present, there is a lack of generally acceptable guidelines or standard operating procedures for all the steps involved in subsurface sampling and handling for MBTs. Because of cost constraints, the physical, geochemical, and microbial data obtained from a contaminated site can be compromised such that the results do not correctly reflect the true geometrical information concerning the pollutants (e.g., direction of plume movement), and the type and activity of microbial communities. Such inadequacy of information can lead to decisions that may adversely affect the remediation effort.

Thorough consideration must be given to every sampling step. For example, a sufficient number of samples must be obtained and handled in a cost-effective manner so that biomarkers remain intact without change until laboratory analysis. The number of samples required is usually dependent on the resolution needed for assessing microbial activity and processes. Statistical methods of analysis such as those used in studies of ecology could be adapted to determine the best sampling frequency. If on-site measurement is necessary, “smart samplers” that require minimal human involvement in sample preparation and concentration may be developed. Reproducible storage and extraction methods for nucleic acid (i.e., DNA and RNA) and other biomarkers, possibly for on-site use, need to be further developed for samples obtained from soil matrices and groundwater. Lastly, the hydrogeology of the contaminated site can greatly influence the selection of sampling methods and frequency. To help guide future efforts, an evaluation is needed of sampling procedures used at sites with different known contamination, possibly using as models those sites that have been studied extensively and where extensive geochemical and physical data are available (e.g., Field Research Center at Oak Ridge, Tennessee).

5.2.2 Signature Identification/Biomarkers

A second long-term need is to discover, monitor, and develop for field use key biomarkers that link target organisms with process performance. At present, nucleic acid-based biomarkers are most commonly used. These include the 16S rRNA genes for phylogenetic identification and functional genes for metabolic pathways of importance in bioremediation processes (e.g., the RDase genes *tceA*, *bvcA*, and *vcrA*). The metabolic activity of important contaminant degraders may be determined by monitoring the expression levels of key phylogenetic and functional gene markers (i.e., rRNA and mRNA). Proteins and metabolites produced from the important metabolic pathways also could serve as important biomarkers. For example, PLFA and respiratory quinones are used to monitor the presence of different microbial populations.

However, the types and numbers of biomarkers currently available for use in bioremediation are limited and in need of further expansion. One need is to minimize the gaps (i.e., type and diversity) in functional biomarkers available for processes that are poorly understood. These

include the genes involved in the anaerobic degradation of aromatics, chlorinated solvents, and energetic compounds. Another critical focus is to obtain larger numbers of indicator genes to establish knowledge of the sequence diversity within each gene group.

Similar to biomarkers, MBTs also serve as a key driving force for advances in bioremediation technology. At present, there is a suite of nucleic acid-based MBTs developed for various medical and biological purposes. MBTs that have been adapted and used in environmental studies can be classified into tools for discovering the microbial and functional diversity of known and novel biomarkers, and tools for rapid field diagnostics. Tools for discovery include the construction of clone libraries for phylogenetic markers (e.g., rRNA genes, ATPases, and DNA gyrases) and metabolic genes (i.e., dehalogenases and oxygenases). The diagnostic tools include qPCR, DNA microarrays, and community fingerprinting methods (e.g., DGGE and T-RFLP). Although these tools are currently used primarily for increasing knowledge regarding the organisms involved in bioremediation, they have the potential to be valuable diagnostic tools that can be used to monitor the presence and possibly the concentration of degraders and to estimate the activity of metabolic functions associated with those degraders. Unlike nucleic acid-based MBTs, tools for monitoring and identifying proteins and metabolites produced in bioremediation (e.g., MALDI-TOF MS, peptide mass fingerprinting, and difference gel electrophoresis) are less frequently used but have shown great potential.

Overall, applications of these MBTs in laboratory and field settings have uncovered several drawbacks associated with currently-used MBTs, such as sensitivity, specificity, and quantitative resolution. The analytical characteristics can vary from one MBT to another. For example, qPCR is able to quantitatively detect a desired gene accurately down to less than 10 copies per reaction, but this technique has lower throughput capability than a DNA microarray. In contrast, fingerprinting techniques like T-RFLP can simultaneously profile the distribution of the total genes present in a sample, but can only detect a given marker at a level higher than 0.1% of the total genes present.

Specificity is usually referred to as the ability of an MBT to differentiate a true signal from a false-positive or a false-negative signal, and it is affected by the types of biomarkers and MBT used and also by interfering compounds present in the sample. For qPCR, the distinction between a perfect-match target and a single-mismatched target can sometimes be challenging because the specific primers used for the target can anneal or bind equally to both target and non-target genes that contain a single mismatch occurring towards the 5' end of the primer. One way to minimize this non-specific binding is to combine two or more MBTs together with physical and geochemical parameters to achieve higher redundancy and specificity. Another aspect of specificity refers to the inability of certain MBTs to discover novel genes. None of the current PCR- and hybridization-based MBTs can effectively detect or discover an unknown but important functional gene within a sample because MBTs rely on prior knowledge of sequence information for primer/probe design.

There is a strong, long-term need to further develop new MBTs for bioremediation. It is anticipated that the next-generation MBTs in bioremediation will be derived from advances in medical diagnostics adapted for use in the environment. Although it is hard to predict which MBT advances will be most useful for bioremediation, it is certain that some will be attractive

and adaptable. For example, environmental metagenomics and proteomics may lead to discovery of novel biomarkers (i.e., genes and protein), which can be further used in quantitative-based or array-based technology to monitor the activity of important metabolic pathways involving bioremediation. Likewise, the sensitivity, specificity, throughput, and robustness associated with an MBT could be enhanced through the development of nanotechnology. The use of quantum dots as a novel fluorescent reporter can improve the detectable fluorescence intensity at least 10 to 50 times higher than through use of a conventional organic fluorophore. The use of gold nanoparticles can improve the specificity in differentiating perfectly matched probe-target duplexes from mismatched duplexes. The throughput capability further could be enhanced by combining PCR with microarray technology to allow parallel amplifications of hundreds to thousands of PCR reactions. The development of micro- or nanofluidic devices such as the “lab-on-a-chip” for integrated sample preparation/concentration and biochemical reactions (i.e., DNA extraction and amplification) may simplify the use of MBTs for engineers and practitioners on-site and produce more consistent results. Additionally, it is desirable to standardize assays (QA/QC) for MBTs so that results obtained among different laboratories at different contamination sites are valid and believable.

5.2.3 Database/Modeling

A third long-term need is to develop good data sharing and modeling systems so that useful and correct information can be rapidly and easily extracted, simulated, and used to improve the effectiveness of MBTs for bioremediation. An initial effort would be to ensure that existing bioinformatics resources, particularly ones related to nucleic acid sequences, are more available and easily retrieved. Bioinformatics resources can be classified into those for phylogenetic marker genes (rRNA and DNA gyrases) and those for functional genes (e.g., dehalogenases, and oxygenases). Although such sequences can be found in primary sequence databases (e.g., GenBank, DNA Data Bank of Japan [DDBJ] and European Molecular Biology Laboratory [EMBL]), they would better be maintained as subsets of databases. For phylogenetic marker-related sequences, the Ribosomal Database Project (RDP) is one of the most commonly used databases for 16S rRNA gene sequences. It is provided in aligned format and organized based on the bacterial taxonomy used in the latest version of Bergey’s manual (Holt, 1994).

Databases for functional gene sequences are less comprehensive than the phylogenetic marker sequences, and it is challenging and time-consuming to assemble the data necessary for developing assays for different functional genes. Pfam and TIGRFAM are two of the most comprehensive databases, but they contain only protein information. To facilitate metagenomic and high-throughput research methods (e.g., DNA microarray), the FGPR (functional gene pipeline/repository) database recently was established by Michigan State University (<http://flyingcloud.cme.msu.edu/fungene/>). It includes many useful bioinformatics features such as constructing a neighbor-joining tree for a subset of sequences, downloading a subset of aligned functional gene sequences, and testing the specificity of probes/primers designed for specific genes.

Current bioinformatics resources further provide software that allow users to extract useful information from sequences. The most commonly used feature is probe/primer design, specificity check, and melting temperature calculation, and can be found in software like RDP, ARB (<http://www.arb-home.de/>), and PRIMOSE (<http://www.cf.ac.uk/biosi/research/biosoft/>).

Still, with the rapid growth of gene sequences, future efforts are needed to improve and expand the breadth of current bioinformatics resources. One suggestion is to establish a database related to the 18S rRNA gene sequences of fungi, and another is to further incorporate 16-23S intervening sequences into the RDP database. With the rapid development in environmental metagenomics studies, it is also important to establish databases for massive amounts of sequence data retrieved from different environments. Although the quality of sequences can be significantly improved through the advance of sequencing techniques, additional actions can further enhance sequence data quality. Filtering chimeric sequences before submitting them into the database is one such step. Since different tasks require different quality and/or comprehensiveness tradeoffs, data should be accessible as subsets of varying quality, such as high-quality sequences from known isolates versus short single-read environmental sequence survey datasets. In addition, new data annotation standards should be developed for environmental sequence submission.

One ultimate goal of data management is to transform the current bioinformatics resources from specialist resources to resources more easily used by those better able to assess bioremediation field needs. For example, it is necessary to continually revise the probe sequences previously designed in order to achieve better specificity. The current approach is to import newly submitted sequences into a database used for probe/primer design, align these sequences with existing sequences, and then perform probe/primer design. This can be very time consuming if thousands of probes require re-evaluation. It should be possible to reduce this difficulty through the development of probe re-evaluation tools that automatically take into account the newly added sequences and automatically redesign probes with updated information. It would be very useful to have hyperlinks between different types of databases so that users could query a specific gene sequence or its placement in a gene tree, ask what probes/primers are available for a set of selected sequences, or know what the functional genes are for a specific metabolic pathway. With the integration of a broad range of bioinformatics tools and databases, it should further ease the challenges currently facing the implementation of effective MBTs for bioremediation, and will eventually play an important role in the understanding of physiology in different microbial ecosystems.

5.2.4 Physiology

Ultimately, achieving the long-term goal we have defined will require the development of tools that can provide a comprehensive understanding of the physiology of microorganisms involved in bioremediation. It is recommended that there be a major effort to continue the isolation of microorganisms responsible for biodegradation of important contaminants. Further efforts are needed to isolate other contaminant degraders such as anaerobic and aerobic oxidizers of vinyl chloride and dichloroethene (DCE), and anaerobic oxidizers for aromatics. Once successfully isolated, key enzymes and genes related to degradation processes can be identified from these pure cultures, and their activities and expression in response to contaminant degradation and environmental perturbation can be studied at a single-gene level to produce informative results for further improvement of bioremediation strategies.

It is also imperative to further elucidate the key metabolic processes involved in bioremediation at a genome-wide level, so that the regulation and expression of key metabolic pathways involved in bioremediation can be better understood. To achieve this, obtaining genome sequences is an essential requirement. Currently, the genomes of *Geobacter* and

Dehalococcoides are readily available, and there are more strains related to *Geobacter* and *Dehalococcoides* that are in production or in the last stages of sequencing. Genome sequencing efforts could be further extended to other important contaminant-degrading isolates and low-diversity microbial communities that show effective degradation ability for contaminants (e.g., anaerobic aromatic oxidizers and polychlorinated biphenyl [PCB] dehalogenators). The latter sequencing approach is also known as environmental metagenomics. It is anticipated that complete or nearly complete genome sequences can be obtained and annotated to provide detailed metabolic maps. Gene expression microarrays may be subsequently developed and used *in situ* to understand which genes are being expressed and how the key metabolic activities are regulated under different environmental conditions.

In addition to genome-wide experiments, which can be very expensive and time-consuming, a systems biology approach is highly recommended as a cost-effective alternative to provide an optimal strategy for bioremediation within a short time. It is recommended to focus systems biology approaches on those important degraders that are slow growing and/or difficult to grow. To pursue this approach requires not only a good understanding of genome information related to the microorganisms responsible for bioremediation, but also a good set of physical and geochemical data regarding the environmental conditions at a contaminated site. With such information, appropriate conceptual models can be developed for input into *in silico* models. Such models have the capability to predict cellular metabolism carried out by the microorganisms that are responsible for the important steps in bioremediation under different environmental conditions. Ultimately, it is anticipated that the outcomes from a systems biology approach can provide information to decision makers that will enable better selection of either a natural attenuation strategy or an engineered strategy to accelerate bioremediation.

6. RESEARCH AND DEMONSTRATION NEEDS FOR ENVIRONMENTAL REMEDIATION APPLICATIONS OF MOLECULAR BIOLOGICAL TOOLS

Molecular biology has had an enormous impact on fields such as agriculture and medicine, and it has led to a much better understanding of processes and organisms involved in bioremediation of hazardous chemicals in the environment. However, to date, MBTs have received little use by practitioners involved in environmental restoration. Workshop participants were optimistic about the potential contributions that molecular biology could make in the near future to the bioremediation field.

MBTs have the potential to provide rapid and reliable measures for both a second and third line of evidence for *in situ* bioremediation (i.e., evidence that indigenous microorganisms have the potential to transform or degrade contaminants, and evidence that transformation or degradation is occurring in the field) (NRC, 2000; USEPA, 1998). Field-scale tools to address the latter line of evidence would be particularly valuable (Smets and Pritchard, 2003). In addition, the panel was enthusiastic about the potential for incorporating MBTs into models that not only demonstrate but also predict bioremediation performance. However, before this promise can be realized, MBTs need to be further developed, evaluated, and demonstrated at the field scale.

The following sections discuss the research and demonstration needs identified by the expert panel assembled at this workshop. This discussion is introduced by a description of the major barriers to field implementation. The final sections then describe those areas for future investment that the panel members believe have the greatest promise to overcome those barriers.

6.1 Major Barriers to Field Implementation

The panel members identified six major barriers to field implementation. In most cases, these barriers include several related topics grouped into broader categories. The major barriers are listed below, roughly in order of priority, and then discussed in the following section. The barriers also provide an introduction into the research and demonstration needs identified in subsequent sections. Major barriers identified include:

- Subsurface sampling difficulties;
- Insufficient knowledge regarding key biomarkers;
- Limited decision-making impact;
- Limited ability to develop rate information;
- Insufficient confidence in results; and
- Limited commercial interest.

6.1.1 Subsurface Sampling Difficulties

Sampling the subsurface is, in general, difficult and inherently uncertain. However, sampling for MBTs involves somewhat unique issues that constitute a significant barrier to the wider use of these tools. Many of the biomarkers of interest are unstable, so complete recovery can be very difficult, if not impossible. Biomarkers are also often present at relatively low concentrations in

environmental samples (compared to medical or food samples, for example), making recovery and quantification difficult. The microorganisms of interest may also be difficult to isolate or grow under laboratory conditions, further complicating their study. Finally, the temporal and spatial heterogeneity in the distribution of biomarkers in the subsurface has received only limited attention, but is expected to be a significant issue that could impact the cost and difficulty of using MBTs and the interpretation of MBT results.

A particularly important concern is the fact that sampling is often focused on the use of conventional monitoring wells and groundwater sampling procedures. However, microbes are generally attached to surfaces within the subsurface, and monitoring well sampling can provide a highly inaccurate assessment of biodegradation potential (Thomas et al., 1987). Also, monitoring wells themselves may change the local environment, so that the results from sampling for MBTs may not be representative of the actual *in situ* condition. The environment within the monitoring well may also be heterogeneous, which can further complicate analysis of samples. The result is that conventional groundwater sampling procedures may grossly underestimate the abundance of target biomarkers, and in many cases important biomarkers may not be detected even though they are present. Such false negatives may be a common result, and in fact can be a deterrent to the use of MBTs by practitioners who become concerned with how to interpret or explain such negative results.

Given the difficulties inherent in subsurface sampling and the embryonic stage in use of MBTs for environmental applications, there have been inadequate efforts made to develop and test standardized sampling procedures for MBTs. Data quality objectives and QA/QC guidelines are generally lacking, and this raises significant concerns regarding the ability to interpret results from use of MBTs. It will be difficult for practitioners and regulators to have confidence in MBT results, in even a qualitative sense, until we understand how to efficiently recover biomarkers for different key functions and how to quantify both the actual recovery achieved and the uncertainty involved.

6.1.2. Insufficient Knowledge Regarding Key Biomarkers

Currently, there are only a few known biomarkers that have become important to environmental remediation. Notably, markers for *Dehalococcoides* and probes for genes involved in chloroethene reductive dechlorination (e.g., *tceA*, *vcrA*, and *bvcA*) are the most commonly used. Even for these relatively well-studied cases, questions remain. For example, the *vcrA* and *bvcA* probes available do not detect all of the vinyl chloride (VC) RDase genes in environmental samples, and may in fact detect only a small percentage of the total.

There was broad support for developing more functional gene probes and for developing a greater understanding of key biomarkers in general. Biomarkers useful for evaluating the degradation of common contaminants such as chlorinated ethanes and methanes, chlorinated aromatics, and explosive compounds were consistently cited as important needs. Development of MBTs for environmental applications is in its infancy, and even though they have already had an impact, more fundamental research and development are needed to more fully realize the potential of these tools for field application.

A related concern has been the lack of knowledge regarding some of the key biological processes involved in contaminant degradation. This lack of knowledge makes it difficult to interpret

MBT data or even design the full suite of needed biomarkers. For example, there appear to be several microbial interactions that can impact the process of reductive dehalogenation, including competition for electron donors and production of needed cofactors or breakdown of organic compounds to produce hydrogen needed by the dehalogenating bacteria. In fact, it is not clear that the organisms responsible for dehalogenation have been fully identified. Without a greater fundamental understanding, it is difficult to develop the needed MBTs or interpret MBT results.

6.1.3. Limited Decision-Making Impact

To date, MBTs have had little impact on restoration decisions regarding remedy selection, design, operational monitoring, or site closure. The tools are not frequently used, though use is increasing. When MBTs are used, they are often employed along with other tests that are performed to demonstrate the biological potential within an environment. The consensus has been that MBTs generally provide confirmation of conclusions based on more conventional biological and geochemical monitoring, and MBT results generally have little impact on site management decisions.

Many participants believed that the “return on investment” for use of these tools has not been clear to site owners and remediation practitioners, and that the value added by spending money for such analyses has been difficult to demonstrate. Part of this difficulty results from the fact that cause and effect relationships often are unclear. As a result, it is difficult to interpret the results of MBT analyses concerning the presence or absence of specific biomarkers or the levels of such markers in a specific sample. Some participants also felt that the use of MBTs could even be a liability in some cases, particularly when key functions of microorganisms could not be detected because of sampling or methodological problems, so that false negatives generated unwarranted concerns and required explanations that would not otherwise be needed.

However, several participants noted that there have been cases in which MBTs were clearly valuable. In particular, detections of key organisms (such as *Dehalococcoides*) or key processes (such as anaerobic TCE oxidation capability) at sites has helped with the selection and continued operation of enhanced bioremediation systems, or allowed natural attenuation to be used at sites where it was difficult to demonstrate slow rates of degradation using conventional chemical analyses. In fact, some participants believed MBTs would be more widely used if more people understood how effective the tools have been in some such cases.

There was general agreement regarding the valuable past contributions of MBTs to more in-depth knowledge about the suite of remediation technologies currently available, as well as to the considerable promise for MBTs to reduce costs and uncertainty at remediation sites in the future. However, the current limitations with respect to the biomarkers available, the difficulties in sampling and quantification, and the difficulties in interpreting the results of MBT analyses have so far severely limited the impact of these tools on the practice of restoration.

6.1.4. Limited Ability to Develop Rate Information

A key issue affecting the potential impact of MBTs is that it is currently extremely difficult to use such tools to evaluate or predict rates of degradation or other key processes in the environment. For many people, the potential value of MBTs is to assess the rates of contaminant degradation rapidly and inexpensively under intrinsic conditions or, if the conditions are modified, to enhance biodegradation. For example, the amount of a given protein in samples

from a well could be used as input data in computer models to derive an estimate of the natural attenuation capacity at a given site and thereby predict whether MNA would be a viable remedy alternative. Currently, such MNA evaluations rely on chemical monitoring over an extended time, and therefore can be costly and time-consuming.

The ability to use MBTs as one of many “lines of evidence,” e.g., to measure the rate of contaminant degradation, was consistently cited as a highly desirable goal. It would allow MBTs to be used more widely to monitor remediation progress or to select appropriate remediation approaches, which would thus be extremely valuable to practitioners. However, at the present time, it is not possible to develop credible and defensible rate estimates from MBT results alone, even if the sampling uncertainties were to be overcome. The inability to use MBTs reliably for rate measurements also stems from the fact that many of the key proteins and/or genes of interest remain unknown. This inability to measure MBT-linked rates constitutes a major barrier to the use of MBTs for remediation because it limits the questions that can be addressed by these tools.

It should be noted that some participants believed that developing credible rate information from MBT data is not feasible and may in fact be asking too much of such tools. In this view, MBTs can be valuable for diagnosis of the reasons that rates are not sufficient, or as a measure of the response of a system to amendments intended to enhance the rates, but it may never be realistic to expect useful rate estimates from these tools alone. Others believed that rate estimates need not be extremely precise to be valuable (i.e., the actual rate may be 2 to 3 times higher or lower than the predicted value, but the estimates would still be of practical value).

6.1.5 Insufficient Confidence in Results

Obtaining quantitative data from many MBTs is more difficult than most people realize, mainly due to the relatively high inherent variability. Even if the sampling difficulties are overcome, the variation in the absolute results obtained for the same samples between different labs can be significant, although others have reported close agreement between results in some cases. Such variation raises concerns about how much confidence should be placed in results from some molecular biological analyses. The lack of confidence also results from the relatively immature state of the practice. As stated earlier, QA/QC guidelines are not developed for environmental applications of MBTs. Few analytical labs even offer molecular biological analyses, and many of the proposed MBTs are not yet commercially available.

Environmental applications of MBTs also involve relatively unique issues that affect the confidence level. In particular, as mentioned earlier, the biomass in soils or aquifers is far lower than in media that have been more commonly sampled for biomarkers (e.g., blood, wastes, or foods). The biomarkers of interest are not as well understood, and may be more variable. Finally, the background of the professionals involved is also a significant issue. Engineers and environmental scientists have relatively little training in molecular biology. As a result, even in cases where the tools have proven to be of benefit, acceptance by practitioners and regulators has been slow and difficult.

The use of MBTs in environmental restoration will continue to be viewed with skepticism until accepted standards of practice and guidelines are developed. In addition, confidence will remain low until the practitioners and regulators receive sufficient training in the technology and its proper applications.

6.1.6 Limited Commercial Interest

In contrast to other fields that have used MBTs more widely (notably medical technology, homeland security applications, and the food industry), environmental restoration constitutes a very small commercial opportunity. Much of the development in this market therefore will have to rely on adaptation of the tools developed for other applications. Many of the tools used to date are not available at commercial laboratories because there is too little commercial interest.

However, the environmental marketplace has important distinctions that need to be addressed, and there is a very limited amount of private or public sector funding available for the research and development needed. Environmental samples generally have far lower numbers of organisms and biomarkers than foods or medical samples, for example. Far more is known about key food pathogens or potential biological weapons than about most of the organisms or genes useful for remediation, and many of the latter are in fact difficult to grow or isolate.

Past history suggests that advances in the use of MBTs in other fields will progress by a type of “punctuated equilibrium,” in which use is relatively slow until a critical mass of available knowledge and experience is developed, after which the use increases very rapidly. In this sense, the limited development to date continues to limit further development, but targeted research has the potential to increase commercial viability, leading to much faster development of MBTs for remediation than would otherwise occur. In particular, the development of QA/QC guidelines and standard protocols should spur commercial interest.

6.2 Research Needs

The panel was asked to “identify promising areas of research and development with the potential to lead to improved cost-effective tools to support remedial design and decisions.” These research needs were then to be designated as either “critical” or “high priority” (see Table 1).

Several specific research needs were identified and later grouped into broader categories. These categories are summarized in the following sections. Specific demonstration needs are briefly identified in bold text and described below.

6.2.1 Research Needs: Critical

6.2.1.1 Sampling Needs

The major goal of the workshop was to develop a road map for research and development that would lead to the efficient use of MBTs for environmental restoration. The workshop participants agreed that research and guidance on sampling is a critical need. The use and evaluation of MBTs requires a better understanding of the effects of all steps in sampling, including sample collection, transport, storage/preservation, and processing. The heterogeneity of field sites and the lack of standard sampling practices have complicated the interpretation of MBT results, but few studies have addressed the effects of sampling procedures on MBTs or of how well groundwater itself is representative of the subsurface matrix.

In order to better understand MBT efficacy, **sampling techniques conducive to MBTs need to be tested** in a systematic fashion for both groundwater and sediments. The temporal and spatial

heterogeneities in the distribution of microbial communities also need to be addressed. In addition, **the processing of retrieved samples needs to be evaluated**, including sterility and fast-freezing in the case of microbiological analyses and the impacts of filtration and improved filtration technologies. An important initiative encouraged by the workshop participants was to **gather and evaluate past data and experiments on sampling practices and outcomes** to minimize redundancy and learn from past experiences.

Post-sampling, the transport and storage of field samples taken for MBT analyses, is crucial and can vary due to different field conditions and lack of standardization. The time of transport and method of storage is particularly important for the use of MBTs because of the susceptibility to degradation and change of biological molecules and cells. Therefore, an **evaluation of different methods for transport and storage is needed**. Workshop participants noted that the type of MBT to be used needs to be considered as part of the transport and storage/preservation methods.

For MBTs, sample processing can vary with the extraction method used, which in turn depends on the molecules of interest. Some comparative studies exist in the scientific literature, but most approaches have intrinsic advantages and disadvantages. An important note by the workshop participants was to adequately **determine the metrics and constraints for each method** and not to accept a default position of a one-size-fits-all approach for sample processing for MBTs in general. Namely, **confidence limits should be determined for each MBT** so that its advantages and disadvantages can be considered for a given field site. The workshop participants agreed that these should include such tangibles as percent recovery determinations for nucleic acids, baseline normalizations, and quantification.

6.2.1.2 Identify Additional Biomarkers

Most work to date on identifying biomarkers (e.g., genes, peptides, enzymes, lipids, etc.) has focused on a narrow spectrum of target organisms or activities. For example, many MBTs have been developed and applied to monitor chlorinated solvent-degrading organisms such as *Dehalococcoides spp.* and a few of their known functional genes, but less work has been performed to develop biomarkers for organisms that support the activity of *Dehalococcoides* or to identify additional functional genes or biomarkers for these organisms. Likewise, processes other than reductive dehalogenation (e.g., anaerobic oxidation) may be important for degrading chlorinated solvents and related contaminants, but few biomarkers have been developed to monitor these processes. **Identifying biomarkers to evaluate community structure and to assess the total degradative potential of a microbial population is critical** to correlating biomarker measurements with functional activity.

In addition, **biomarkers are needed for organisms involved in degrading other contaminants of concern** and their associated microbial communities. Target contaminants include PCBs, polycyclic aromatic hydrocarbons (PAH), chloroethanes, methyl-tert-butyl-ether (MTBE), chlorobenzenes, and emerging contaminants of concern such as N-Nitrosodimethylamine (NDMA) and 1,4-dioxane. Identification of additional biomarkers would also be useful for evaluating community structure and/or assessing the total degradative potential of a microbial population. Such work is critical to correlating biomarker measurements to functional activity.

A key need is to correlate the detection or quantification of biomarkers with *in situ* activities (e.g., degradation rates). *In situ* activity measurements are essential for estimating

contaminant risk, in particular for evaluating natural attenuation rates, and for assessing the success and progress of active bioremediation efforts. Correlating activity to biomarker measurements is, therefore, critical to the successful and widespread adoption of MBTs. The ability to correlate biomarker measurements to *in situ* activity will likely require a better understanding of the relationship between key degradative organisms (e.g., *Dehalococcoides*) and their supporting microbial communities and an expanded knowledge of homologous or related functional genes/enzymes that ultimately determine overall *in situ* activity.

Improved culturing and metagenomic/proteomic methods also are needed to identify additional biomarkers. Given that only a small percentage of natural microbial communities can commonly be cultured in the laboratory, it is likely that such improved methods will lead to the development of more and/or improved biomarkers for analyzing microbial communities and correlating MBT results to *in situ* activity. Innovative techniques are needed to improve the cultivation of contaminant degrading microbes and their supporting communities from environmental samples. Likewise, additional development is needed in the area of metagenomics and proteomics to improve our analysis and characterization of natural microbial assemblies. Metagenomic and proteomic approaches would improve our understanding of microbial relationships and interactive capacities and would aid in identifying new, potentially important organisms and/or activities.

Finally, the panel also recommended the **development of software tools to manage functional biomarker data, including archiving and annotation of source/site characteristics**. Most environmental applications of MBTs are performed in individual laboratories, or they are collected as part of commercial remediation projects and the resulting data are not readily disseminated throughout the scientific community. Notably, there are currently few, if any, software tools or databases to collect and store environmental MBT data, and few reports have correlated biomarker measurements with site hydrogeology, physicochemical or biological data from the site at which the tools have been applied. Consequently, **improved software tools and databases are needed** to compile, store, and analyze MBT-derived data, and to assist in correlating data between sites and research groups. Ultimately, the software should provide an easy to use depository for biomarker data and allow annotation and comparison of biomarker data from multiple studies and/or laboratories. **A long-term goal may be to create and maintain a central database and web site** that is regularly updated as new MBT data are published or submitted.

6.2.1.3 Microbial Interactions

Most biological processes in the subsurface are not fully dependent on a single organism but rather depend on the combined interactions of several organisms. Further, direct, and indirect interactions with other populations can play a major role in the ability of a microorganism to survive and thrive in a given habitat. **A more thorough understanding of key microbe-microbe interactions is needed** to increase our ability to predict and optimize biological remediation processes.

For example, reductive dechlorination of chlorinated ethenes may be mediated by *Dehalococcoides spp.*, but the activities of numerous other bacteria impact the process. In fact, there is some doubt that *Dehalococcoides* is the only, or even the primary dechlorinating organism active *in situ*. *Dehalococcoides* uses hydrogen as the electron donor for

dechlorination, but the overall rate of dechlorination may be controlled by other organisms that produce hydrogen through the fermentation of organic compounds. In addition, other organisms may produce vital cofactors, such as vitamin B12, that are needed by *Dehalococcoides*. Other organisms, such as methanogens, may compete for the hydrogen, limiting the efficiency of the desired activity. Still other organisms, such as sulfate reducers, may be needed to alleviate potential inhibitory conditions and allow *Dehalococcoides* to flourish.

The use of MBTs has had a dramatic impact on the field of microbial ecology, and has revolutionized how microorganisms can be identified and detected in the field. MBTs can also help us understand field sites as interdependent systems, where interactions between microbial populations ultimately control inputs and outputs. But a great deal of work is still needed to develop a general understanding of how microbial populations interact and respond to changing conditions. **Research is needed to identify key organisms, to understand the positive and negative interactions between them, to understand the critical aspects of community structure and function, and to identify biomarkers** that can be used to predict and monitor key activities and to indicate potential problems.

6.2.2 Research Needs: High Priority

6.2.2.1 Improved Sampling/Analysis Methods

Three related topics that were deemed important, but not critical in the near-term, were the development of novel subsurface sampling strategies, the comparison and contrast of groundwater and sediment samples, and the development and adaptation of high-throughput methods for MBT analysis. These are described briefly below.

There was a substantial concern that **groundwater samples may not adequately represent the surrounding sediments** (i.e., geomatrix) in some cases. Many organisms are predominantly attached to solids, and wells may alter the local groundwater geochemistry and hydrology. However, it can be difficult and costly to obtain intact sediment samples, particularly during and post-treatment. As a result, monitoring wells are generally used for groundwater sampling for MBT analyses, and the magnitude and impacts of any potential differences have not been sufficiently addressed.

The difficulties in obtaining samples are particularly important for biomarker monitoring, because many samples may be needed to overcome the temporal and spatial heterogeneities, and many biomarkers may be sensitive to changes in environmental conditions that can occur during sampling and sample handling. The **development of new methods for obtaining representative sediment samples** would certainly have a major impact on the field and could facilitate the use of MBTs to better understand the system as a whole.

Finally, high-throughput methods (e.g., methods to automate sample preparation) have been developed for other industries, but these will need adaptation before they can be used for environmental samples. High-throughput methods are currently used in some laboratories to create and analyze large biomarker libraries (e.g., environmental clone libraries), and they have been proven to be essential for such large-scale efforts. **Development and adaptation of high-throughput methods** should reduce the volumes needed for analysis, help produce more accurate and reproducible results, and hasten the adoption of MBT analyses by contract

analytical labs. High throughput methods that can generate quantitative results that can aid in correlating biomarker measurements to *in situ* activities would be most desirable.

6.2.2.2 Systems Biology

Systems biology is an interdisciplinary approach that attempts to integrate high-throughput biological data at all levels of information (i.e., inputs). An understanding of a system is only as complete as the fraction of the parts that are studied, and multiple measurements better capture how an entire system may work. Systems biology can be applied to a single microorganism or a community of organisms. In the case of a single microorganism, systems approaches attempt to measure all possible molecules in a cell and coordinate the data for a complete description of regulation and metabolism. This approach may include genomics, transcriptomics, proteomics, lipidomics, metabolomics, and phenomics. The data represents levels of all major molecules (transcripts, proteins, metabolites) over time. The ultimate **goal is to develop *in silico* models that can be used to predict outcomes** with various inputs and outputs under various growth conditions (Lovley, 2003).

In the case of a community, the proportion of different populations is related to both biotic and abiotic conditions, and the proportions of specific populations can sometimes be related to specific biochemical activities. With systems approaches, energy and carbon flux can be accounted for as it moves through the different trophic levels of the system. The workshop participants deemed systems approaches worthy of further study and recommended that these approaches should be explored once the sampling and standardization issues were addressed. Ultimately, systems approaches show the greatest promise for integration with modeling techniques.

6.3 Demonstration Needs

The panel also was asked to “identify the most promising areas that are ready for and could benefit from rigorous field-scale demonstrations.” As with the research needs, these demonstration needs were categorized as “critical” and “high priority” (see Table 1). Several specific needs were identified and later grouped into broader categories. These categories are summarized in the following sections, and specific demonstration needs are briefly identified in bold text and described below.

6.3.1 Demonstration Needs: Critical

6.3.1.1 Standardization and Validation of Methods

The panel identified a need to standardize the methods used to extract biomarkers. Recovery of biomarkers from the environmental matrix is critical for obtaining reliable and reproducible results. In some cases, commercial kits are available for biomarker extraction, but the reliability and reproducibility of these products for different environmental samples remains largely untested. The current lack of standardization in extraction methods makes it difficult to compare biomarker data between studies and analytical laboratories. **Research is needed to establish and verify biomarker extraction systems and to establish a set of standardized procedures** that can be applied in analytical laboratories to ensure accurate and precise biomarker analysis. Standard QA/QC procedures similar to those used for chemical analyses should be established and verified for biomarker extraction and analysis.

Standardization is also needed in several other components of MBT methodology. For example, many laboratories currently perform PCR and/or gene probing analysis of *Dehalococcoides*-like organisms, but often the primers and probes used to do the analyses differ between studies, or they are not disclosed. It is difficult to ascertain how these differences affect the measurements made, or how the resulting data can be compared between studies or laboratories. Standardizing these tools between groups is complicated by patents, publications, and proprietary interests, and also by the rate at which new tools are identified or developed. Furthermore, different analytical equipment (e.g., PCR machines) is used in the different labs performing analyses. **Research is needed to evaluate differences between the different analytical methods and to develop a standardization process** that will allow comparisons between studies or applications. Such standardization also will ensure that results obtained from MBTs will be accurate and precise.

The panel also recognized the **need to develop a validation process for new biomarkers**. Although MBTs are used widely in academic research and in clinical settings, their use in environmental monitoring has been limited. The relatively slow adoption of these technologies stems in part from the inability to correlate biomarker measurements with field-related activities. Further, it is difficult to validate the results obtained with MBTs. Additional research is needed to establish and test biomarker validation procedures to ensure that the results of MBT analyses are accurate and precise and that the results can ultimately be correlated to *in situ* activities. Validation procedures should address analyses of various environmental matrices and multiple biomarkers to confirm the adaptability of MBTs to a diversity of environmental samples.

Finally, the panel recommended an **assessment of the precision, accuracy, and reproducibility of MBT analytical methods and eventual establishment of accepted QA/QC protocols**. MBT analyses are currently performed commercially by a few analytical laboratories and performed widely in academic research. To date, however, no standardized QA/QC procedures have been established to ensure reliability and reproducibility. Such procedures are well-established and routinely applied in chemical analytical laboratories. The chemical analysis QA/QC procedures provide recommendations for sample handling, instrument calibration, analytical protocol, and methods for evaluating analyte recovery and measurement. Development of similar QA/QC procedures for MBT analysis is needed. Separate procedures will likely be needed for each class of biomarker analyzed or method of analysis.

6.3.1.2 Integrated Field Demonstration

The panel members strongly supported the **need for an integrated field demonstration of MBTs focused on the bioremediation of chlorinated ethenes**. Such a study would apply currently available and developing MBTs to a suite of sites with varying conditions to demonstrate the potential for the tools to diagnose possible problems and to replace or augment current monitoring and characterization approaches. Conceptually, MBTs would be used in addition to the current geophysical and chemical methods for site characterization, and the results would be used to assess the value added by MBTs and the real and potential future return on investment by the use of these tools.

For example, using MBTs at one or more sites that are exhibiting “failure” of reductive dechlorination (i.e., accumulation of *cis*-DCE or vinyl chloride) may provide valuable insight into the reasons for such failure and the methods that could be used to allow complete

dechlorination to occur. However, such a use has not been demonstrated, and many are skeptical that MBTs can be effectively used for such diagnosis, or that MBTs can replace the use of conventional analyses for this purpose. Other potential diagnostic uses include the identification and characterization of slow and poorly understood natural attenuation processes, such as the anaerobic oxidation of *cis*-DCE or vinyl chloride.

A multi-site demonstration was favored so that the demonstration could elucidate the conditions under which MBTs may or may not be valuable. Such research could also indicate the improvements in available MBTs that would be needed in order to broaden their applicability.

6.3.2 Demonstration Needs: High Priority

Currently, programs and algorithms exist for modeling environmental processes at contaminated field sites, and experimental data have been compared to simulations (e.g., Brandt et al., 2003; Schreiber et al., 2004). However, due to the heterogeneity of field sites, microbial communities, and contaminants, different models may be needed. There is broad agreement on the evidence needed to demonstrate *in situ* bioremediation (NRC, 2000; U.S. EPA, 1998), and MBTs could provide cost-effective methods to generate such evidence. However, few studies have documented the **incorporation of MBT data into models that demonstrate, and more importantly, predict bioremediation performance or failure in the field.** Microbial populations and communities can be responsible for most transformations, and the integration of microbial processes into field-scale, predictive models is needed for successful assessment of long-term natural and enhanced attenuation.

Incorporating MBT results into accepted analytical models could increase our confidence in model predictions and reduce the costs for collecting the data needed for such models. However, there was considerable discussion regarding the ability of MBTs to provide the data needed to develop rate estimates. Some participants believed MBTs could provide useful measures of the rates of biological activities *in situ* while others believed that was asking too much from MBT assays, particularly those that are currently available. All agreed that reasonable rate estimates based on MBTs would be highly valuable, and that correlations between MBT measurements and *in situ* degradation rates need to be established and tested under field conditions. Workshop participants noted the **need for the development of new tools that formulate and predict rates that integrate MBT measurements.**

An added value of MBTs may be the ability to evaluate and address remediation failures as well as remediation success. More work is needed to **demonstrate the use of MBTs to predict and circumvent bioremediation shortfalls,** and this use of MBTs may well also involve incorporation of the results into appropriate models. Ultimately, models are needed that can integrate conventional, geophysical, and biological techniques from varying field sites with different contaminants of interest.

6.4 Outreach/Technology Transfer

The panel also identified a need for technology transfer. Most practitioners and regulators in the environmental restoration field have little experience or background in molecular biology.

Despite the fact that its use is increasing in this field, as well as in many others, there is relatively little knowledge about its potential or its recent applications. Education could be valuable in increasing awareness and providing guidance to professionals who must decide whether to use such tools or how to interpret results from MBTs. However, any technology transfer effort will be a difficult enterprise at this point, because the use of MBTs is still in such an early stage of development and the practice is evolving so rapidly.

The panel recommended development of a “living document” on the state of the practice.

A web site providing background information, summaries of the advantages and limitations of various types of MBTs, examples of MBT uses, and limited guidance on appropriate uses and developing standards of practice could be a valuable resource. The panel also recommended collection and assessment of existing data from MBT uses, and this living document would provide an outlet for such information, with the potential for regular updating as new information becomes available.

Finally, the panel also briefly discussed **other related outreach efforts**. One example of such an effort is the formation of a team within the ITRC focused on MBTs (discussed further in Section 3.2.2). As the use of MBTs increases, the need for education, guidance, and standardization of approaches will increase. This type of technology transfer would be a natural extension of the research and demonstration work funded by SERDP and ESTCP and would be a valuable service to the user community.

7. CONCLUSION

There are more than 9,000 sites on former and current DoD installations requiring environmental restoration because of groundwater, soil, and sediment contamination. Assessing the human and ecological risk of this contamination and monitoring remediation performance can be difficult and costly given the currently available tools. SERDP and ESTCP, as DoD programs that promote the development and demonstration of innovative, cost-effective environmental technologies, must determine how their limited funds can best be invested to improve DoD's ability to effectively address its cleanup requirements in consideration of and in collaboration with past, present, and planned initiatives of other funding organizations and research programs.

While advances in molecular biology have had a profound effect on the understanding of biological remedial processes and are used extensively in the research community, use of MBTs in the operational cleanup community is limited at present. There is, however, tremendous potential for these tools to improve the design, implementation, field performance, and monitoring of remediation technologies. The state of the science and technology of MBTs with applicability to environmental cleanup was explored in a series of background papers, highlighting current tools and techniques, future applications, and the field perspective.

Applications of these tools and barriers to their use were assessed through overview presentations and group discussions. A summary of the state of current applications for various MBTs is provided in Table 2 whereas potential applications for remediation are reviewed in Table 3. With the exception of qPCR 16S rRNA genes, all tools were cited as "low frequency of use." While applications of these tools for degradation potential and specific organism distribution are fairly well-established, use for assessing organism activity, process adequacy, environmental limitations, operational improvements, and continuous monitoring/process control is less certain. Technical barriers to field implementation include insufficient knowledge of key biomarkers, limited ability to develop rate information, limited understanding of physiology, and limited availability of databases. Other barriers include subsurface sampling difficulties, limited decision-making impact, insufficient confidence in results, and limited commercial interest.

To address these barriers and support future implementation of MBTs at DoD contaminated sites, research, demonstration, and technology transfer needs were identified and prioritized based largely on the timing needed to accomplish goals. Critical research needs included sampling techniques, identification of additional biomarkers, correlation of detection or quantification of biomarkers with degradation rates, and understanding of microbial interactions. High priority research needs focused on sampling/analysis methods and systems biology approaches. Critical demonstrations included standardization and validation of methods used to extract biomarkers and application of tools across multiple field sites for chlorinated ethenes. High priority demonstrations focused on the incorporation of MBT data into models that demonstrate, and more importantly, predict bioremediation performance or failure in the field. Technology transfer needs included web-based resources for sharing information and data as well as education, guidance, and standardization of approaches among stakeholders.

The result of this workshop is a strategic plan to guide SERDP and ESTCP investments in MBTs over the next 5 to 10 years, ultimately impacting environmental restoration efforts at DoD sites.

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APPENDIX A

GLOSSARY

Direct PCR: Direct Polymerase Chain Reaction uses primers specifically amplifying the target gene(s) in a sample.

Nested PCR: For increased sensitivity, two successive PCR amplifications are performed, in which with the amplicons from the first PCR take the role as template for the second amplification. The primers used for the second amplification round bind to internal sites of the amplicons generated in the initial PCR.

DGGE: Denaturing Gradient Gel Electrophoresis separates PCR amplicons based on their melting behavior in polyacrylamide gels under increasingly denaturing conditions.

Cloning: Transfer of a gene of interest into a foreign host, typically *E. coli*.

T-RFLP: Terminal Restriction Fragment Length Polymorphism is a comparative fingerprinting technique that only analyzes the terminal fragments generated in the restriction digests of PCR amplified target gene(s).

qPCR: Real-Time PCR is a quantitative approach that monitors the fluorescence emitted during the amplification of a target gene at each PCR cycle (in real time). Different detection chemistries are available including Taqman probes and SYBR Green.

qRT-PCR: Real-Time Reverse Transcription PCR is a quantitative approach that evaluates transcription of an indicator gene by detecting mRNA transcripts.

FISH: Fluorescent In Situ Hybridization uses fluorescently labeled probes to visualize cells that possess the target 16S rRNA gene(s). Variations of FISH such as Catalyzed Reporter Deposition (CARD)-FISH can increase the sensitivity and allow the detection of functional genes and mRNA.

PhyloChip: PhyloChips or phylogenetic oligonucleotide arrays are DNA microarrays consisting of rRNA-targeted oligonucleotide probes. Multiple oligonucleotide probes are included that target 16S rRNA gene sequences of organisms at different or the same phylogenetic levels ("multiple probe concept").

Functional gene array: Functional gene arrays contain probes corresponding to genes encoding key enzyme systems (catalysts) involved in the processes of interest. Both PCR-amplified DNA fragments and oligonucleotides derived from functional genes can be used to fabricate Functional gene arrays.

MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry is a fast, sensitive, high-throughput technology to detect process-specific biomarkers using whole cells or minimally processed cells.

PLFA: Phospholipid Fatty Acid biomarker analysis is a quantitative approach to measure viable biomass and metabolic activity.

CSIA: Compound-Specific Stable Isotope Analysis is an in situ monitoring tool that characterizes the natural abundance of stable isotope signatures (C, N, H, and O) of individual dissolved contaminants.

SIP: Stable Isotope Probing involves the incorporation of stable-isotope-labeled substrates (typically ^{13}C) into process-specific biomarkers (DNA, RNA, proteins, lipids).

APPENDIX B
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APPENDIX C

AGENDA



MOLECULAR BIOLOGICAL TOOLS WORKSHOP



TUESDAY, AUGUST 9, 2005		
0700	Continental Breakfast and Registration	
0800	Welcome and Introduction	Dr. Jeffrey Marqusee SERDP Technical Director ESTCP Director
0815	Overview of Current Tools and Techniques	Dr. Andrea Leeson SERDP/ESTCP Environmental Restoration Program Manager
0845	Future Perspective: Potential Application of Microarrays	Dr. Frank Löffler Georgia Institute of Technology
0855	Potential Application of Proteomics	Dr. Darrell Chandler Argonne National Laboratory
0905	Potential Application of Proteomics	Dr. Rolf Halden Johns Hopkins University
0915	Systems Biology Approach to Bioremediation	Dr. Derek Lovley University of Massachusetts
0925	Next-Generation Real-Time PCR	Dr. Syed Hashsham Michigan State University
0935	Potential Application of Nanoparticles	Dr. Wen-Tso Liu Singapore National University
0945	Potential Applications from Other Fields	Dr. Suresh Pillai Texas A&M University
0955	Role of Bioinformatics in MBT Implementation	Dr. James Cole Michigan State University
1030	Field Perspective: Current Use of MBT and Limitations	
1045	Breakout Session I Discussions: Key Issues	Mr. Patrick Haas P. E. Haas & Associates, LLC
1200	Break	
1300	<ul style="list-style-type: none"> Near-Term MBT (located in Salon A) Long-Term MBT (located in Ashlawn/Highlands) Field Considerations (located in Lewis/Clark) 	Breakout Groups
1430	Working Lunch (Lunch Provided)	
1500	Breakout Session I Discussions (cont'd)	Breakout Groups
1600	Break	
1700	Reports from Breakout Session I	Breakout Group Chairs
1830	Identification and Discussion of Key Issues	Dr. Paul Johnson Arizona State University
1900	Reception in Atrium (through 1830)	

WEDNESDAY, AUGUST 10, 2005		
0730	Continental Breakfast	
0800	SERDP/ESTCP Investments: R&D and Field Demonstrations	Dr. Andrea Leeson, Environmental Restoration Program Manager
0820	Army Investments: R&D and Field Implementation	Dr. John Cullinane, U.S. Army Corps of Engineers-ERDC
0840	Navy Investments: R&D and Field Implementation	Dr. Linda Chrisey, Office of Naval Research Mr. Cliff Casey, NAVFAC Southern Division
0900	DOE Investments: R&D and Field Implementation	Dr. Todd Anderson, NABIR
0920	EPA Investments: R&D and Field Implementation	Dr. Mitch Lasat, NCER
0940	Break	
0955	Breakout Session II Discussions: Molecular Biological Tools RDT&E to Impact Environmental Remediation <ul style="list-style-type: none"> • Group 1 (located in Salon A) • Group 2 (located in Lewis/Clark) • Group 3 (located in Ashlawn/Highlands) 	Breakout Groups
1130	Working Lunch (Lunch Provided)	
1230	Breakout Session I Discussions (cont'd)	Breakout Groups
1330	Break	
1400	Reports from Breakout Session II	Breakout Group Chairs
1500	Prioritization of RDT&E Needs	Dr. Hans Stroo, HGL Inc.
1645	Concluding Remarks	Dr. Andrea Leeson
1700	Adjourn	

THURSDAY, AUGUST 11, 2005		
0730	Continental Breakfast for Breakout Session Chairs and Rapporteurs (i.e., Working Group)	
0800	Discuss and Prepare Draft Sections of Summary Document (Working Group)	
1200	Adjourn	